Biochemistry and Biophysics Reports 40 (2024) 101852

Contents lists available at ScienceDirect

Biochemistry and Biophysics Reports

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

Recent advances in aptamer discovery, modification and improving performance

Arezoo Fallah [a], Abbas Ali Imani Fooladi [b], Seyed Asghar Havaei [c], Mahdieh Mahboobi [b], Hamid Sedighian [b,*]

[a] Department of Bacteriology and Virology, Faculty of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran
[b] Applied Microbiology Research Center, Biomedicine Technologies Institute, Baqiyatallah University of Medical Sciences, Tehran, Iran
[c] Department of Microbiology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran

ARTICLE INFO

Keywords:
Aptamer
Post-SELEX optimization
Aptamer modifications

ABSTRACT

Aptamers are nucleic acid (Ribonucleic acid (RNA) and single strand deoxyribonucleic acid (ssDNA)) with a length of approximately 25–80 bases that can bind to particular target molecules, similar to monoclonal antibodies. Due to their many benefits, which include a long shelf life, minimal batch-to-batch variations, extremely low immunogenicity, the possibility of chemical modifications for improved stability, an extended serum half-life, and targeted delivery, they are receiving a lot of attention in a variety of clinical applications. The development of high-affinity modification approaches has attracted significant attention in aptamer applications. Stable three-dimensional aptamers that have undergone chemical modification can engage firmly with target proteins through improved non-covalent binding, potentially leading to hundreds of affinity improvements. This review demonstrates how cutting-edge methodologies for aptamer discovery are being developed to consistently and effectively construct high-performing aptamers that need less money and resources yet have a high chance of success. Also, High-affinity aptamer modification techniques were discussed.

1. Introduction

Aptamers are usually short single-stranded oligonucleotides (ssDNA) or RNA (25–80 bases) that have a high affinity for specific target molecules. They are believed to be an alternative to monoclonal antibodies in many biotechnology applications such as the therapeutic and detection of diseases [1–3]. It has needed a lot of labor, time, and limited reproducibility to develop an aptamer through an experimental method employing the systematic evolution of ligands by exponential enrichment (SELEX) technique. In the last several years, much effort has been made to develop innovative techniques that may consistently and efficiently produce high-performing aptamers with fewer resources and, most significantly, a higher chance of success [4]. Nevertheless, the intrinsic flaws of those primary-screened aptamers usually limit their use for chemical detection under complex settings. The native (deoxy) ribonucleotides impose some limitations, such as restricted physical space or low specificity, affinity, and affinity for binding targets [5,6]. Aptamers also acquire other needs, such lower production costs, improved serum stability, and nuclease resistance, for clinical uses. To address the shortcomings of pre-selected aptamers and enhance their binding affinity, specificity, and other characteristics, pre-SELEX or post-SELEX optimization is required.

We start this review by discussing the cutting-edge technology used in aptamer discovery. Next, go over the main variables that affect aptamer functionality or characteristics and emphasize the many aptamer alterations that increase binding affinity to the target. It will aid in the direction of the upcoming generation of highly affinity modified aptamers for the diagnosis and treatment of disease.

2. Conventional aptamer selection system

In order to separate oligonucleotide aptamers from a random library, Tuerk introduced Systematic Evolution of Ligands by Exponential Enrichment (SELEX) [7]. SELEX is still the preferred technique for generating nucleic acid aptamers. Regardless of the application of the selection cycle to proteins, DNA or RNA sequences, cellular levels, or live organisms, three fundamental phases are required: (I) allowing a target to be incubated with a library of randomized sequences (II)

* Corresponding author.
E-mail address: osedighian@gmail.com (H. Sedighian).

https://doi.org/10.1016/j.bbrep.2024.101852
Received 3 September 2024; Received in revised form 6 October 2024; Accepted 16 October 2024

distinguishing between bounded and unbounded sequences, and (III) collecting the bound sequences and amplifying them via Polymerase chain reaction (PCR) [8]. The cycle of selection is then repeated until the sequence is enriched for the desired affinity (Fig. 1). Aptamer selection was accomplished primarily using the same traditional SELEX methods. Conversely, other SELEX technologies, such as Cell-SELEX [9–11], High-fidelity SELEX [12], GO-SELEX [13,14], Capture-SELEX [15,16], Magnetic bead-SELEX [17], Microfluidic SELEX [18], and Staggered Target SELEX (ST-SELEX) [19], have been created. The effectiveness and success rate of aptamer isolation for a range of targets are greatly increased by these technologies. Previous research has effectively isolated aptamers that target a variety of substances, including heavy metals, insecticides, proteins, viruses, ions and even entire cells [20].

2.1. Crucial strategies for post-SELEX optimization

Several pre/post-SELEX techniques and approaches have been developed to improve the effectiveness of appropriate aptamers. Here, we discuss the advantages and disadvantages of these numerous optimization strategies and give an overview of them (Table 1).

3. Principal elements influencing aptamer efficacy

The aptamer application performance is influenced by several aspects, including sequence length, nucleotide composition, and spatial structure, which all affect the inherent properties of the aptamer. In order to improve the aptamer's stability, specificity, affinity, and applicability, these crucial elements must be examined and optimized during the SELEX and post-SELEX phases.

3.1. The length of sequence

Typically, the initial aptamers produced using SELEX consist of a randomized region consisting of about 30–50 nucleotides and two fixed primer sections. Constant primer regions are mainly employed for PCR amplification, and the randomly area selected by SELEX assume distinct roles in interacting with target molecules. Consistent primer sequences barely slightly contribute to the aptamer-target binding, according to bioinformatics tools used to examine the contribution of the various regions of over 2000 aptamers to target recognition. Additionally, certain non-primer domains are not necessary for aptamers to recognize their targets [39–41]. Additionally, unnecessary nucleotides in aptamers may increase free energy and perhaps obstruct the way aptamers and target molecules interact [39–41]. In fact, eliminating primer binding sites and further reducing the aptamer sequence can often result in a simpler structure with the same or even higher affinity to the target [42–45]. Therefore, in order to maximize aptamer performance, numerous research have shortened those unnecessary nucleotides. On the other side, adding nonspecificity nucleotides might enhance aptamer's capacity to bind targets, be thermally stable, and resist nuclease degradation [46–48]. Additionally, aptamers were engineered by including a short hairpin DNA fragment, which improved thermal stability and stabilized aptamers against nucleases. The insertion of extra G-C pairings enhanced the stem region of aptamers. Sequence length affects the signal conduction in aptamer-based devices assays as well as the intrinsic features of aptamers. Using aptamer-wrapped gold nanoparticles (AuNPs), Tian et al. created an aptasensor. Also, they discovered that aptamers with flanking nucleotides could stick to AuNPs very nearly, leading to separation of aptamer from AuNPs, but not completely. Therefore, AuNP couldn't aggregate and discolor. The aptamer's colorimetric sensitivity was increased by 3.3 times by eliminating these superfluous bases [49]. Consequently, length optimization is needed to lower the cost of synthesis and enhance the performance of pre-selected aptamers.

