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Aptamer chemistry

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ABSTRACT

Aptamers are single-stranded DNA or RNA molecules capable of tightly binding to specific targets. These functional nucleic acids are obtained by an *in vitro* Darwinian evolution method coined SELEX (Systematic Evolution of Ligands by EXponential enrichment). Compared to their proteinaceous counterparts, aptamers offer a number of advantages including a low immunogenicity, a relative ease of large-scale synthesis at affordable costs with little or no batch-to-batch variation, physical stability, and facile chemical modification. These alluring properties have propelled aptamers into the forefront of numerous practical applications such as the development of therapeutic and diagnostic agents as well as the construction of biosensing platforms. However, commercial success of aptamers still proceeds at a weak pace. The main factors responsible for this delay are the susceptibility of aptamers to degradation by nucleases, their rapid renal filtration, suboptimal thermal stability, and the lack of functional group diversity. Here, we describe the different chemical methods available to mitigate these shortcomings. Particularly, we describe the chemical post-SELEX processing of aptamers to include functional groups as well as the inclusion of modified nucleoside triphosphates into the SELEX protocol. These methods will be illustrated with successful examples of chemically modified aptamers used as drug delivery systems, in therapeutic applications, and as biosensing devices.

1. Introduction

In most modern drug discovery approaches, chemocentric methods strive to develop small molecules that are capable of modulating the activity of biological targets such as enzymes (particularly kinases and proteases) and other proteins such as receptors or ion channels. Often guided by chemoinformatic tools [1], thousands of small organic molecules are synthesized, characterized, and subjected to a battery of *in vitro* and *in vivo* mechanism-of-action assays. This rather lengthy and uncertain approach -one in 10,000 compounds elicits a desired biological response- has led to the identification of numerous synthetic small molecules that have been commercialized as efficient drugs [2]. Nonetheless, alternative methods for the development of synthetic drugs and biological ligands are in dire need in order to reduce the cost associated with drug discovery and concomitantly improve the efficiency of the process. In this context, the advent of SELEX (Systematic Evolution of Ligands by EXponential enrichment) [3,4] and related combinatorial methods of *in vitro* selection impelled the identification of a novel class of biological ligands –aptamers- as well as catalysts —ribozymes and DNAzymes [5–11]. Among these functional nucleic acids, aptamers have the capacity of binding to specific targets with very high affinity and selectivity, and are therefore often considered as the DNA or RNA pendants of antibodies [12]. These inherent properties have propelled aptamers to the forefront of numerous applications which eventually culminated in the development of the first FDA-approved commercialized oligonucleotide-based drug (pegaptanib sodium; Macugen®) [13–16]. Despite their propensity at binding a broad variety of targets with high affinity and specificity, commercial success of aptamers still proceeds at a weak pace. This scarcity of commercial DNA and RNA aptamers might be ascribed to a number of factors including the strong domination of antibodies in therapeutics, biosensing, and point-of-care diagnostics in terms of industrial infrastructure, knowledge in their pharmacokinetic properties and formulation, and investment as well as early intellectual property right protection of the SELEX method (the first patent expired in 2011 [17]) [14]. Also, since natural nucleic acids are rather functionally-deprived biopolymers, their propensity at binding to targets -particularly to more demanding targets such as glycosylated proteins or single enantiomers of small organic compounds- is inherently inferior to that of their proteinaceous counterparts [18]. Moreover, aptamers are rather small-sized entities consisting of unmodified nucleic acid scaffolds which are prone to both a rapid renal filtration and an efficient nuclease digestion.

This review presents the chemical approaches available to improve the bespoken shortcomings of aptamers. First, the post-SELEX introduction of chemical modifications by standard automated solid-phase synthesis of oligonucleotides will be discussed. This approach is particularly suited to the synthesis of larger quantities of aptamers equipped with functional groups that confer an increased stability and nuclease

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resistance as well as for the development of drug delivery systems and nanomaterials [19,20]. However, introduction of chemical modifications into aptameric scaffolds after their isolation by SELEX is often a tedious and uncertain process that might cause an unwelcome decrease in binding affinity, especially in the absence of structural and mechanistic information. Indeed, the small structural alterations induced by the post-SELEX introduction of chemical modifications often lead to marked decreases in binding affinity of aptamers and in catalytic activity of DNAzymes and ribozymes [12,14,21]. In order to by-pass these difficulties, modified nucleoside triphosphates (dN*TPs) can be included directly into the SELEX protocol [22]. The usefulness and complementarity of both strategies will be highlighted with the description of successful examples. Particularly, recent chemically modified aptamers used as drug delivery systems and biosensors will be covered.

2. Overview of aptamers

2.1. Production and properties of aptamers

Aptamers are rather short (20–100 nucleotide long) nucleic acid sequences that are obtained by application of the SELEX protocol and other related combinatorial methods of *in vitro* selection (Fig. 1) [3,4,23]. Since the advent of SELEX the original method has evolved and improved in terms of cost optimization, efficiency, and time required to generate potent aptamers. A comprehensive description of the most established methods has recently been reviewed [24]. However, the main principles of the SELEX process remain the same throughout most of these new selection protocols. Briefly, large libraries of oligonucleotides (typically around 10^{14} molecules) are created by primer extension reactions (PEX) or PCR. The design and the quality of the initial libraries are key components for successful SELEX experiments since only pools with equal distribution motifs of all four nucleotides maximize the sequence space to be explored [25]. The choice of the length of the random region is still dictated by empirical rules and limited by the amount of molecules in the starting pool (0.2–1 nmol; ~10^{14} molecules) [26]. Short randomized regions (N20) will ensure complete coverage of the sequence space (*i.e.* more than one molecule of each sequence will be represented in the pool) during the selection experiment albeit at the expense of a reduction of possible structural motifs. On the other hand, larger randomized sections (N40) provide a higher complexity of possible three-dimensional structures and folds but only a small fraction of the sequence space will be covered during selection experiments [27]. As a result, most selection experiments are carried out with randomized regions comprised between 20 and 60 nucleotides, even though aptamers sequences as short as 8 nucleotides [28] and as long as 228 nucleotides have been isolated [29].

The random libraries are either incubated with targets immobilized on a solid support to ensure physical separation from unbound molecules or directly with cells in the case of cell-SELEX [30]. The species capable of binding to the target are then retained, PCR-amplified, and the resulting enriched populations are then injected into subsequent rounds of selection. The selection pressure or stringency can be increased through iterative cycles by gradually modulating physicochemical parameters such as the temperature, the ionic strength, the concentration of the target, the incubation time, or by adding a negative selection step [31]. When no further enrichment is detectable (which typically occurs after 5–15 cycles), the surviving binders are cloned and sequenced and the different species characterized. The binding capacity of aptamers is usually best described by their equilibrium dissociation constants (K_d) which often reside in the low nanomolar range [22,32,33]. The K_d values are gauged at by biophysical methods such as surface plasmon resonance (SPR) or microscale thermophoresis (MST). In view of their high binding affinities and selectivities, aptamers have been engaged in numerous practical applications including therapeutics [14,34,35], diagnostic applications [36–38], drug delivery systems [39,40], and even start to infiltrate the fields of medical imaging and DNA nanotechnology [11].

2.2. Challenges facing aptamers for practical (in vivo) applications

Aptamers present a number of advantages compared to their proteinaceous antibody counterparts including the binding affinities and selectivities, a rapid penetration into target tissues, the relative ease of synthesis on large scale, the small size (<30 kDa, ~1–2 nm diameter *vs.* >150 kDa, ~10–15 nm diameter for antibodies) [14,20], limited batch-to-batch variation, and low immunogenicity. However, despite these favorable assets, aptamers suffer from two major shortcomings that often thwart their applications as therapeutic and diagnostic agents: [41] limited stability and insufficient binding affinity and specificity.

2.2.1. Binding affinity and specificity

Low K_d values and high specificity are crucial parameters, especially with respect to therapeutic applications and for the construction of drug delivery systems. However, not all selection experiments yield aptamers displaying both characteristics. For instance, difficulties in isolating high affinity-binding aptamers can arise because nucleic acids are negatively charged and functionality-deprived biopolymers that have a low inherent propensity at binding to protein targets devoid of cationic

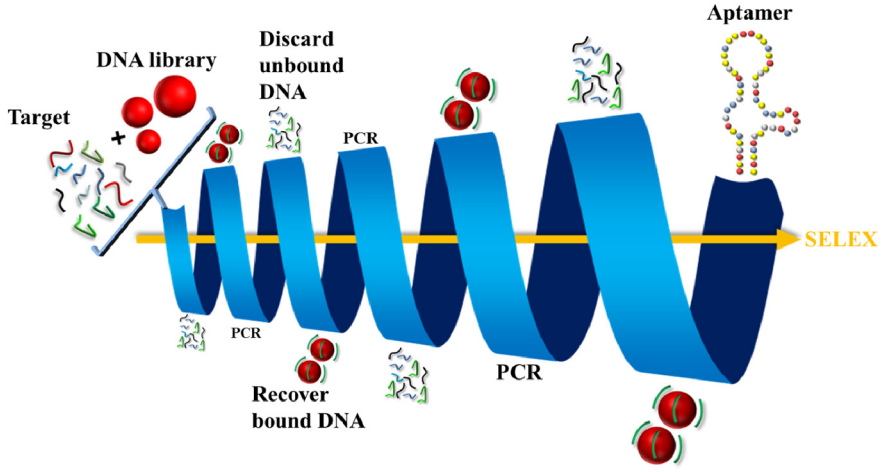

Fig. 1. Schematic representation of the SELEX method. An initial DNA library (of typically 10^{14} molecules) is incubated with the solid support-bound target. Unbound DNA molecules are discarded while the active species are recovered, amplified by PCR, and injected into subsequent rounds of selection. The stringency of the selection protocol can be modulated by altering physicochemical parameters such as concentration, pH, temperature, or buffer composition. At the end of the protocol, the enriched population is sequenced and the individual aptameric sequences evaluated for their capacity at binding to the target.