3.2. The composition of dimensional

Another essential component that governs the target affinity, stability and specificity of aptamers is their spatial structure or configuration. After all, the correct aptamer-target interaction depends on their spatial structures being compatible. Adaptive folding allows aptamers to form a better-suited spatial structure for binding with target molecules [50]. Aptamers, such as [d(TTA GGG)] [51] or [d(TTG AGG)] [52], which are composed of all parallel-stranded tetrameric G-quadruplex DNA, alter their initial spatial structure upon binding with theme. Aptamers often have secondary features such hairpins, pseudoknots, bulges, stems, loops, triplexes, and quadruplexes [5]. Eliminating or creating these secondary structures can have a significant effect on the characteristics of aptamers. For instance, they discovered that the aptamer's physical skeleton's extreme flexibility hindered the process of adaptive folding, damaging the affinity. Nevertheless, they were able to successfully enhance the stability by giving the aptamer a triple helix shape [53]. Additionally, spatial structure is essential for the creation of aptamer-based biosensors since most aptasensors are developed based

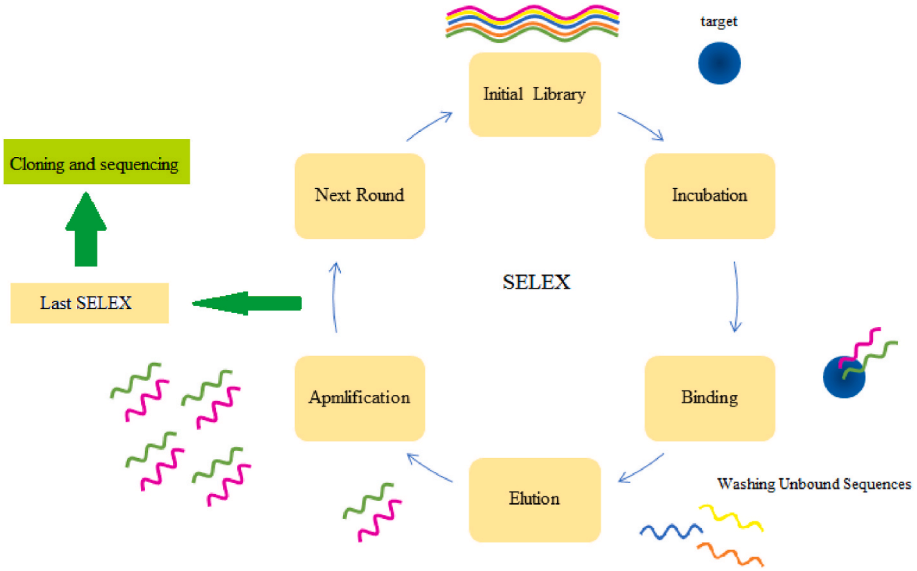

Fig. 1. Schematic illustration of the conventional SELEX process.

Table 1
Various SELEX techniques that have been created.

SELEX methods	Description	Ref.
Conventional SELEX	For aptamer selection, a chemically generated oligonucleotide library is often utilized. Up to 60 oligonucleotides in random sections can be found in this collection. These regions are flanked by brief constant portions that are typically utilized to anneal primers during PCR. During traditional SELEX, many selection processes are introduced based on the desired aptamer type. However, this approach is well-known and efficient, but it requires a lot of time and labor.	[7,21]
Cell-SELEX	This technique uses entire living cells as targets to choose appropriate aptamers. This method can be used to produce highly specialized aptamers from whole, prokaryotic or eukaryotic cells. It's not necessary to know the goal beforehand. Aptamers are chosen against native-state molecules. There are a lot of possible targets on the surface of cells. Purification of proteins is not necessary. Ideal for targets on the cell surface. also calls for a high degree of technical proficiency. Expensive. It takes time. Target identification post-Selex is necessary.	[11,22]
Animal-SELEX	Aptamers are chosen directly from models of living animals. Targets are bound by specific aptamers in their native habitats. However, prior awareness of the objective is not necessary. Not much optimization is required. and time-consuming (need multiple cycles).	[23]
Capillary Electrophoresis SELEX (CE-SELEX)	involves using electrophoretic mobility to separate the ions. By using this strategy, the number of selection rounds can be decreased from approximately twenty in a standard SELEX to one or four, allowing for the selection of aptamer candidates with high affinity.	[24]
Artificially expanded genetic information system (AEGISs)	makes use of libraries containing genetic code that has been intentionally enlarged; one such library has two nonstandard nucleotides (Z and P) in addition to four standard nucleotides (G, A, C, and T). However, natural DNA recognizes abnormal bases Polymerases function badly.	[25]
Microfluidic SELEX (M-SELEX)	a versatile, automated method for producing aptamers quickly that have great affinity and specificity at the microscale level. The most noteworthy enhancements to M-SELEX are the bead-based acoustophoresis technique, sol-gel technique, microarray-integrated microfluidic chip technique, and a micro-magnetic separation device to boost separation efficiency.	[26–33]
Capture-SELEX	a different approach to in vitro selection that works well for separating aptamers from tiny compounds. To facilitate the immobilization of the oligonucleotides onto the solid matrix (Agarose beads), the Capture-SELEX library was created. During the aptamer selection procedure, magnetic beads) are used in place of the target molecules.	[15]
	the restrictions that some targets are hard to immobilize and that RNA and DNA aptamers can be effectively chosen to target tiny molecules.	
Graphene oxide (GO)-SELEX	GO has been utilized to provide a unique immobilization-free screening technique in the separation process. Due to its ability to adsorb single-stranded oligonucleotides through π-π stacking, GO.	[34]
Fluorescence activated cell sorting (FACS-SELEX)	fluorescence-labeled oligonucleotide library incubated with a variety of cell types. Using the signal for calcein AM staining the cells at the start of the experiment, cells are separated by flow cytometer into dead and living cells. After that, the aptamers that are exclusively bound to the target cells are eluted, purified, and amplified. beneficial for aptamer selection against primary or suspension cells (as opposed to cell-SELEX), However, the workflow and instrumental setup are tough.	[35]
Photo-SELEX	Tymidine (T) from regular DNA is typically replaced with 5-bromo-2′-deoxyuridine in photoaptamer libraries. This allows the aptamers to crosslink to specific protein analyte sites, especially tyrosine residues, when exposed to UV light. This has made it possible to use a modified selection procedure whereby oligonucleotides that do not crosslink with the protein are removed from the pool and those that do so are amplified in a manner akin to the conventional SELEX approach.	[36]
Atomic force microscopy (AFM)	Creates a three-dimensional image of the sample surface using AFM. The adhesion or affinity force between a sample surface and a cantilever can be dynamically detected using AFM. This characteristic is helpful in selecting aptamers that bind to their targets quickly and with a high affinity just three to four rounds are needed for this process. however, expensive equipment is needed. Target and aptamers must be immobilized.	[37]
High-Throughput Sequencing SELEX (HTS-SELEX)	Through an iterative process of Darwinian selection, aptamers are found by starting with a random pool of billions of nucleotides. The primary and most important feature of HTS-SELEX is that it enables library sequencing during each selection phase. As a result, it takes less time to see enriched sequences at a much earlier stage. Additionally, fewer rounds of selection prevent any potential PCR bias brought on by over-selection.	[38]
Staggered Target SELEX Staggered Target SELEX (ST-SELEX)	This technique uses ambiguous targets in a periodic schedule and This method require preliminary studies such as in silico analysis or experimental data to find specific sequences. The results obtained from this technique confirm that this is able to isolate non-cross-reactive aptamers against two homologous proteins. However, time-consuming and labor-intensive some disadvantages this method.	[19]

on the conformational changes that aptamers experience upon ligand interaction. In the absence of targets, aptamers often combine with fluorescent dyes to provide a powerful signal. However, when targets bind with aptamers, changes in spatial structures are induced, which lowers the fluorescent signal and can be used to identify or measure the concentration of the target molecules [54]. The fluorescence signal is reduced and may be used to determine or quantify the quantity of the target molecules when targets interact with aptamers, which induces changes in spatial structures.

3.3. The nucleotide's makeup

DNA or RNA aptamers bind ligands by non-covalent acting forces, such as hydrogen bonding, van der Waals forces, electrostatic contact, and hydrophobic interaction, between target molecules and nucleotides [55,56]. Therefore, the interaction between aptamers and target molecules may be significantly impacted by the makeup of the nucleotide. The nature and intensity of the aptamer-target interaction are directly determined by certain nucleotides present at the critical binding sites. For instance, stable thymine-Hg^{2+}-thymine base pairs can be formed when thymine bases interact with mercury ions (Hg^{2+}). These base pairs have been utilized for Hg^{2+} detection in a range of sensor platforms [57,58]. Furthermore, although not directly in contact with the target molecules, specific nucleotides contribute considerably to aptamer-ligand interactions, determining aptamer performance. For instance, aptamers with a high G base content can fold into G-quadruplex structures, which improve the immobilization of target molecules [59]. Therefore, modifying or adjusting significant nucleotides can also improve an aptamer's efficacy.

4. Limitations of unmodified aptamers

Despite the many benefits of nucleic acid aptamers, their inherent physicochemical characteristics can result in inappropriate pharmacokinetic curves due to rapidly occurring renal clearance, instability to nuclease degradation, rapid distribution from plasma to particular tissues (the spleen or liver), polyanion effects, and non-specific immune response [60]. These disadvantages may lessen the medicinal potencies in vivo. Significant changes to aptamer chains and conjugations with functional molecules are necessary to make aptamer-based therapies more realistic.

4.1. Renal filtration and renal clearance

Aptamers with masses of 6–30 kDa typically have a diameter of less than 5 nm [61]. Renal filtration removes unformulated small aptamers with stabilizing backbone modifications quickly from the circulation. Therefore, the bulky moiety change prolongs the circulatory half-life and helps prevent renal filtration. Recently, several techniques have been described to avoid the renal clearance procedure by attaching a big moiety, including proteins [62], liposomes [63], polyethylene glycol (PEG) [64], cholesterol [65] and organic or inorganic nanomaterials [66] at the 3′/5′-end of aptamers. Among these strategies, PEGylation [67] is the most traditional tactics. The United States Food and Drug Administration (FDA) has cleared PEG, a synthetic homopolymer, for use in injectable medications and drug compounds can be conjugated with PEG to increase their half-life. Additional tactics are employed to enhance the pharmacokinetic and metabolic stability of therapeutic nucleic acid aptamers. These tactics include conjugating the aptamers with bioactive natural protein [68] or lipid nanoparticles delivery system [69]. On the other hand, it has also been observed that low molecular weight coupling agents (LMWCAs) and long-half-life proteins increase the half-life of aptamers in circulation. Nevertheless, all of these modifications significantly raise the cost of manufacturing of the aptamers.

4.2. Toxicity of aptamers

Toxicity is another important factor that affects how aptamer-based medications evolve clinically. Unspecific immunological reactions or chemical toxicities can result from the usage of synthetic nucleotides. Nucleic acids modified with Locked nucleic acid (LNA) were reported to exhibit extreme hepatotoxicity [70], and 2′-F-modified RNAs had an impact on pattern recognition receptor activation [71]. As a result, adjustments should be applied carefully, and the aptamers' real application environment should be taken into account. Another potential source of unwanted reactions that need to be taken seriously is the composition of therapeutic aptamers. Serious immunological responses have been documented in the phase III clinical of aptamer-based treatment caused by PEG moieties. Adoption of high-molecular mass PEG moieties in aptamer modifications clearly raises safety issues [72]. Therefore, in order to create long-acting, effective therapeutic aptamers, it is still preferable to look for a low molecular-weight coupling agent that does not cause allergic reactions.

4.3. Sensitivity to nucleases

Apart from excretion through renal filtration owing to the intrinsic characteristics of oligonucleotides, aptamers are rapidly degraded by ubiquitous nuclease [73]. Unmodified nucleic acids typically have half-life as brief up to 16 h [74].In vivo, it was demonstrated that an anti-thrombin DNA aptamer had a half-life as brief [75], whereas an RNA aptamer against keratinocyte growth factor was quickly degraded in 90 % human serum [76]. Consequently, many techniques have been devised to prevent nucleases from degrading aptamers. As discussed in Section-5 "Current approaches for Aptamer modifications" this issue might be solved by adding changed nucleotides to the aptamer sequence.

5. Current approaches for aptamer modifications

Various approaches have been developed to optimize the performance of aptamers. In the following, we summarize some different strategies (Figurer 2).

5.1. Truncation strategy

Aptamer truncation is an effective technique that reduces the cost of synthesis and increases the use of aptamers by eliminating unnecessary regions from ssDNA sequences [77–80].Additionally, truncation becomes crucial if assembling a multivalent aptamer is required. For this reason, several attempts have been made to shorten the aptamers' sequences, including deleting the primer region [81], For example, fixed sequences make up around 50 % of the entire length of a ssDNA library, and are employed as primer binding areas in PCR. However, it has occasionally been demonstrated that these random sequences are detrimental to the overall architectures of aptamers and can therefore be truncated analyzing the secondary structure of the aptamers' sequences and binding sites prediction [82] and the conserved sequences [83]. However, these approaches still suffer from blindness or inefficiency. Through the use of molecular docking techniques, targeted and efficient methods for truncating aptamer sequences have been developed [84].

5.2. Splitting strategy

Similar to split protein systems, which require accurate fragmentation of the protein domain [85], recently, split aptamers which consist of two or more short nucleic acid strands have been developed to create systems that are less complex to assemble and have less negative charges per strand [86]. According to this theory, a designated aptamer is separated into two or more distinct, non-functional pieces that can preferentially combine when the target is present. The ligand's robust

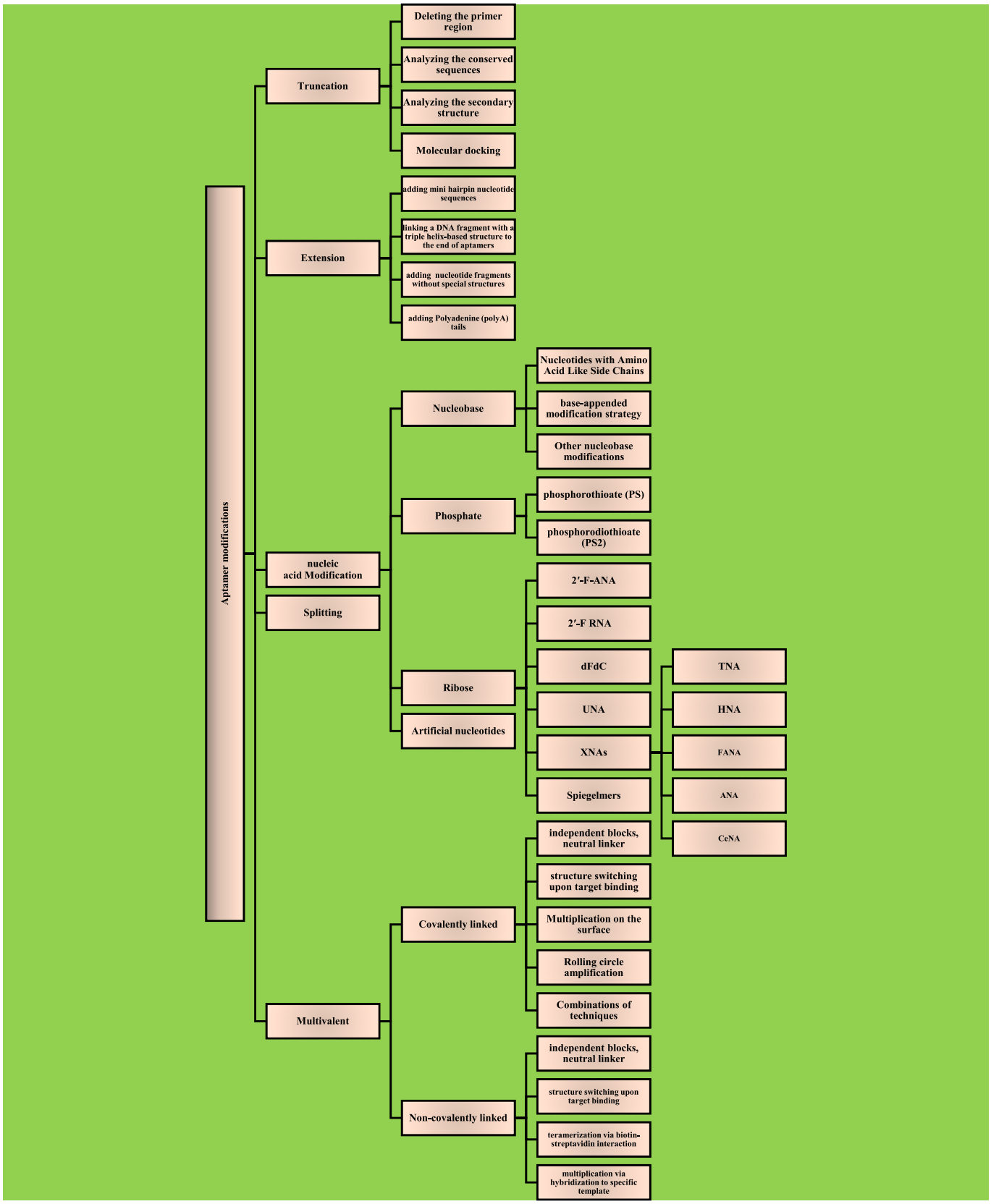

Fig. 2. Schematic summary of approaches aptamer Modification.