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regions or to hydrophobic pockets [41,42]. Other compounds such as glycoproteins or single enantiomers of small organic molecules are also known to be problematic targets in selection experiments [25,43]. Limited selectivity or perturbations in specificity might arise due to a change in the environment: an aptamer will recognize its target more readily in the buffer used during the *in vitro* selection experiment than in the crowded environment of a cell [44]. Lastly, selection experiments that favor high affinity binders often result in species with limited selectivity (and *vice versa*) since high specificity can be achieved by including one or multiple negative selection steps in order to discard compounds presenting structural and/or chemical similarities to the intended target, thus potentially excluding species with high binding capacities [12]. Consequently, the low K_d values for more difficult targets and the change in specificity are important concerns for numerous practical applications of aptamers.

2.2.2. Stability of aptamers

Aptamers, like any natural oligonucleotides, are prone to rapid nuclease-mediated degradation [45]. Early work on the development of therapeutic antisense oligonucleotides has shown that unmodified nucleic acids generally survive about 5 min in serum [46] and less than an hour in living cells [47,48]. Similarly, unmodified aptamers do not fare any better and are rapidly hydrolyzed by *endo*- and exonucleases *in vivo*. For instance, a well-known DNA aptamer against thrombin was shown to have a half-live as short as 108 s *in vivo* [49], while an RNA aptamer recognizing the keratinocyte growth factor is degraded within seconds in 90% human serum [50]. Lastly, temperature is an additional parameter that can have a negative impact on the binding efficiency of aptamers and can accelerate their degradation by nucleases [51]. Indeed, aptamers form intricate secondary and tertiary structures that enable binding to their target. An increase in temperature (for instance to 37 °C) might disrupt these secondary and tertiary structures and impede binding, as exemplified by the thermolabile cocaine-binding aptamer [52–54].

In addition to a rapid nuclease-mediated metabolic clearance, aptamers are prone to an efficient renal clearance. Indeed, due to their rather small size, aptamers are rapidly excreted by the kidneys which are capable of eliminating substances with a molecular weight < 50 kDa [55]. Taken together, the total plasma clearance –*i.e.* the sum of metabolic clearance, renal filtration, and irreversible tissue uptake [56]– of unmodified aptamers is rather high and thus represents a serious concern for therapeutic *in vivo* applications.

3. Chemical modification of aptamers

Different strategies have been devised to alleviate the aforementioned shortcomings of aptamers and to enhance their therapeutic and diagnostic efficiency. For instance, the change of binding affinity due to altered pharmacokinetics or conformational variations caused by a different environment has been addressed by the development of *in vivo* SELEX [57]. In this approach, the selection experiments are carried out directly in *in vivo* relevant media such as live cells or animals. *In vivo* SELEX enabled the identification of aptamers that selectively recognize hepatic colon cancer metastases [57] and the RNA Helicase Protein DHX9 [58] as well as aptamers capable of crossing the blood–brain barrier (BBB) [59]. Alternatively, traditional *in vitro* SELEX can be combined with *in vivo* SELEX to increase both the binding affinity and the selectivity [60].

The introduction of chemical alterations to the nucleotide scaffold is another very alluring strategy to enhance the properties of aptamers. Unlike other improved or alternative protocols, the chemical modification of aptamers can address the nuclease degradation, thermal stability, renal clearance, off-target binding and other specificity issues of aptamers as well as enhance binding to more demanding targets [18,61–64]. Chemical modifications can be incorporated into aptamers either after the *in vitro* selection by application of standard automated solid-phase synthesis or during SELEX by including dN*TPs directly in the selection protocol.

3.1. Post-SELEX modification of aptamers

Once aptamer sequences have been identified by SELEX, chemical modifications can be incorporated using solid-phase oligonucleotide synthesis at internal positions and/or at the 3′ and 5′ termini of oligonucleotides [65]. In automated oligonucleotide solid-phase synthesis, activated modified phosphoramidite building blocks are successively appended on the growing oligonucleotide chain starting from a first, immobilized nucleoside (Fig. 2) [66,67]. After completion of the synthesis, oligonucleotides are detached from the solid support and the protecting groups of the nucleobase are removed concomitantly by treatment with aqueous ammonia. Subsequent purification by HPLC or gel electrophoresis then yields the pure oligonucleotides.

In this section, we describe the different modifications introduced by this post-SELEX optimization method as well as application of the resulting modified aptamers in the fields of drug delivery and biosensing.

3.1.1. Improvement of nuclease resistance

Early chemical modifications introduced into aptamers stem from progresses obtained in the development of therapeutic antisense oligonucleotides and mainly aimed at improving the resistance to nuclease-mediated degradation [68,69]. Initial efforts focused on the introduction of modifications at the level of the 2′-position of the (deoxy-)ribose sugar unit as well as the phosphate groups (Fig. 3). Substitution of the 2′-hydroxy residue of RNA with either a fluorine atom (2′-F) or with methoxy (2′-OMe) and amino (2′-NH_2) groups, markedly improves the resistance of aptamers against nuclease degradation (Fig. 3A–C) [70]. However, the 2′-NH_2-modification is not often used anymore due to modest coupling efficiencies during solid-phase synthesis and for its preference for the C2′-endo ("DNA-like") ribose conformation [71]. An early and prominent example of a sugar modified aptamer obtained by post-SELEX optimization is pegaptanib (Macugen®). Pegaptanib is the only FDA approved oligonucleotide-based drug to be currently marketed [72] and is used for the treatment of neovascular age-related macular degeneration [73]. This aptamer was obtained by *in vitro* selection using the human vascular endothelial growth factor ($VEGF_{165}$) as a target and a mixture of unmodified purine NTPs and 2′-F-pyrimidine N*TPs. The remaining unmodified purine nucleotides of the 27 nucleotide long aptamer were converted into the corresponding 2′-OMe units after the selection by solid-phase synthesis [74]. In addition to the post SELEX 2′-OMe modifications, a 40 kDa poly(ethylene glycol) moiety was appended to the 5′-end to prolong renal clearance (*vide infra*) and the sequence was terminated with a 3′-3′-linked deoxythymidine residue to further slowdown nuclease degradation [74]. Despite this impressive amount of chemical modifications present on the aptameric scaffold, pegaptanib binds to its $VEGF_{165}$ target with an extremely high affinity (K_d = 50 pM) [75] and survives 20 h long incubations in human umbilical vein endothelial cells containing medium. Another related and important example is the anti-factor IXa RNA aptamer RB06 and its antidote RB007. The aptamer was also initially selected using a mixture of unmodified purine NTPs and 2′-F-pyrimidine N*TPs [76]. After truncation to a 34 nucleotide long species and appendage of a 5′-40 kDa-PEG moiety and a 3′-inverted deoxythymidine by solid phase synthesis, mutant RB06 was obtained which was still capable of binding to its target (factor IXa) with high affinity (K_d = 2.8 nM) [76]. This modified aptamer was shown to be stable *in vivo* and to produce a strong anticoagulation activity in human and animal plasma [55]. RB007 is a fully 2′-OMe-modified sequence complementary to the 5′-section of RB06 and serves for the modulation of the anticoagulation activity of the aptamer: hybridization of RB007 to RB06 precludes binding of the aptamer to factor IXa and hence terminates the anticoagulation activity of the aptamer. The aptamer and antidote pair has successfully

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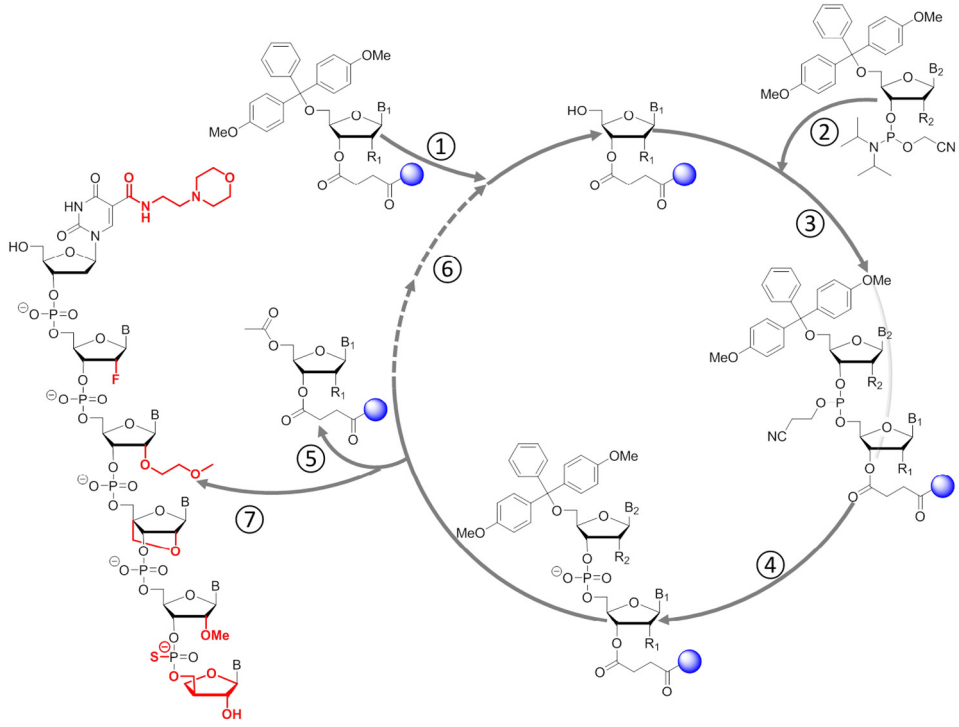