interactions with the aptamer-based structure alter the thermodynamic equilibrium and facilitate the fragments' assembly (Fig. 3). One way to further improve the stability of the formed structure is to add functions to particular strands that, when folded, would cause the fragments to covalently bond. Assembly places the two reactive functions near together, enabling the creation of an extra natural or modified internucleosidic bond following chemical activation [87]. This sophisticated strategy has been widely used to detect a wide range of targets, particularly small molecular targets, utilizing a variety of transduction techniques, such as colorimetric, fluorescence, and electrochemical approaches [88,89]. Only a small number of the more than 100 small molecule-binding aptamers that have been found so far have been effectively divided [90,91]. Because of the parent aptamer's required 3-dimensional (3D) structure, which can be severely disrupted if the division occurs at the incorrect location, such as in a hairpin binding pocket, split aptamers are challenging to design. Aptamers are simpler to split if the target molecule binds in the stem region. Regarding aptamers based on three-way junctions, it is simple to split their arms into brief segments while maintaining their contact with the target [92].

5.3. Extension strategy

Extending aptamer sequences has the potential to increase the range of aptamer-based applications by improving other features including nuclease resistance and thermal stability, in addition to optimizing affinity and specification and reducing sequence length. A wide variety of nucleotide fragments can combine to create unique structures that can improve the use and functionality of aptamers. For instance, the heat and nuclease stability can be improved by using micro hairpin nucleotide sequences [93]. According to a different study, an aptamer's skeleton that is too flexible will harm its folding and reduce its functionality [94]. Aptamer conformation was fixed and skeleton flexibility was considerably decreased by attaching a DNA fragment with a triple helix shape to the end of the aptamer. This resulted about ten-fold increase the binding affinity of the aptamers. Additionally, nucleotide fragments with no distinctive structures may be added to aptamers for a range of applications. For instance, the steric hindrance brought on by modified groups or other chemicals indicated on aptamers has been lessened by the use of numerous thymine nucleotides (T(n)) as a spacer arm [47]. Physical adsorption allows polyadenine (polyA) tails to physically bond with metal nanoparticles including AuNPs, AgNPs, and CuNPs [95–97]. In order to create AuNP-based aptasensors, some research connected polyadenine (polyA) tails to aptamers [98].

5.4. Bivalency or multivalency strategy

Multivalent aptamer strategies often seek to increase binding avidities toward their target ligands. Multivalent aptamers have been demonstrated to cooperate to enhance the avidity of interactions by connecting two or more aptamer units. Avidity is a term used to characterize the overall affinity of an aptamer's interactions with its target [99,100]. A greater likelihood of interaction between aptamers and cell-surface ligands is associated with higher local aptamer concentrations. Furthermore, adjacent aptamers can be easily recruited to promote further binding after an aptamer and its ligand have first bound, increasing avidity. Moreover, aptamers exhibit challenges under physiological conditions; in their monomeric state, they have been shown to be rapidly degraded by nuclease, a noteworthy drawback that limits their application in vivo environments [101]. However, there is no direct relationship between the amount of monomers and the activity of multivalent aptamers. Because of the large molecular weight and steric hindrance, the activity against the target may not increase with the amount of monomers [75]. Additionally, a rise in molecular weight will drastically reduce the diffusion rate, which is likely to result in less activity [102]. For this reason, the binding strength of the multivalent aptamer can significantly outperform that of its monovalent counterpart when the linker type, length, and flexibility are selected appropriately [103,104]. Furthermore, heterovalent aptamer techniques, often known as aptamer cocktails, combine two or more aptamer types to target several target moieties. Heterovalent aptamer approaches have been demonstrated to cooperatively boost overall aptamer performances when compared to single type aptamer approaches. For instance, an aptamer cocktail-modified electrode (three separate aptamers targeting various moieties on the surface of *E. coli* cells) was found to provide an 18-fold improvement in limit of detection (LOD) over an electrode modified with a single-type aptamer [105].

5.5. Nucleic acid modification strategy

Two common examples of modification strategies are genetic alphabet expansion and nucleobase alterations. Aptamers are intended to operate better and interact with more targets by the inclusion of these new chemical moieties or bases. Aptamers' three-dimensional structure is instantly impacted by these modifications [106]. The following are some of the most prevalent nucleic acid aptamer modifications:

5.5.1. Ribose modifications

5.5.1.1. 2′-Fluoro-RNA oligonucleotides (2′-F-RNA). The binding affinity may be increased by ribose portion of the nucleic acid modifications, such as 2′-fluoro (2′-F), 2′-fluoro Arabino, 2′,2′-difluoro-2′-deoxycytidine (Fig. 4). Unmodified aptamers frequently have a poor affinity for their targets; however, 2′-fluoro (2′-F) optimized aptamers have a better binding affinity and resistance to nucleases [38,107]. (Fig. 4A).

5.5.1.2. 2′-Fluoro Arabino nucleic acid (2′-F-ANA). The 2′-deoxy-2′-fluoro-arabinonucleic acid (FANA) (Fig. 4B) often contain a fluorine group in the β conformation rather than the α conformation, and its sugar ring generally assumes a C2′/O4′-endo conformation as opposed to the C3′-endo conformation of 2′-fluorinated ribonucleotides [108,109].

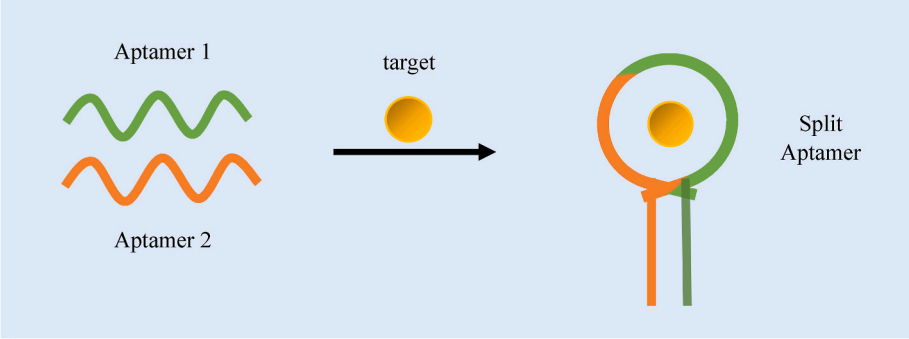

Fig. 3. General scheme of the split aptamer concept.

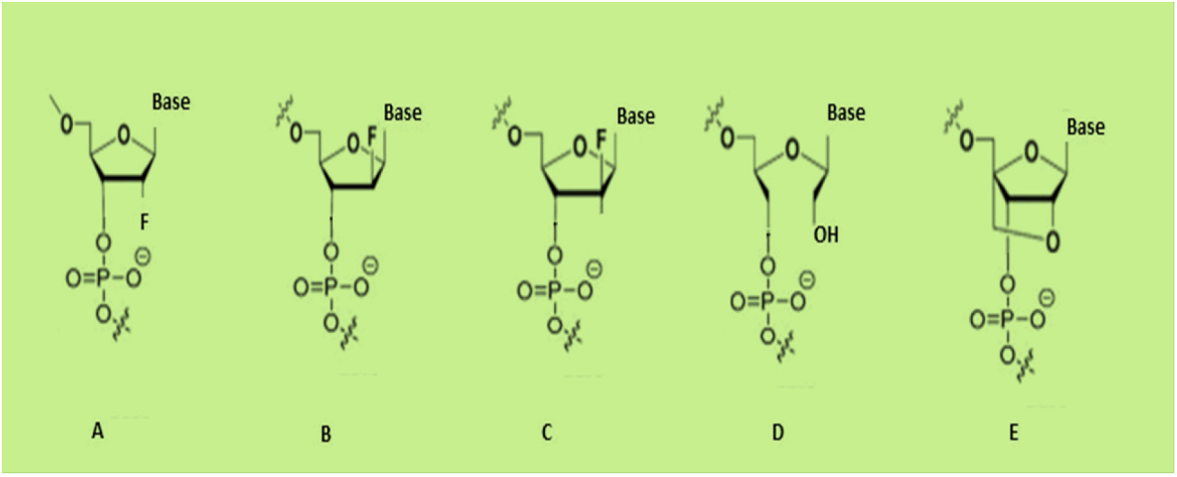

Fig. 4. Ribose Modifications: A 2′-fluoro (2′-F) modification 2′-Fluoro Arabino nucleic acid modification; C 2′, 2′-difluoro-2′ deoxycytidine modification; D Unlocked Nucleic Acid modification; E Locked Nucleic Acid modification.

Recently, using SELEX, it was possible to identify (2′-F-ANA) modified aptamers that have affinity for the HIV human integrase enzyme. This enzyme plays an essential role in the virus's infection cycle because it integrates viral DNA into the host's genome [110]. In comparison to previously published unmodified DNA and RNA aptamers, the two (2′-F-ANA) modified aptamers developed after the selection procedure have dissociation constants in the 50–100 pM range, which is more than two orders of magnitude stronger. The binding affinity was significantly decreased when the (2′-F-ANA) nucleotides were replaced with their fluorinated RNA counterparts. This finding suggests that the nucleotides are causing the aptamer to adopt a unique conformation, which is essential for high affinity binding to the integrase enzyme [111]. Additionally, the F-ANA modified thrombin aptamer (TBA) demonstrated much greater affinity and nuclease resistance than the untreated TBA [112,113].

5.5.1.3. 2′,2′-Difluorocytidine (dFdC, gemcitabine). Originally employed for its antiviral Gemcitabine [2′,2′-difluoro-2′-deoxycytidine (dFdC)] (Fig. 4C) contains a fluorine substituent in the pentose ring and involves the most significant deoxycytidine analogs [114,115] Gemcitabine [2′, 2′-difluoro-2′-deoxycytidine (dFdC)] contains a fluorine substituent in the pentose ring and involves the most significant deoxycytidine analogs [116]. dFdC has been approved as a prodrug and pyrimidine antimetabolite for the treatment of non-small cell lung cancer, pancreatic cancer, bladder cancer, ovarian cancer, colon cancer, and breast cancer [117]. Concentrated nucleoside transporters (hCNT3, hCNT2, and hCNT) and Nucleoside transporters (hENT2, hENT1) may be involved in the internalization of gemcitabine into cells [118]. Prior research has demonstrated that the G-quadruplex's loop region is essential for nucleolar protein binding. Therefore, chemical alteration in the loop region may alter the modified aptamer's capacity to attach to proteins [119]. An AS1411 (APTA-12) augmented with gemcitabine, for instance, has been built [120]. The data on binding affinity show that the alteration of gemcitabine (KD = 14.37) may somewhat improve the modified AS1411's ability to bind to nucleolin (KD = 16.36).

5.5.1.4. Unlocked nucleic acid (UNA). In order to prevent nucleases from degrading modified oligonucleotides in cells, a structurally stiff change known as unlocked nucleic acid (UNA) (Fig. 4D) that eliminates the connection between the sugar ring's C2 and C3 positions makes aptamers more flexible [121]. UNA's flexibility could help relieve tension in constructions with tight loops. Thermodynamic stability is affected by UNA alterations on the loop sections of a 15-mer thrombin-targeted DNA aptamer. Nevertheless, alterations in the G-quartet structures are detrimental to the creation of quadruplexes [122]. It has also been shown that UNA can be positioned in a variety of ways without compromising the aptamer's ability to bind thrombin or function as an anticoagulant [122].

5.5.1.5. Locked nucleic acid (LNA). The conformational structure of locked nucleic acid (LNA), a nucleic acid analog, is "locked" by a methylene bridge connecting to the 2′-oxygen and 4′-carbon atoms. The redesigned aptamers had improved nuclease resistance, stable base pairings, and increased heat stability thanks to the fixed C3' internal conformation of LNA (Fig. 4E) [123]. The most promising modified nucleotide, LNA, is widely used in aptamers, siRNAs, and antisense oligonucleotides. Numerous studies have been conducted on its binding affinity and nuclease resistance properties [124]. The G-quadruplex-forming thrombin aptamer was the subject of the first investigation using LNA-modified aptamers [125]. LNA was implanted in a variety of sites to determine how it affects biological activity. The aptamer's thermal stability was always improved by the addition of the LNA moiety, but the binding affinity was not improved. LNA aptamers exhibited a lower or similar biological affinity to the unmodified aptamer, depending on the location. Since this study, several aptamers have been changed with LNA, including the (strept) avidin binding aptamer [124,126], the Sgc8 aptamer [127] and the TD05 aptamer [128], with consistent outcomes in each case. Generally speaking, the location and quantity of mutations on an LNA determine the structural alterations and biological impacts. If one is just concerned with binding affinity, alterations in regions critical for target binding are rarely tolerated since they typically cause binding to diminish or cease completely. Furthermore, aptamers that undergo significant structural alterations during target binding frequently have a reduced threshold for LNA mutations. As a result, adding LNA changes to aptamers is a challenging procedure that takes patience, experimentation, and fine-tuning. But when done properly, these aptamers' characteristics can be superior to those of conventional aptamers in terms of binding affinity, nuclease resistance, and thermal and structural stability [111].

5.5.1.6. Xenogenic nucleic acids (XNAs). Beyond the initial 2′-alterations brought to the ribose unit, as discussed above, sugar modifications can confer nuclease resistance to oligonucleotides. However, of greater significance, these modifications can result in orthogonal artificial nucleic structures, XNAs or Xeno-Nucleic Acids, which can be used for therapeutic and targeting applications [129,130]. This strategy might work with sugar scaffold alterations involving 2′-deoxy-2′-fluoro-arabinonucleic acid (FANA), arabinonucleic acid (ANA), Hexitol

nucleic acid (HNA), Threose nucleic acid (TNA), and Locked nucleic acid (LNA) chemistries)Table 2([131]. The first time, adding a change in which a sulfur atom took the place of the sugar unit's 4′-oxygen atom (Fig. 5A) [132]. 4′-Thioribonucleoside was shown to help increase the modified aptamer's stability without causing any loss [133]. Kato et al. synthesized 4′-thiouridine (4′-thioUTP) and 4′-thiacytidine (4′-TTP) triphosphate using 4′-thioribose. These compounds were subsequently utilized for the in vitro screening of anti-thrombin thioRNA aptamers [132]. In comparison to wild-type RNA, the resultant 4′-thio-modified aptamer exhibited a 50-fold improvement in resistance to RNase A and bound the thrombin target with great affinity. Furthermore, 4′-thioRNA aptamers were chosen by maximizing the concentration of nucleoside triphosphates (NTPs), and the most effective aptamers produced by this selection process exhibited high affinity binding to thrombin [134].

5.5.1.6.1. Hexitol nucleic acid (HNA). The backbone of 1′,5′-anhydrohexitol is the basic structural feature of HNA. It is the source of HNA's versatility, as it can form stable duplexes with DNA, RNA, and HNA, as well as alternate base pairs, such as HNA-A/HNA-A [139]. Three structurally distinct 2′-O-methyl-ribose-1, 5-anhydrohexitol nucleic acid (MeORNA-HNA) aptamers (Fig. 5B) that target the rat VEGF164 target protein were effectively screened by the researchers among the other XNA aptamers [137]. Rat VEGF164 could be bound by the HNA/2′-OMe modified aptamer with a low nanomolar affinity (KD = 1.1 nM). Furthermore, utilizing customized polymerase variations, Arangundy Franklin et al. report the synthesis and reverse transcription of uncharged P-alkyl phosphonate nucleic acids (phNA) under complete substructure (Fig. 4D) [138]. The outcomes of the experiment demonstrated that the low millimolar range was where the affinities of phNA aptamers T1-20 and T5-20 to streptavidin (SA) occurred.