Fig. 2. Key steps in automated oligonucleotide solid-phase synthesis for the incorporation of chemical modifications into aptamers. Step 1: the synthetic cycle starts with the removal of the 5′-DMTr protecting group of the first nucleoside bound to a solid support (*e.g.* CPG) through a succinyl linker; step 2: the incoming phosphoramidite building block (modified or not) is activated (usually with 5-(ethylthio)-1*H*-tetrazole); step 3: the activated phosphoramidite is coupled on the growing chain; step 4: oxidation of the phosphoramidite (P^{III}) to phosphate (P^{V}) with iodine; step 5: the unreacted 5′-hydroxy groups are capped with acetic anhydride to prevent coupling with the next building block; step 6: the next cycle restarts with the detritylation of the last appended nucleotide; step 7:after completion of the synthesis, the oligonucleotide is detached from the solid support and the protecting groups of the exocyclic amines of the nucleobases are removed by treatment with ammonia.

undergone clinical trials as anticoagulation agent for patients undergoing elective percutaneous coronary intervention [77–79], however commercialization was halted due to the occurrence of severe allergic reactions in patients [80]. In a more recent example, Mallikaratchy et al. performed a systematic truncation and incorporation of 2′-OMe units of the KH1C12 aptamer which specifically recognizes the myeloid leukemia cell line HL60 [81]. This analysis allowed the identification of a 2′-OMe-modified version of KH1C12 which had an improved half-life in human serum without negatively impacting the binding affinity.

In phosphorothioates (Fig. 3D), one non-bridging oxygen atom of the phosphate units is replaced with sulfur [82]. This well-established modification provides oligonucleotides with an efficient protection against nuclease-mediated degradation and hence markedly improves their half-lives in serum and cell extracts [83]. Phosphorothioate modifications have also been incorporated into aptameric scaffolds in order to increase their nuclease resistance and serum stability [84,85]. For instance, aptamer AR1779 was selected with 2′-OMe N*TPs to target the multimeric plasma protein von Willebrand factor (vWF), which triggers platelet adhesion and aggregation [86]. The original sequence was then subjected to a post SELEX modification strategy during which some of the 2′-OMe-modified nucleotides were converted to natural deoxynucleotides, a single phosphorothioate linkage was included, and a 3′-inverted deoxythymidine unit and a 5′-20 kDa-PEG moiety were introduced. The resulting aptamer AR1779 was capable of binding to the vWF with high affinity (K_d = 2 nM) and efficiently inhibited platelet aggregation. Clinical trials (phase I and II) have been completed with ARC1779 for the treatment of the von Willebrand disease [87–89]. In the phosphorodithioate modification (PS2), both non-bridging oxygens are replaced with sulfur atoms, which ablates the question of diasteromeric phosphoromonothioate mixtures (see Section 3.2.3) [90,91]. Recently, PS2 modifications were introduced into the scaffolds of RNA aptamers selected for binding to $VEGF_{165}$ [92] and thrombin

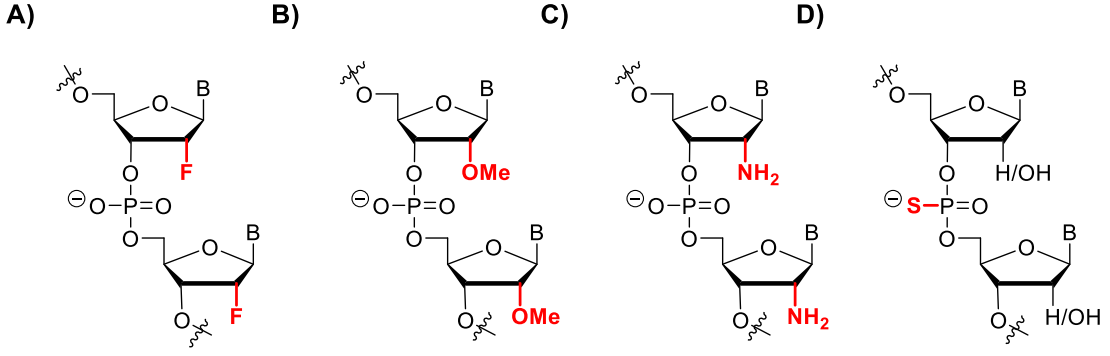

Fig. 3. Chemical structures of modifications for the improvement of nuclease resistance of aptamers: A) 2′-fluoro-RNA (2′-F); B) 2′-methoxy-RNA (2′-OMe); C) 2′-amino-RNA (2′-NH_2); D) phosphorothioate-DNA/RNA.

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[93]. Some mutant aptamers containing a single PS2 modification presented an impressive three-order of magnitude increase in binding affinity (K_d values were lowered from 961 pM down to 1 pM and from 1871 pM down to 1.8 pM for the anti-$VEGF_{165}$ and thrombin aptamers, respectively) along with an improved nuclease resistance compared to the parent aptamers [94]. The increase in binding affinity was ascribed to a favorable interaction between a sulfur atom and the aromatic moiety of phenylalanine residues of the thrombin target.

Following these early modifications, other chemical alterations of the nucleotide scaffold were introduced post-SELEX in order to improve the resistance to nuclease degradation (Fig. 4). A prominent example is the RNA-mimic Locked Nucleic Acid (LNA; Fig. 4A) [95,96]. In LNA, the sugar is maintained in a C3′-endo configuration by a methylene linker connecting the O2′ to the C4′ position of the sugar. The introduction of LNA units into DNA or RNA oligonucleotides significantly increases both the thermal stability of their respective duplexes as well as their specificity for complementary sequences [97]. In addition to this increase in stability, LNA-containing oligonucleotides have been shown to display an improved resistance against nucleolytic degradation [98,99]. As for other sugar or phosphate modifications, LNA units can be introduced into aptamer sequences during a post-SELEX optimization step requiring structure–activity relationship (SAR) studies. In these SAR studies, the LNA building blocks are incorporated into various structural elements of aptamers such as stems or loops and the impact of these alterations on binding affinity is assessed. The mutants with the best binding affinities are then retained for further *in vivo* assays [100,101]. In this context, various nucleotides constituting stem 1 of the Tenascin-C binding RNA aptamer TTA1 were substituted with LNA units and one particular mutant displayed an improved binding affinity ($K_d = 2.0$ *vs* 5.8 nM) and thermal stability as well as a prolonged half-life in human plasma (53 h *vs* 42 h) compared to the unmodified aptamer. Interestingly, the Tc-99m-radiolabelled LNA-modified aptamer TTA1.2 displayed a three times higher U251-tumor uptake in mice compared to the unmodified counterpart [101]. Similarly, LNA modifications were introduced into the scaffold of DNA aptamer sgc8c which is capable of recognizing leukemia cells [102]. Particularly, the serum stability of sgc8c could be markedly improved when the first three base pairs at the end of the stem were substituted with LNA moieties and loop-internal nucleotides were replaced with two PEG-linkers [103]. More recently, 40 LNA-containing mutants of a streptavidin-binding aptamer were synthesized and evaluated for their binding affinity and enhancement of nuclease resistance. Various mutants could be identified that displayed a modest improvement in binding affinity and an important increase of stability in serum [104]. Related to this post-SELEX approach, LNA-DNA chimeric oligonucleotides have been used as synthetic antigens for the detection of antibodies specific of the disease lupus erythematosus in patient samples [105]. Also, multimeric scaffolds consisting of LNA-modified aptamers interconnected with PEG linkers have been shown to have an increased nuclease resistance while maintaining their specificity for B-cells *in vitro* and *in vivo* [106].

As an alternative strategy to post-SELEX modification, LNA units can be incorporated into the primers flanking the randomized region of libraries and used in SELEX experiments to identify LNA-aptamers. This approach suppresses the need for engineered polymerases to incorporate modified triphosphates since the LNA modifications need only to be read by the polymerase during the PCR amplification step. Application of this SELEX protocol has allowed for the identification of nuclease resistant aptamers against recombinant human CD73 [107] and the human thrombin [108,109].

Other sugar-modified nucleotide analogs such as HNA (Hexitol Nucleic Acid) [110] or modified LNA nucleotides [111] have also been successfully incorporated into aptamers by the post-SELEX method. Among these modifications, unlocked nucleic acid (UNA; Fig. 4B) has advanced as an excellent candidate for the post-SELEX modification of aptamers. UNA is an acyclic analog of RNA where the C2′-C3′ bond is missing, giving rise to an incomplete sugar ring [112]. This distinctive structural feature confers an increased flexibility to UNAs compared to parent unmodified RNAs but is also responsible for the rather large decrease in thermal stability of the modified duplexes [113]. The flexibility of the UNA modification along with its limited structural impact on duplexes were exploited to improve the binding affinity and the blood clotting activity of a DNA thrombin-binding aptamer *via* a thermodynamic stabilization of the G-quadruplex structure [114]. Similarly, modification of the G quadruplex-based scaffold of an anti-VEGF aptamer with UNA and LNA monomers resulted in different aptameric constructs that were capable of inhibiting breast cancer cell proliferation with a higher efficiency than the unmodified counterpart [115]. Particularly, a modified sequence containing three LNA units (coined RNV66) was capable of inhibiting cancer cell proliferation in a mouse breast cancer model due to a remarkable stability in serum originating from the presence of the LNA nucleotides and the G quadruplex structure. The NMR structures of these LNA and LNA/UNA modified anti-VEGF aptamers have also been determined [116].