5.5.1.6.2. Threose nucleic acid (TNA). The RNA of threose nucleic acid (TNA) (Fig. 5C) is a kind of XNA that replaces natural ribose with synthesized threose. It is a potent candidate system for the development of therapeutic and diagnostic medicines due to its high binding affinity, resistance to nuclease digestion and thermal stability [137,135,136].

5.5.1.7. Spiegelmers or mirror image aptamers. Another workable tactic is to add artificial nucleotides to the oligonucleotide chain in order to increase the stability of the aptamers [140]. When non-natural nucleotides are added to aptamers, they become less vulnerable to hydrolysis by nucleases. Because the nucleases are often chiral, they can only detect the substrate in a certain stereotypical manner. D-nucleotides are used to make natural nucleic acids, but L-nucleotide oligonucleotides are resistant to identification and destruction by common enzymes. "Spiegelmers" are target-recognizing L oligonucleotides, which are the mirror image of natural oligonucleotides (Fig. 6) [1]. The requirement for the enzymes to identify the L-nucleotides was crucial in the development of Spiegelmer, as the enzymes were required for the PCR or sequencing process. The technology for the selection of L-aptamers using mirror-image DNA polymerase was created by Zhu's group [141]. They chose L-aptamers with the potential to inhibit and detect thrombin in environments rich in nucleases. Additionally, a large number of L-aptamers with high binding affinities were produced in these years [142]. Other spieglemers, such as and NOX-A12 (anti-CXCL12) [143], NOX-H94 (anti-hepcidin) [64] and NOX-E36 (anti- CCL2) [144], are now being investigated for medicinal purposes. In phase 1 investigations, all of these candidates have shown good safety in healthy volunteers. Furthermore, in phase 2 investigations, NOX-H94 and NOX-A12(bind stromal-cell-derived factor-1) have both shown intriguing efficacy. Additional clinical trials for assessments are presently underway, and several more spiegelmers are planned.

Table 2
Xenogenic nucleic acids (XNAs).

Modification	Target	Result KD	Reference
Threose nucleic acid (TNA)	S1 protein in SARS-CoV-2	KD values of 3.1 ± 1.0	[135]
Threose nucleic acid (TNA)	HIV reverse transcriptase (HIV RT)	KD = 0.4–4.0 nM	[136]
2′-O-methyl-ribose-1,5 anhydrohexitol nucleic acid (MeORNA-HNA)	VEGF164 target protein	KD = 1.1 nM	[137]
P-alkyl phosphonate nucleic acids (phNA)	streptavidin (SA)	affinities in the low millimolar range.	[138]

5.5.2. Nucleobases modifications

To improve the interaction between the protein and aptamer, nucleobases can have a wide variety of functional groups added to them. This modification will be discussed in the following.

5.5.2.1. Nucleotides with amino acid like side chains. Nucleobases with side chains that resemble amino acids may provide modified aptamers additional benefits due to the intrinsic characteristics of both proteins and nucleic acids. This is because the side chains of amino acids can actively engage in interactions with proteins. For the first time, the conformational flexibility of nucleic acids and the functional variety of proteins were combined at the C5 position of deoxyuridine triphosphate (dUTP) with some hydrophobic group, benzyl-(BndU), naphthyl-NapdU), tryptamino-(TrpdU) or isobutyl derivative (iBudU). This significantly increased the binding affinity of modified aptamers to proteins [145]. Additional hydrophobic interactions with hydrophobic protein pockets can be formed by dUTP modification at C5. These aptamers, which were named SOMAmers (Slow O-rate Modified Aptamers) due to the strict selection criteria utilized in their isolation, embodied slow off-rate to proteins. In fact, from the original DNA library, only high affinity aptamers with slow dissociation rates (t1/2 > 30min) were chosen. Furthermore, developing "next-generation" SOMAmers which are distinguished by changes at the C5 position of two pyrimidine bases deoxycytidine triphosphate (dCTP) and deoxyuridine triphosphate (dUTP) and elaborating on this idea [146,147].

Günter Mayer et al. created a triazole linker via click chemistry between alkynemodified uridine (5-ethynyl-deoxyuridine (EdU)) and hydrophobic group azide rather than thymidine as in the SOMAmers, which employed an amido linker between the hydrophobic group and dU (Fig. 7A). By utilizing click chemistry's high efficiency and EdU's compatibility with DNA polymerase in the SELEX technique, they were able to screen an indole-modified aptamer against cycle 3 GFP with a KD value of 18.4 nM [148,149]. In addition to hydrophobic alterations, hydrophilic adjustments could help ion-ion interactions and the formation of new hydrogen bonds. As an illustration, consider the amido linkages that conjugate the histamine to C5 of the thymine base in the aptamer [150]. The 14mer double helix's core was occupied by a single modified thymine at four different locations, from which the interaction between imidazole and the double chain and its impact on imidazole pKa were examined. Unrestricted molecular dynamics and nuclear magnetic resonance are used to construct a structural motif that involves the establishment of hydrogen bonds between imidazole and the Hoogsteen side of two adjacent GC base pairs. It has been demonstrated that these bonds considerably improve the DNA Double strands' stability at temperature. Base changes have been shown to support synthetic DNA duplexes' heat stability [151]. It was demonstrated by unconstrained molecular dynamics (MD) modeling that imidazole and the adjacent guanosine might create attributable hydrogen bonds (Some studies are listed Table 3).

5.5.2.2. Base-appended method of modification. Base-appended bases, a different modified technique (Fig. 7B), where in, after multiple selection cycles, a high affinity aptamer could be identified using a modified SELEX (Table 3) [158].

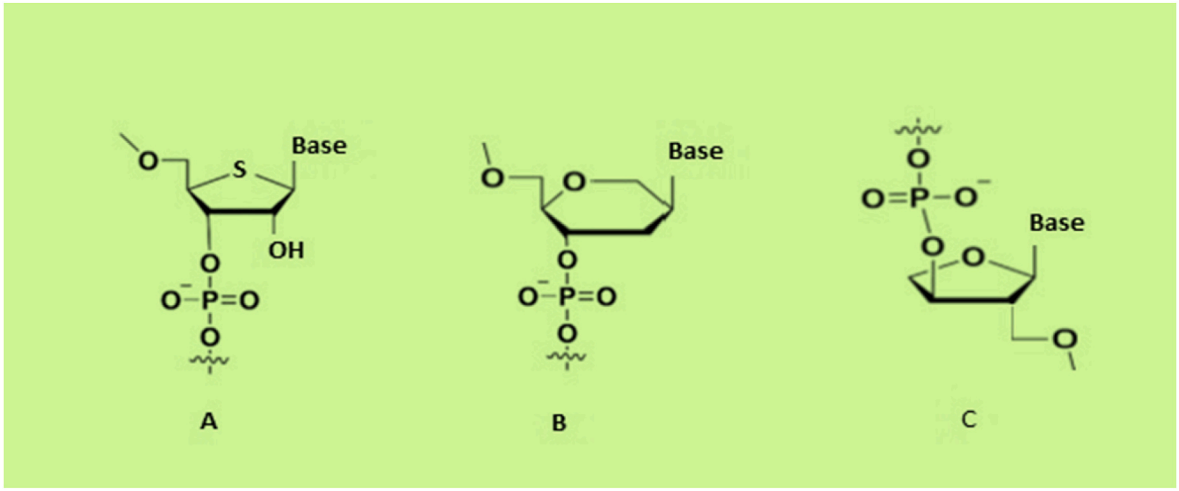

Fig. 5. The ribose modifications. A: 4′-S modification; B: HNA modification; C: TNA modification.