Another interesting and important modification of aptamers is the so-called Spiegelmers [117]. Spiegelmers are aptamers consisting of L- rather than D-DNA and RNA scaffolds and are thus mirror images of wild-type nucleic acids [118,119]. The incorporation of L-nucleotides confers a high plasma and serum stability as well as immunological passivity to aptamers since mirror image nucleic acids are not recognized by nucleases (and enzymes in general) or by the immune system [120]. These inherent, highly potent properties of these modified nucleic acids are reflected by the three Spiegelmer aptamers that are currently in clinical trials and another four in preclinical testing [14,18,121]. Until the recent engineering of D-polymerases capable of recognizing L-modified nucleoside triphosphates (see Section 3.2.1)

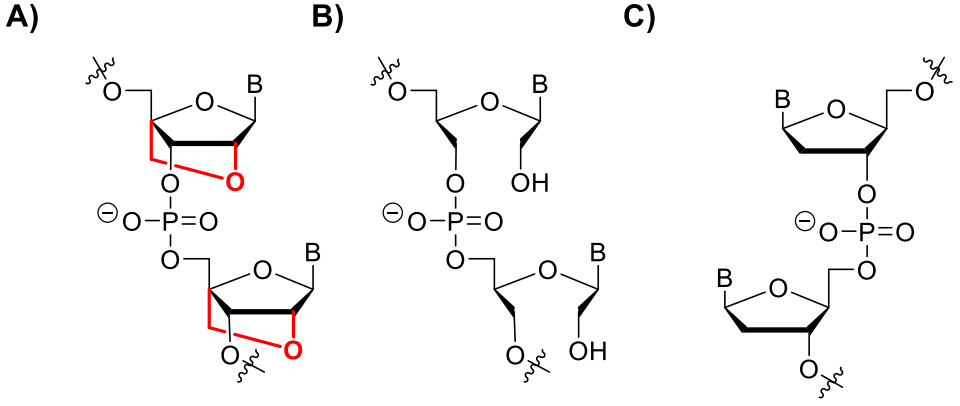

Fig. 4. Chemical structures of second generation modifications for the improvement of nuclease resistance of aptamers: A) LNA; B) UNA; C) Spiegelmers.

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[122,123], Spiegelmers are usually obtained by application of a two-step protocol involving selection of a D-aptamer by conventional SELEX using synthetic targets that are enantiomers of the intended target and followed by a post-SELEX conversion to the corresponding L-oligonucleotide (using L-phosphoramidites). Other recent examples obtained by this SELEX-post-SELEX protocol include Spiegelmers targeted against the signaling molecule sphingosine 1-phosphate as angiogenesis inhibitor [124], the neuropeptide (α-) calcitonin gene-related peptide for the treatment of migraine [125], glucagon for the treatment of diabetes [126], and the small peptide hepcidin for the treatment of anemia of chronic inflammation [127]. Besides numerous proteins, Speigelmers have also been raised against natural D-RNA targets [128]. This is not a trivial undertaken since L-nucleic acids are not capable of binding to natural D-sequences by Watson-Crick base pairing. Therefore, in order to isolate Spiegelmers against the D-enantiomer of the HIV-1 trans-activation responsive (TAR) RNA target, Joyce et al. used the mirror-image of the target to isolate a D-aptamer by SELEX. The natural D-aptamer was then converted to the corresponding Spiegelmer by post-SELEX solid-phase synthesis. The resulting L-aptamer bound to D-TAR RNA with an appreciable affinity ($K_d = 100$ nM) through the formation of tertiary interactions [128]. A similar strategy was also applied by the same authors to identify Spiegelmers against the D-enantiomers of the precursor microRNAs pre-miR-10b, pre-miR-33a, and pre-miR-155 in order to block the formation of the corresponding mature microRNAs [129]. Related to this, a base-modified Spiegelmer specific for the oncogenic precursor microRNA 19a (pre-miR-19a) has also recently been raised by SELEX using an L-miRNA target and the modified D-allyl-amino-UTP ($U^{aa}TP$) to construct the D-RNA libraries used in the selection rounds [130]. In the context of cross-chiral recognition of nucleic acids, peptide nucleic acids (PNAs) have been suggested as an achiral interface between L- and D-DNA to mediate toehold-mediated strand-displacement reactions –a strategy that might have implications for the selection of Spiegelmers [131]. Lastly, the Spiegelmer concept was recently expanded to catalytic nucleic acids since 1) the L-enantiomers of a Cu^{2+}-dependent DNA-cleaving DNAzyme and a Pb^{2+}-dependent RNA-cleaving DNAzyme were both shown to be capable of catalyzing the hydrolysis of their respective L-substrates [132] and 2) a D-ribozyme catalyzing the ligation of L-RNA oligonucleotides (and *vice versa*) has recently been identified by *in vitro* selection [133].

3.1.2. Renal clearance

Due to their small size, aptamers are prone to rapid elimination by filtration by the kidneys which concomitantly wreaks a serious threat on their therapeutic usefulness. However, the inclusion of different modifications into aptamers can remediate this shortcoming by efficiently retarding renal clearance. Typical backbone or nucleobase modifications such as 2′-F or 2′-OMe are not sufficient or even capable of mitigating the rapid renal clearance of aptamers [14]. On the other hand, the phosphorothioate modification is known to prolong the residence time of antisense oligonucleotide based-drugs through non-specific binding to plasma proteins which prevents their rapid renal clearance [134–136]. While no precise pharmacokinetic and biodistribution data comparing unmodified and phosphorothioate-modified aptamers are available, this modification might help in reducing the renal filtration of these functional nucleic acids [56]. However, the most commonly used strategy for the retardation of renal filtration and increasing the circulating half-lives of aptamers is the conjugation with larger molecules in order to exceed the 30–50 kDa molecular mass cut-off for the renal glomerulus. Among these modifications, the appendage of high molecular mass PEG units (20 or 40 kDa) [137] at the 3′- or 5′-termini of aptamers is no doubt the most popular and widespread approach [92,138]. PEG is an amphiphilic, non-toxic polymer that has an estimated plasma circulating half-live of 10 h in mice and has been used in numerous formulations to increase the bioavailability of drugs [139]. In this context, pegaptanib is undoubtedly the best example of a pegylated aptamer. In pegaptanib, the incorporation of a 40 kDa PEG unit at the 5′-end vastly increased the residence time in circulation, the plasma half-life, and the inhibition of the vascular leakage, albeit at the expense of a slight (4-fold) decrease in binding affinity [73,74,137]. This clearly illustrates that the choice of the molecular weight of PEG is a crucial parameter that is often the result of a compromise between altered pharmacokinetic properties, reduced binding affinities for the target, and enhanced *in vivo* residence times (circulating half-lives of up to 24–48 h have be observed) [140,141]. New pegylation strategies such as the symmetrical branching 2-cyanoethyl-*N*,*N*-diisopropyl phosphoramidite PEGylation [141] or *comb*-shaped PolyPEG [142] have been proposed to improve the circulating half-lives without impacting other pharmacokinetic properties.

Other successful post-SELEX modifications introduced into aptamers to reduce the rates of renal excretion include cholesterol [143,144], 3′-biotin-streptavidin (SA) bioconjugates [145], liposomes [146–148], proteins [149,150], dendrimers [151], and inorganic nanoparticles [11,152,153].

3.1.3. Drug delivery systems with modified aptamers

In terms of applications, modified aptamers have mainly been used as ligands for therapeutic purposes to enhance selectivity and reduce side effects [14]. However, the high binding affinity and the low off-target interactions of aptamers make them excellent supports for the guidance of drugs to their intended targets. In this context, aptamer-drug conjugates (ApDCs) have been shown to enhance the site-selective delivery of drug payloads as well as target engagement and are thus believed to be promising candidates for targeted therapies [11,12,34,154]. In a vast majority of ApDCs, unmodified DNA or RNA aptamer sequences are coupled either directly to the drug cargo or indirectly through a linker unit [155]. However, ApDCs -like any system containing unmodified aptamers- are prone to *in vivo* degradation by nucleases and to rapid renal clearance (*vide supra*). The stability of aptamers in ApDCs can be improved by the post-SELEX inclusion of chemically modified units such as 2′-F, 2′-OMe, or LNA nucleotides. For instance, Bagalkot et al. have constructed an ApDC consisting of a 2′-F pyrimidine modified aptamer targeting the prostate specific membrane antigen (PSMA) coupled to the surface of a quantum dot. This system can carry doxorubicin (DOX) through intercalation in the double-stranded stem region of the aptamer and thus efficiently suppresses tumor growth. The use of the modified aptamer allowed increasing the serum stability over 72 h incubation times while maintaining cell specificity of LNCaP over PSMA negative PC3 cells [156]. A similar approach was used by Wang et al. to target PSMA but by coupling the 2′-F pyrimidine modified aptamer to thermally crosslinked superparamagnetic iron oxide nanoparticles (TCL-SPION) loaded with DOX. The resulting bioconjugate acted as a stable drug delivery system and MRI agent due to the presence of the aptamer and the SPION moiety, respectively [157].

Interestingly, the half-life of oligonucleotides used for drug delivery purposes *in vivo* is not directly limited by plasma stability but mainly by renal clearance. The renal clearance of ApDCs generally depends on the nature of the conjugate, but the appendage of relatively small molecules to the aptamer, such as PEG [77] or cholesterol [55] is often sufficient to substantially prolong the *in vivo* residence time. Recently, Kuhlmann et al. reported the conjugation of the anti-factor IXa aptamer to recombinant human albumin through a dsDNA section. The aptamer was equipped with a 2′-F-modified unit and 2′-OMe-nucleotides and the resulting albumin-aptamer complex maintained the binding affinity and blocking capacity of the parent aptamer and seriously improved the resistance to nuclease-mediated degradation. Such a construct could easily be converted into a drug delivery platform for future targeted therapeutic applications [158].

Small interfering RNAs (siRNAs) are a promising class of therapeutics due to their specificity and relatively low immunogenicity [159]. However, they lack therapeutic efficiency mainly because of delivery

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problems from the plasma into cells as well as off-target effects [160]. In the last decade, several groups tried to overcome these hurdles using various strategies and siRNAs have regained momentum as therapeutic agents. Particularly, the chimeric conjugation of siRNAs and (modified) aptamers was found to be a potentially useful approach to increase the therapeutic potency. In a first example, McNamara et al. conjugated the anti-PSMA RNA aptamer A10 with siRNAs targeting the polo-like kinase 1 and B-cell lymphoma 2 genes [161]. The resulting chimeric RNAs were successfully delivered to prostate cancer cells expressing PSMA and the siRNAs triggered the cleavage of the cognate mRNAs and thus specifically caused gene silencing. When the chimeric RNAs were injected into the tumors in a mice xenograft model of prostate cancer, an efficient tumor regression was observed. Dassie et al. further improved this system to allow systemic administration which is required for therapeutic purposes. Particularly, the size of the aptamer was markedly reduced, the RNA sense strand was equipped with 2′-F nucleotides to further improve nuclease resistance, 2-nucleotide UU 3′-overhangs were included in order to favor recognition of the guide strand by the endoribonuclease Dicer, and a 20 kDa PEG unit was appended to the 5′-end to improve the circulation half-life. The resulting construct indeed led to a marked tumor regression in mice after systemic administration [162]. However, Berezhnoy et al. suspected that the therapeutic effect of this and previously validated siRNA targeted delivery methods were often compromised when conjugated to an aptamer [163]. The authors then conducted systematic experiments based on the thermal stability of the 2′-F pyrimidine modified aptamer-siRNA conjugates and showed that siRNAs with lower melting temperatures are not affected by the conjugation compared to those with higher melting temperatures [163]. Consequently, siRNAs with lower thermal stabilities (T_m ≤ 50 °C) seem to be more amenable to conjugation to aptamers for their targeted delivery.

Related to these approaches, a three-way junction RNA was extended into an arrow-shaped construct by appendage of a truncated version of the 2′-F-modified anti-PSMA aptamer A9 (which had been selected at the same time as A10). The resulting construct was then affixed on the surface of extracellular vesicles through a cholesterol moiety which inserts spontaneously into the membrane of the vesicles. Survivin siRNA was then loaded as a cargo into the vesicle and could be selectively delivered to cancer cells. This system was capable of reducing tumor growth of three cancer models in mice [164]. Currently, a number of siRNA-aptamer conjugates bearing modified nucleotides have been developed to target specifically various cells (see Table 1).

A very different approach using modified aptamers for drug delivery was followed by Kruspe et al. They prepared a variant of the AIR-3 aptamer which binds to the human interleukin-6 receptor replacing natural uridine with 5-fluoro-2′deoxyuracil -a drug with cytostatic properties. This modification caused a tolerable loss in affinity (K_d value of 151 nM instead of 21 nM for the parent aptamer). After binding of the aptamer to the surface receptor and subsequent endogenous internalization, the aptamer is degraded by lysozymes resulting in the release of the cytotoxic drug in the target cell exclusively [174].

Table 1
Modified aptamers for the targeted delivery of therapeutic siRNAs.

Target	Drug	Modification	Reference
Anti-apoptotic gene, Bcl-xL	DOX/shRNA	2′-F pyrimidines/3′-invT	[165]
TAR of HIV-1 genome	siRNA	LNA/2′-OMe pyrimidines	[166]
Human papillomavirus E6 and E7	siRNA	Boranophophates/2′-F	[167]
HIV-1	siRNA	2′-F pyrimidines	[168]
Ovarian cancer	DOX/siRNA	Phosphorothioate	[169]
EpCAM	siRNA	LNA	[170]
Adenosine kinase	siRNA	2′-OMe pyrimidines	[171]
Antimicrobial AgNP delivery to *S. aureus* cells	AgNP	2′F G, 2′OMe A,C,T	[172]
Nucleolin	SSO	2′-OMe/Phosphorothioate	[173]

3.1.4. Biosensing with modified aptamers

Aptasensors are an important class of biosensors that have found a growing importance in the field of diagnostics and in detection technologies. An aptasensor typically consists of an aptameric part which is modified with a transductive element that converts the binding event of a target to the recognition element into an analytically detectable and measurable signal. The transducitve elements of aptasensors are versatile and consist of simple fluorescent probes such as fluorescein [175] or more sophisticated luminescent probes such as Tb^{III} [176], redox active probes such as ferrocene [177], or Ru^{II} complexes [178]. The progress and challenges in the field of aptasensors has been well reviewed and will not be covered herein [179]. Most strategies involving the chemical, post-SELEX functionalization of aptamers for biosensing purposes mainly strive at anchoring or immobilizing these oligonucleotides on surfaces [11]. These methods encompass biotinylation [180] or the appendage of 3′- or 5′-thiol or amino residues for direct coupling on gold nanoparticles [181], quantum dots [182], graphene oxide [183], or pyrene labeling for noncovalent coupling to graphene [184]. Recently, we have proposed an enzymatic method for the immobilization of aptamers on solid supports for the rapid development of biosensing platforms. This method relies on the polymerization of an imidazole-modified nucleoside triphosphate [185] to generate a his-tag mimic that can interact with Ni- and Eu-NTA agarose columns [186]. Besides the appendage of functional groups to the termini of aptamers to facilitate their immobilization on various surfaces, some nucleobase and sugar modified sequences have been used to enhance the capacity and the properties of the resulting biosensors. Indeed, Spiegelmers have been used in certain instances that required nuclease or base resistant nucleic acids. For instance, an L-RNA aptamer specifically recognizing L-arginine was immobilized on a chromatographic support through the formation of a biotin-avidin complex. The resulting RNA stationary phase then served as an affinity probe for the separation of the enantiomers of arginine. The mirror-image of the aptamer had to be used instead of the D-RNA sequence to prevent the rapid degradation by RNase A [187]. A similar approach has recently been reported for the specific detection of glucagon and murine amylin [188]. The biostability of aptamer gold nanoparticle bioconjugates can also be improved by using Spiegelmers. In a recent example, Han and co-workers affixed the L-enantiomer of a DNA aptamer targeting nucleolin onto the surface of gold nanoparticles through a 3′-C-rich oligonucleotide. The resulting bioconjugate revealed to be completely immune against DNase I degradation and was as efficient for cell-type-specific imaging as the D-DNA based construct [189]. Paralleling these efforts, biosensing platforms with mirror image DNAzymes and ribozymes have also been constructed [132,190].

Although it is known that aptamers equipped with nucleobase modifications such as the SOMAmers [191] can increase the binding affinity to targets [192–194], only a small number of examples of aptasensors relying on sequences with nucleobase modifications have been reported. Li et al. demonstrated that an aptasensor system comprised of a fluorescent analog of deoxycytosine -pyrrolo-dC (P-dC)- as a signaling probe results in a cost inexpensive and highly selective system [195]. The devised aptasensor is based on earlier studies which showed that the P-dC analog served as an excellent probe to sense slight structural changes in the conformation of oligonucleotides [196]. In the system devised by Li et al., a P-dC-containing competitor sequence forms a duplex with the unmodified aptamer in the absence of the target and the intrinsic fluorescence of the P-dC units is therefore quenched. Upon binding to the target, the competitor sequence is released from the duplex leading to a detectable fluorescent signal. This system was employed for the sensitive detection of ATP, thrombin and platelet-derived growth factor [195]. Similarly, in a systematic study by Manderville and co-workers various fluorescent base analogues such as 8′-furyl-2′-deoxyguanosine (^{Fur}dG) [197], 8′-thienyl-2′-deoxyguanosine (ThdG) [198] or 5-furyl-2′-deoxyuridine (^{Fur}dU) [199] were incorporated at different loci of the thrombin binding aptamer (TBA) by post-SELEX solid-phase

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synthesis. The working principle of these aptamers relied on the emission property of the fluorescent base modifications to sense structural changes in the microenvironment. Not only could they show that this set up was suitable to sense thrombin binding but also to sense solvent polarity changes due to rotor rigidity effects of the furanyl moieties attached to the modified nucleobases [199].

Development of aptamer-based sensing platforms that display low limits of detections, sensitive and accurate readouts in biologically relevant media are particularly relevant for point-of-care diagnostic applications.