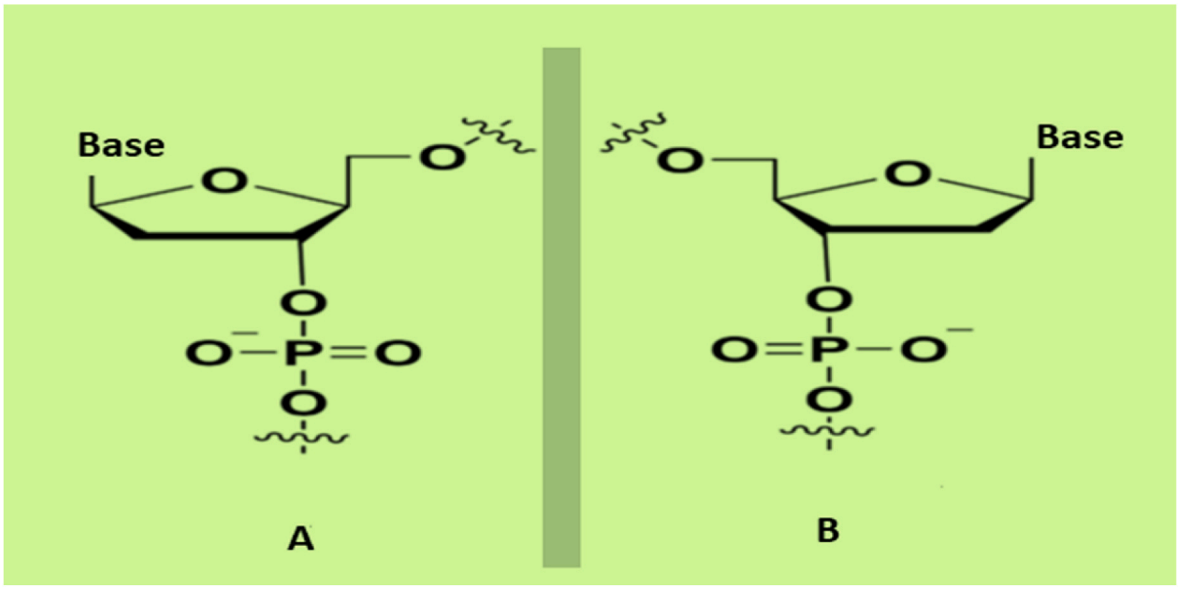

Fig. 6. Spiegelmers. A: L-configuration; B: D-configuration.

5.5.2.3. Other nucleobase modifications. The other study used post-SELEX modification to add amino acids, like Leu, to the nucleobase of TBA [159]. They provided evidence that these adjustments might raise the anticoagulant's potency and improve binding affinity. The crystal data showed that the target proteins and modified aptamers have more interaction regions thanks to changed bases) Table 3) [106].

5.5.3. Phosphate linkage modifications

A valuable tactic for increasing affinity is to modify the phosphate portion of the aptamer; this alteration can also aid in the aptamer's resistance to nuclease in vivo (Table 4) [160]. Phosphate linkage modifications can also be introduced into aptamers for stabilizing the chains of nucleic acids By substituting sulfur-containing phosphate ester bonds [161–163], for traditional phosphate (PO) backbones, such as phosphorothioate (PS) bonds and more recently phosphorodiothioate (PS2) bonds (Fig. 8) [164,165]. Phosphate linkage modifications can also be added to aptamers to stabilize the chains of nucleic acids. Chemical modification of oligonucleotide (ODN) dithiophosphates involves substituting two sulfur atoms for the non-bridging oxygen atoms at the phosphate moiety [166]. This modification technique was employed to increase nucleic acid drug binding affinity and prevent nuclease degradation. It should be mentioned that, like in natural DNA, the substitution of sulfur atoms (PS2) for two nonbridging oxygen atoms in a single phosphate ester unit is achiral at phosphorus. On the other hand, chiral substitution (Sp or Rp configuration) occurs in PS links, which may have a negative impact on biological function [1].

5.5.4. Artificial nucleotides or unnatural nucleotides

Extending the genetic code with uncommon base pairs is another way to create customized nucleic acid libraries that are more varied and valuable. Thus far, various artificial base pairs have been created for various objectives [170] and over time, in PCR amplification and sequencing projects, these building blocks have been refined for high fidelity inclusion by polymerases [171]. A version of the standard SELEX that employs an artificial nucleotide-modified random library of sequences was introduced through the use of genetic alphabet expansion SELEX. Predetermined 1–3 highly hydrophobic unnatural bases Ds (7-(2-thienyl) imidazo [4, 5-b] pyridine) were added in all of the SELEX library's oligonucleotides (Fig. 9). These bases may improve the hydrophobic interactions between the aptamer and target proteins [172].

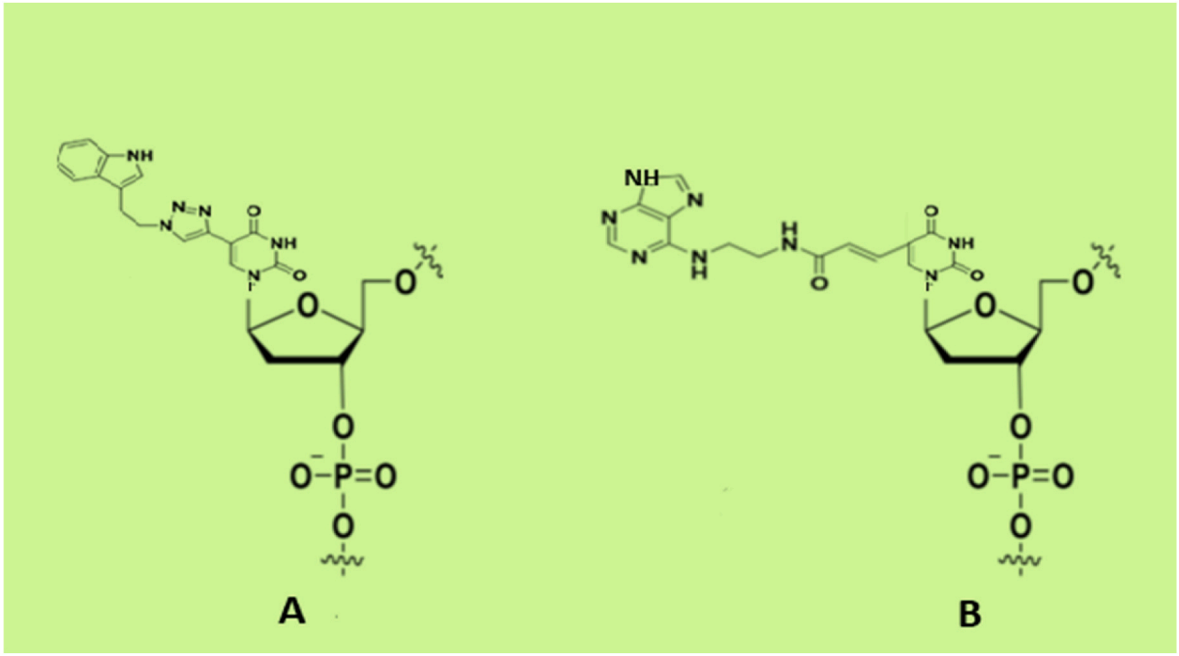

Fig. 7. A. Modified uridine by click chemistry; B. Base-appended bases modification.

Table −3
Some studies by Nucleobases modifications.