3.2. SELEX with modified nucleoside triphosphates

As described in the previous section, chemical modifications can be brought to the scaffold of aptamers by application of solid phase synthesis. This post-SELEX modification method is very convenient, especially when large scale production of aptamers is considered. However, the rather tedious and uncertain SAR studies required to identify modified aptamers displaying similar K_d values as the unmodified counterparts along with the intolerance of certain functional groups to the rather harsh conditions involved in some of the synthetic steps are non-negligible shortcomings. Also, solid-phase synthesis of oligonucleotides is restricted to rather short sequences (≤100 nucleotides). The enzymatic polymerization of dN*TPs represents a valid alternative to solid-phase synthesis for the introduction of chemical diversity into nucleic acids. Indeed, (d)N*TPs can be used as vectors for the modification of DNA and RNA provided they are accepted as substrates by polymerases. The polymerization of dN*TPs has allowed to decorate nucleic acids with a broad variety of functional groups including amino acid-like residues [200–204], ligands for transition metals [205] and metal complexes [206,207], carborane [208], side chains mediating organocatalysis [209], constrained and modified sugar nucleotides [210,211], oligonucleotides [212,213], and even enzymes [214]. Both double and single stranded sequences can be synthesized by this method [215]. Alternatively, dN*TPs can be used to generate oligonucleotide libraries adorned with chemical functionalities which can then be directly injected into the SELEX protocol to identify modified aptamers (Fig. 5). Modified aptamers using N*TPs have also been selected despite the lower tolerance of RNA polymerases to unnatural substrates and the restricted number of engineered RNA polymerases. This modified SELEX approach is a particularly convenient method since the resulting aptamers do not necessitate any subsequent SAR studies. In addition, recent progress in organic synthesis and polymerase engineering has augmented the chemical diversity that can be explored during SELEX experiments with modified libraries [64,216]. This is reflected by a strong increase in publications reporting aptamers obtained by modified SELEX since 2010 (Fig. 6B), even though post-SELEX modification is still the preferred strategy (Fig. 6A). In this section, we describe and discuss the different modifications introduced into aptamers by modified SELEX. Obviously, aptamers obtained by modified SELEX can be further functionalized by the post-SELEX strategies described in the previous section.

3.2.1. Base modifications

The choice of the location of the modification on the nucleobase is mainly dictated by empirical rules: functional groups appended at the C5-position of pyrimidines and the *N*7 of 7-deaza-purines *via* rigid alkynyl or alkenyl linker arms often result in higher substrate acceptance by numerous DNA polymerases [201,209,217]. Alternatively, the C2-position of purines was recently shown to be a good site for the enzymatic introduction of small functional groups into the minor groove of DNA [218]. Modifications introduced at the level of the nucleobase can enhance the affinity of the resulting aptamers *via* increased contact interactions with their intended targets and through the creation of additional secondary and tertiary folds and structures [219]. Moreover, the appendage of hydrophobic residues on the nucleobase positively impacts the stability of aptamers to nuclease-mediated degradation (*e.g.* a nine-fold increase in half-life of base modified SOMAmers was observed in 90% human serum compared to the corresponding unmodified sequences) [220,221]. The presence of hydrophobic side-chains was also shown to reduce the plasma clearance of aptamers [56].

The first *in vitro* selection with a base-modified dN*TP was performed by Latham et al. who used a pentynyl-modified dUTP (**1** in Fig. 7) to isolate aptamers against thrombin [222]. This initial experiment paved the way for numerous SELEX experiments with base-modified dN*TPs, most notably the so-called SOMAmers (Slow Off-rate Modified Aptamers) [191,220]. SOMAmers are prepared by including dUTP analogs equipped mainly with hydrophobic C5-amide linked side chains (**2**–**5** in Fig. 7) [223] *in lieu* of the natural counterpart into the SELEX protocol. The presence of the modified side-chains creates hydrophobic surfaces on aptamers which can interact with hydrophobic regions of amino acids on the protein targets. The creation of these

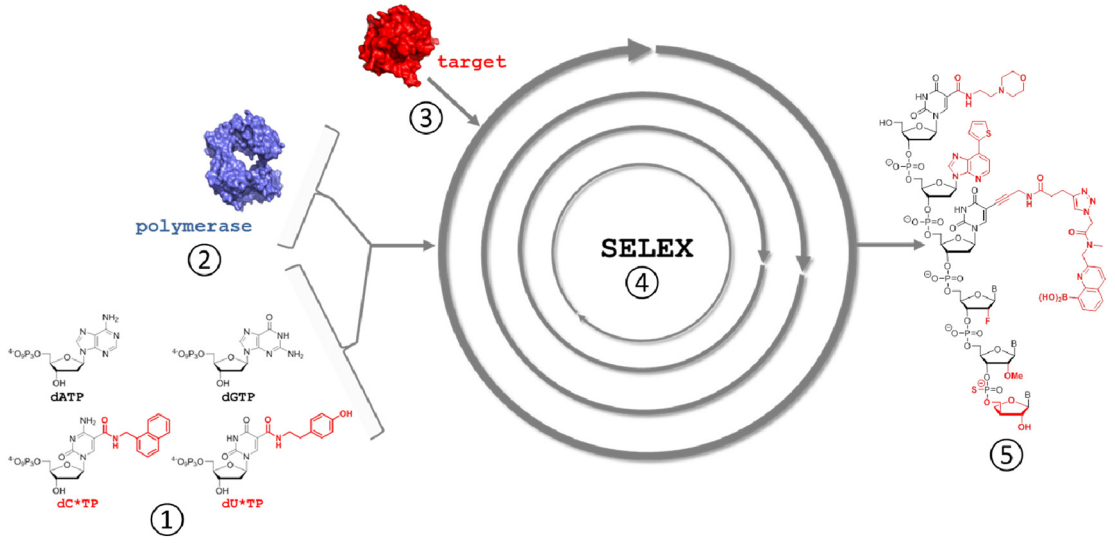

Fig. 5. Selection of aptamers with modified nucleoside triphosphates. Step 1: after synthesis and biochemical characterization of the dN*TP, the modified analogs are used *in lieu* of their natural counterparts; step 2: a modified library is generated by primer extension reactions or PCR with the dN*TPs; step 3: the population of modified oligonucleotides is incubated with the desired target and the binders are recovered and PCR amplified; step 4: as for conventional SELEX, the cycles are repeated until no further enrichment is observed; step 5: after sequencing, the modified aptamer can be obtained by solid-phase synthesis.

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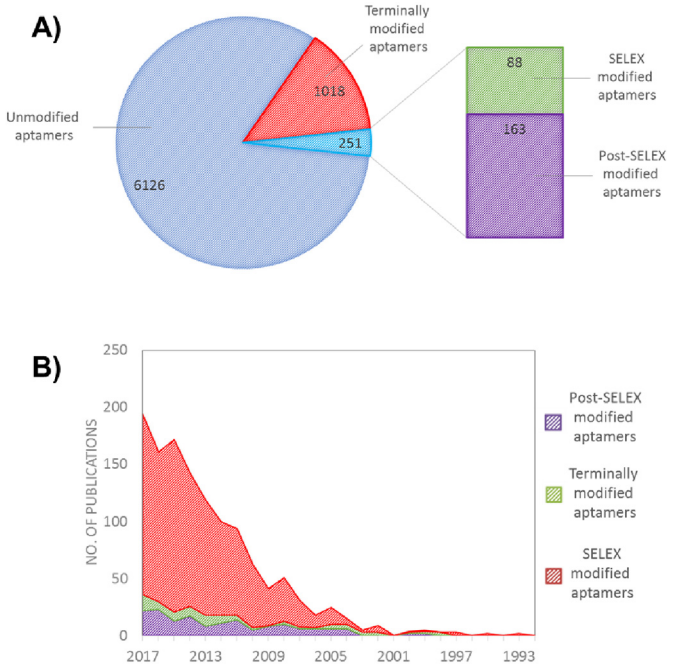

Fig. 6. Publication analysis of modified aptamers using the PubMed database. A) Pie chart representation of all the published articles from 1993 to 2017 with the starting queries "aptamers" or "modified aptamers". The subset of modified aptamers was then manually screened for sugar, base or phosphate backbone modifications that were introduced either by post-SELEX functionalization (shown in violet) or directly by SELEX with (d)N*TPs (shown in green). Articles that could not be attributed to one of each category corresponded to terminal (3′ or 5′) modifications, labels, linkers or review articles (shown in red). B) Annual frequency of the articles using "modified aptamers" as starting query. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

protein-protein-like interactions has been confirmed by recent crystal structure analyses of SOMAmers with their respective targets [42,224–226]. Importantly, the newly created hydrophobic interactions favor long lasting complexes by lowering the k_{off} rates down to ~10^{-5} s^{-1} [221]. These inherently highly interesting properties have led to the isolation of SOMAmers against over 3000 proteins and to the concomitant development of aptamer-based proteomic screens (the so-called SOMAscans) [227–229]. Recently, the chemical diversity of SOMAmers was expanded by evaluating the synergetic effect of two hydrophobic residues borne by two different nucleotides (dCTP and dUTP) in SELEX for the isolation of SOMAmers against the proprotein convertase substilisin/kexin type 9 [230]. Indeed, modified libraries bearing a dual modification pattern, single hydrophobic side-chains, or consisting of unmodified DNA were subjected to *in vitro* selection and the affinities of the resulting ligands were compared. The libraries created by the *co*-polymerization of modified dCTPs and dUTPs (particularly the Tyr-modification (**2** in Fig. 7) on dU and the Nap- or Pp-modifications (**3** and **4** in Fig. 7, respectively) on dC) led to the highest binding species. The dual modification pattern also enhanced the stability in serum and offered a larger epitope coverage compared to aptameric species containing single or no modifications.