	Aptamer	target	Affinity (Kd)	Reference
Base modification	AMYm1 aptamer	Salivary α-amylase (sAA)	<1 nM	[152]
	Aad1 aptamer (Adenine-appended modification strategy)	human β-defensin 2	6.8 nM.	[153]
	SARS-CoV-2 aptamer	receptor-binding domain (RBD) and	1.2 nM	[153]
	SARS-CoV-2 aptamer	spike trimer of the SARS-CoV-2	1 nM	[153]
Nucleotides with Amino Acid Like Side Chains	modified thrombin aptamers (TBA)	antithrombin activity	2.94 nM	[154]
	indole modified aptamer	cycle 3 GFP	18.4 nM	[155]
Other nucleobase modifications	cubanemodified aptamers (cubamers)	Plasmodium vivax lactate dehydrogenase (PvLDH)	670 nM	[156]
	added triphosphate analogs modified by 5-chlorouracil and 7-deazaadenine bases	β-secretase 1 (BACE1- the therapeutic target of Alzheimer's disease	10 nM	[157]

Table-4
Some studies by Phosphate linkage modifications.

	aptamer	modifications	result	Reference
3Phosphate linkage modifications	adipocyte-specific aptamer (Adipo8)	PS	higher binding affinity high specificity in vitro.	[167]
3Phosphate linkage modifications	AP613-1	PS	Improving binding affinity of APS613-1 was improved to 15.48 nM	[168]
3Phosphate linkage modifications	*E. coli* single stranded DNA binding protein (SSB)	PS2-ODN	binding affinity	[169]

1–3 dDs modified aptamers were chosen to target the vascular endothelial growth factor VEGF-165 [173], the interferon-cytokine (IFN-γ) [173] and the VonWillebrand Factor (VWF) [172]. The former two demonstrated a binding affinity (pM range) that was more than 100 times higher than that of the aptamer sequences that contained only natural nucleobases. The suitability of a few chosen dDs-modified aptamers for use in Enzyme-Linked Oligonucleotide Assay) ELONA (tests was investigated [174]. A laboratory in vitro evolution system comprising A, C, G, T, Z and P was also built by Benner's group [175]. They produced protective antigen (PA) PA63, an aptamer binding PA with a dissociation constant of about 35 nM. Additionally, Benner and his colleagues discovered a number of aptamers, including unusual nucleotides targeting glypican 3 (GPC3), which was expressed on the surface of liver cells, by merging cell engineering technology and a laboratory in vitro evolution system [176]. The SELEX library's complexity could be raised by artificial nucleoside inclusion, which also made aptamers more similar to proteins and enabled them to bind target proteins with a high degree of affinity. The eight-letter Hachimoji DNA/RNA was recently reported, which could greatly advance the aptamer sector in the future [177].

6. Conclusion

Aptamers are versatile molecules used in drug delivery, bioimaging, biosensing, therapies, and diagnostics [178,179]. Aptamers face issues such rapid renal elimination and decreased in vivo stability. An important area of research to improve aptamer performance is chemical modification to boost aptamer resistance to nucleases and in vivo stability. Modification can be carried out through solid-phase chemical synthesis after the SELEX approach has been finished, or by adding functional groups to random libraries during the SELEX operation (selection process). When adding phosphorothioate units that are resistant to nuclease digestion and impede rapid renal filtration, the latter

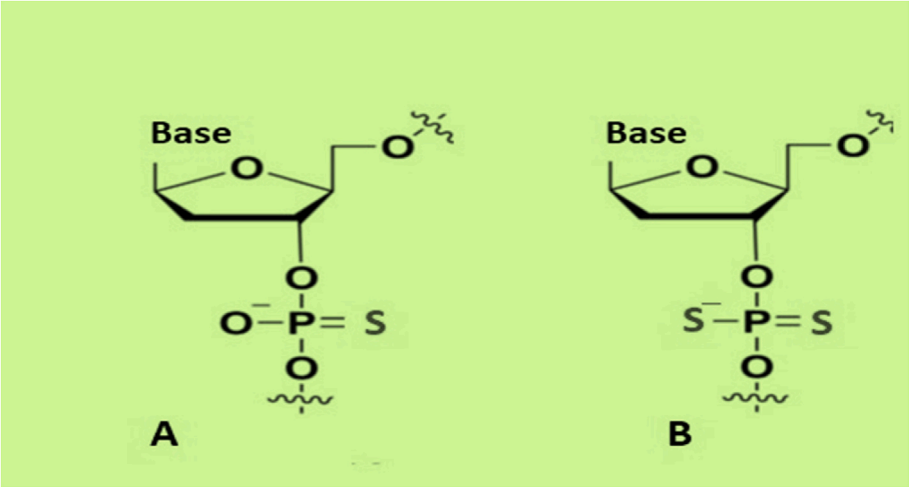

Fig. 8. A: Phosphorothioate (PS) modification; B: phosphorodithioate (PS2) modification.

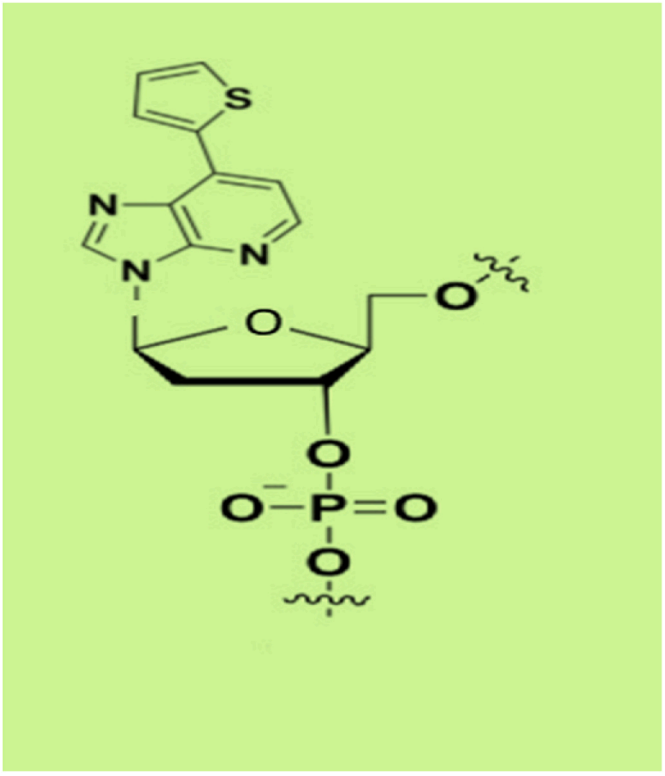

Fig. 9. The (7- (2-thienyl) imidazo [4, 5-b] pyridine) (Ds) artificial nucleotide.

method works particularly well. Due to the restricted half-life extension offered by PEG components, compliance may be decreased by the need for frequent subcutaneous injections. Therapeutic efficacy can be increased by creating aptamers that include a larger percentage of the active aptamer component and by using low molecular weight modification techniques. Despite recent advancements in modified aptamers, challenges remain to be addressed. Many possible target binders can be identified as aptamer candidates thanks to next-generation sequencing. But the processes for validating and characterizing improved aptamer-target binding, as well as the throughput of binding affinity and specificity tests, continue to be labor- and time-intensive. Aptamer offers an enormous variety of alteration sites and modification kinds. Aptamers that have been altered can identify several sorts of molecular targets. However, there is currently little use for them in vivo, and more research is needed. Aptamer research in both basic and applied biosciences has a strong foundation and a new paradigm thanks to recent notable advancements in the aforementioned major fields [179]. To enhance the production of high-quality aptamers and promote the usage of aptasensors, post-SELEX and pre-SELEX optimization may be expedited in the future by fully understanding the mechanism of aptamer-target interaction and extensively using diverse optimization strategies. However, it is not possible to carefully characterize every interaction between the changed aptamers and the target. Consequently, an efficient virtual prediction technique has to be developed to optimize the aptamer alteration types and modification locations for improved binding affinity. With great optimism, we hope that the above-mentioned challenges can be addressed by the improvement of the methodologies outlined or the development of novel approaches. Using advanced machine and deep learning models, it has recently become possible to anticipate the binding capabilities of ligands and targets in drug development. Therefore, these models could offer a trustworthy and precise way to forecast how aptamers and targets would bond. More could be done using machine/deep learning to predict aptamer binding. Since modified nucleic acid aptamers are still a relatively new field of study, there is much potential ahead for further investigation. We can now make significant scientific progress in the construction, characterization, and biological applications of modified aptamers owing to recent technological advances. We are really optimistic that we will be able to address the aforementioned concerns through the improvement of the techniques discussed or the development of other approaches.

CRediT authorship contribution statement

Arezoo Fallah: Writing – original draft. **Abbas Ali Imani Fooladi:** Writing – original draft. **Seyed Asghar Havaei:** Writing – original draft. **Mahdieh Mahboobi:** Writing – review & editing, Visualization, Conceptualization. **Hamid Sedighian:** Writing – review & editing, Supervision.

Ethics approval and consent to participate

Not applicable.

Funding

Not applicable.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We thank our colleagues from Applied Microbiology Research lab especially Dr. Mozhgan Kheirandish for comments that greatly improved the manuscript.

Data availability

No data was used for the research described in the article.

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