SELEX experiments with dN*TPs bearing amino acid-like side chains other than those of SOMAmers have also been reported. In a recent example, Perrin et al. have used a C5-phenol modified dUTP in a whole cell-SELEX experiment in an effort to impart the structural resemblance of the hypervariable domains of certain antibodies to aptamers [231]. The resulting modified aptamer bound *E. coli* DH5α cells with a 10-fold higher binding affinity than an unmodified RNA aptamer selected against the same target. Similarly, DNA and RNA aptamers bearing cationic amine residues, reminiscent of the side-chain found in the amino acid lysine, have been selected to bind to ATP [232,233] and to the (*R*)-enantiomer of thalidomide [234]. Instead of amino acid-like side chains, triphosphates equipped with additional nucleic acid bases (the base-appended base (BAB) modification) have also been used in SELEX [193,235]. The rationale behind the inclusion of an additional adenosine nucleobase on dUTP (**6** in Fig. 7) was to endow modified DNA libraries with additional stacking interaction possibilities as well as hydrogen bond networks in order to improve the binding efficiency of aptamers to small molecules. The *in vitro* selection experiments conducted with dUTP **6** resulted in highly potent aptamers capable of selectively recognizing the small molecule camptothecin [193] and the enzyme salivary α-amylase [235]. More exotic, non-amino acid- or nucleic acid-like, functional groups have also been included in *in vitro* selection experiments. For instance, dUTP was decorated with a boronic

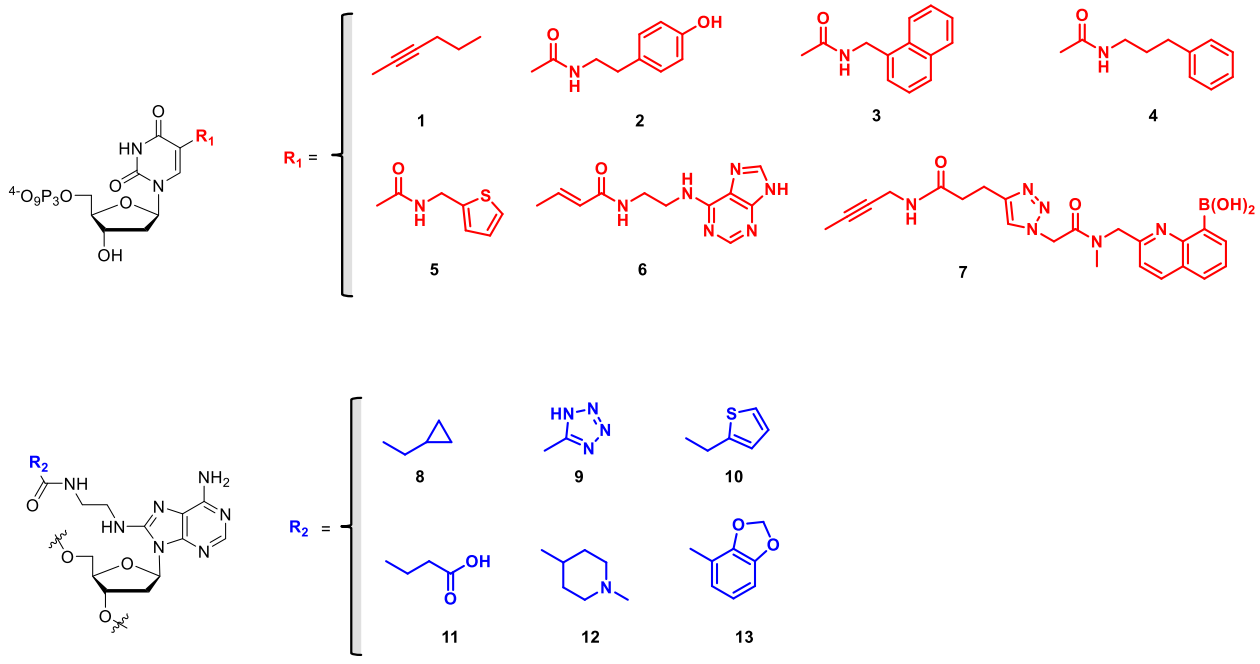

Fig. 7. Chemical structure of base-modified dN*TPs and nucleotides used in *in vitro* selection experiments for the isolation of modified aptamers.

acid residue (**7** in Fig. 7) in order to promote binding of aptamers to glycoproteins –a notoriously difficult target for aptamers- through the reversible formation of covalent bonds with 1,2-diol containing sugar units such as mannose. The screening of a boronic acid-modified DNA library indeed enabled the identification of an aptamer capable of recognizing and tightly binding (aptamers with K_d values in the low nM range) to the glycosylation site of fibrinogen [43]. An alternative method to mimic carbohydrate-protein interactions is the so-called SELMA approach (SELection with Modified Aptamers) [236]. In SELMA, modified DNA-libraries are generated by substituting dTTP with 5-ethynyl-dUTP (EdUTP) [237] in primer extension reactions on randomized templates of DNA hairpins. The ensuing modified population is thus equipped with a synthetic handle which serves for the introduction of large glycans onto the nucleotide scaffold by application of the copper (I)-catalyzed alkyne-azide cycloaddition (CuAAC or click reaction). The glycan-DNA chimeric sequences can then be subjected to a traditional SELEX experiment after carrying out a second primer extension reaction with natural dNTPs which causes strand displacement of the modified stretch. The advantages of SELMA consist of the disconnection between phenotype and genotype (which ablates the necessity of converting modified DNA (mDNA) to unmodified DNA) and the possibility for the covalent appendage of large modifications on oligonucleotides [238]. Application of the SELMA strategy has allowed the identification of various DNA and RNA aptamers capable of identifying the monoclonal antibody 2G12 with K_d values as low as 1.7–16 nM [239–241]. In a similar approach, a DNA library equipped with the same ethynyl units was further modified by the click reaction with an indole azide. The modified DNA library was then subjected to numerous rounds of selection using cycle 3 Green Fluorescent Protein as a proof-of-principle target. The resulting aptamer (a so-called "clickmer") bound to its target with a similar binding affinity than an unmodified RNA aptamer [242].

Base-modified DNA libraries can also be constructed by DNA ligases instead of polymerizing dN*TPs. In the LOOPER method (Ligase-catalyzed OligOnucleotide PolymERization), 5′-phosphorylated pentanucleotides containing a single C8-modified adenosine nucleotide per codon (see **8**–**13** in Fig. 7) are incorporated by the T4 DNA ligase with high fidelity (>95%) [243,244]. C8-modified adenosine nucleotides have been preferred in LOOPER because they adopt a *syn*-configuration around the *N*-glycosidic bond which causes a decrease in duplex thermal stability and a concomitant increase in the fidelity of the ligation reaction [243,245]. This approach was recently employed in SELEX for the identification of aptamers against α-human-thrombin using a library consisting of 16 different functional groups located on the pentanucleotide codons [246]. This SELEX protocol still requires the conversion of mDNA to unmodified DNA by PCR which was successfully accomplished by the KOD polymerase known to be rather permissive to 8-substituted purine triphosphates [247,248]. After 6 rounds of selection, a heavily modified aptamer was isolated which presented a remarkable affinity (K_d = 1.6 nM) and selectivity for the target. This proof-of-principle SELEX experiment was recently followed by the identification of aptamers capable of binding the two protein targets convertase subtilisin/kexin type 9 (K_d = 3 nM) and interleukin-6 (K_d = 12 nM), highlighting the generality of this approach [249].

3.2.2. Sugar modifications

Sugar-modified dN*TPs are not well tolerated by naturally occurring DNA polymerases due to the presence of a bulky steric gate which prevents entry of NTPs –present in large excess compared to dNTPs- into the active sites [216,248,250]. However, engineered polymerases lacking this steric gate or carefully selected by directed evolution can incorporate triphosphates with modified sugar moieties. Initial selection experiments with altered sugar nucleotides involved the small modifications 2′-F [74,251,252], 2′-OMe [253,254], and 2′-NH_2 (Fig. 3A–C) [255,256]. These early efforts culminated in the discovery of pegaptanib, obtained as mentioned previously by selection with a combination of unmodified purine NTPs and 2′-F-pyrimidine N*TPs. Numerous aptamers containing 2′-F modifications have been isolated including an aptamer capable of crossing the BBB [59] but all these selection experiments rely on the use of the T7 RNA polymerase followed by a reverse transcription step to convert the modified RNA into natural DNA or on DNA polymerases that have a moderate acceptance for these modified nucleotides. In order to evade these drawbacks, the Romesberg laboratory has evolved a thermally stable variant of the Stoffel fragment of the Taq DNA polymerase, SFM4-3 that readily accepts 2′-OMe-modified nucleoside triphosphates in transcription and reverse transcription polymerization reactions [257]. Impressively, SFM4-3 was capable of PCR amplifying oligonucleotides partially modified with 2′-OMe- and 2′-F-nucleotides. The engineered polymerase SFM4-3 was used to evolve aptamers against the human neutrophil elastase (HNE) using 2′-F-modified purine nucleotides in the SELEX protocol [258]. This initial selection experiment with SFM4-3 was followed by the identification of fully modified 2′-OMe aptamers against HNE using two variants of SFM4-3 [259]. The binding affinity of the modified aptamers stemming from these two selection experiments was comparable (K_d values of 20 nM and 45 nM for the 2′-F- and 2′-OMe-containing aptamers, respectively). The substrate repertoire of SFM4-3 was recently extended to 2′-chloro (2′-Cl), 2′-amino (2′-Am), 2′-azido (2′-Az), and arabinose (Ara) modified triphosphates (Fig. 8) [260]. In this context, 2′-Az-ATP could efficiently be incorporated into DNA by the SFM4-3 polymerase and this nucleotide thus decorated the duplex with a synthetic azide handle for the incorporation of modifications by the click reaction. The introduction of a single alkyne-modified oligonucleotide on the 2′-Az-modified DNA could be exploited to generate bottlebrush-DNA [261]. Hybridization with a biotinylated primer and extension of the primer with the OneTaq DNA polymerase and natural dNTPs led to new dsDNAs which could interact with avidin-coated gold nanoparticles to form gold clusters (see i) in (Fig. 8). Alternatively, bottlebrush-DNAs could be generated by the appendage of two alkynylated DNA primers that are specific for different sequence stretches of the 2686 bp long plasmid pUC19. PCR with the two bottlebrush-DNAs and the plasmid led to the formation of DNA hydrogels (see ii) in (Fig. 8).

Another interesting and important 2′-modification is the 2′-deoxy-2′-fluoro-β-D-arabinose (FANA, **15** in Fig. 9) substitution [262]. The substitution pattern in FANA locks the sugar in a B-DNA-like 2′-*endo* (south) pucker and modified FANA oligonucleotides have an improved affinity for both complementary DNA and RNA sequences [263]. These interesting properties have mainly been exploited for the development of therapeutic oligonucleotides [264] and for the improvement of the thermal stabilization and the resistance against degradation by nuclease of aptamers [265,266]. The FANA-triphosphates are incorporated into DNA by commercially available polymerases such as 9°N_m or Vent (*exo*$^-$) with an efficiency comparable to that of the epimeric 2′-F-ribonucleotides [267,268]. On the other hand, a mutant of the TgoT polymerase, D4K, obtained by compartmentalized self-tagging (CST) can synthesize fully modified FANA oligonucleotides and transcribe the mDNA back to natural DNA [269]. Importantly, polymerase D4K could be used to identify catalytic FANA sequences (FANAzymes) [270] and aptamers against the HIV-1 reverse transcriptase (HIV-1 RT) that bound the intended target with a very high affinity (K_d = 4 pM) [271]. Synthetic genetic polymers consisting of sugar modifications such as FANA, LNA, HNA, and TNA are often referred to as XNAs (xeno nucleic acids) [272,273].

1,5-anhydrohexitol nucleic acids (HNA) represents a rather drastic sugar modification since the (deoxy)ribose is replaced by a six-membered hexitol moiety [274]. HNA forms very stable duplexes with complementary HNA, RNA, and to a certain extent DNA sequences and is resistant to nuclease degradation [275]. Unsurprisingly, due to the important sugar modification, HNA triphosphates (**16**) are rather poor substrates for reverse transcriptases and DNA polymerases [276]. On the other hand, another evolved variant of the TgoT polymerase,

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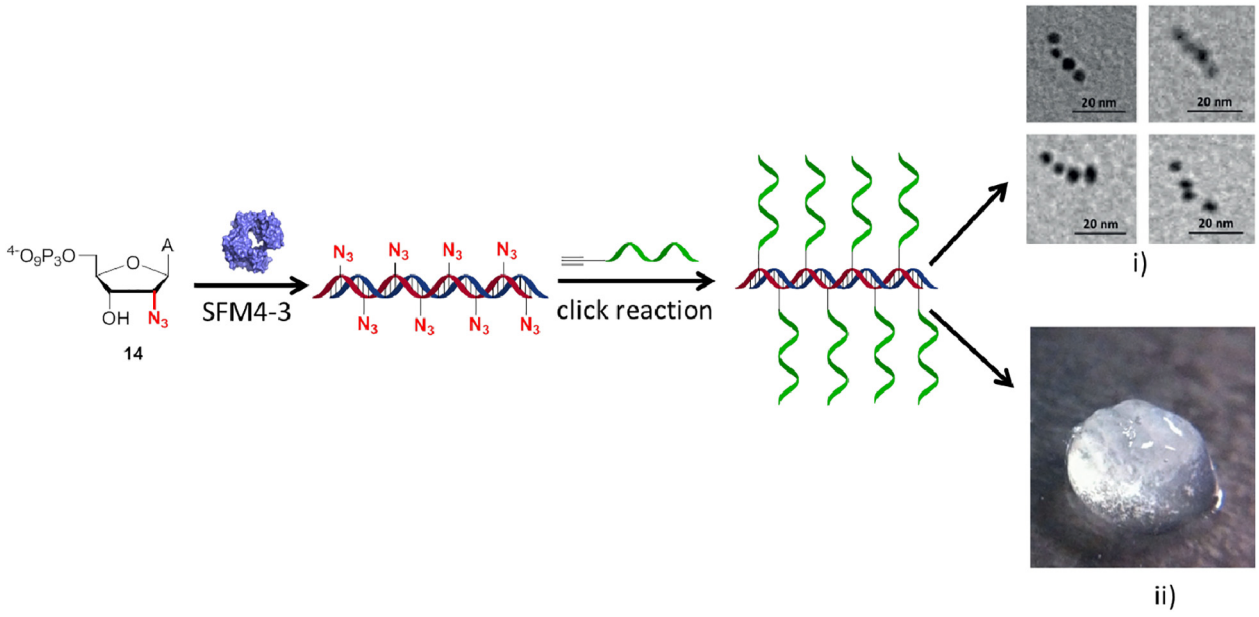

Fig. 8. Practical application of a polymerase recognizing 2′-AzATP. i) The DNA-modified DNA sequence is annealed with a 5′-biotinylated primer which is extended by the OneTaq DNA polymerase. Avidin gold nanoparticles were then assembled to form clusters. ii) two DNA primers recognizing different sequences on a plasmid are clicked on 2′-Az-modified DNA. PCR with the plasmid leads to the formation of large hydrogels. Reproduced with permission [260]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Pol6G12, readily accepts HNA triphosphates as substrates in both transcription and reverse transcription reactions. This capacity at incorporating HNA triphosphates was exploited to isolate HNA aptamers against the trans-activating response RNA (*s*TAR) and the hen egg lysozyme [269]. Similarly, the polC7 polymerase accepts LNA (Fig. 4A) triphosphates and has been used for the generation of LNAzymes [270] but not yet for the identification of LNA aptamers.

Shortening of the backbone repeat by one atom creates the RNA analog TNA (α-L-threose nucleic acid **17**) which contains an unnatural threose sugar. Despite the unusual 5-atom backbone and an A-DNA-like structure, TNA forms stable duplexes with DNA, RNA, and itself and displays a remarkable biological stability (TNA oligonucleotides are stable in serum over 7 days) [277,278]. TNA triphosphates (tNTPs) are readily incorporated into DNA by the engineered polymerase Therminator on defined sequences containing A, T, and C nucleotides but DNA templates containing all 4 nucleotides lead to truncated sequences caused by tG:dG mispairings in the active site of the enzyme [279]. Also, Therminator is not capable of transcribing TNA back to unmodified DNA. Despite these limitations, a Darwinian evolution strategy was devised that disconnects phenotype from genotypes (and thus avoided the transcription of mDNA into unmodified DNA) and that used the 2,6-diamino-TNA-ATP instead of the adenine TNA triphosphate to enhance the stability of the TNA-DNA duplexes formed in primer extension reactions. The library used for the SELEX experiment was exempt of G nucleotides to avoid truncated products and to ensure full sequence coverage. This strategy enabled the selection of a fully modified TNA aptamer against thrombin (K_d ~ 200 nM) [280]. Recently, the Chaput laboratory has developed a new strategy for engineering polymerases called droplet-based optical polymerase sorting (DrOPS). In DrOPS, a library of genes coding for polymerase variants is expressed in *E. coli* cells and then encapsulated in emulsion droplets. Following heat lysis, the polymerases are incubated at the desired temperature for primer extension reactions. Successful polymerase candidates can be detected by a fluorescence read-out since a downstream donor-quencher pair is only liberated in the case of a full extension of the primer. Application of DrOPS allowed the identification of a Mn^{2+}-dependent polymerase, Kod-RI, capable of synthesizing fully modified TNA oligonucleotides in 5-fold faster primer extension reactions than Therminator. In addition, sequencing after an entire replication cycle (DNA → TNA → DNA) revealed a 20-fold higher fidelity compared to Therminator but that Kod-RI still stalls when encountering multiple G-nucleotides in the template [281]. On the other hand, Kod-RI also accepts TNA triphosphates bearing fluorescent nucleobase analogs [282]. A recent crystal structure analysis of the TNA polymerase Kod-RI revealed an imperfect positioning of the incoming TNA triphosphate that might account for the still relatively slow rates of incorporation [283]. Taken together, the identification of polymerases capable of processing TNA building blocks combined with efficient synthetic routes toward TNA triphosphates [284] and base-modified TNA-N*TPs [282,285] will certainly enable the selection of new TNA aptamers with enhanced properties.

As mentioned in Section 3.1.1, many Spiegelmers consisting of unnatural L-scaffolds are currently in various stages of clinical trials and represent a very important class of aptamers. These Spiegelmers are mainly obtained by an indirect selection protocol using a mirror image of the target and natural DNA or RNA libraries followed by the post-

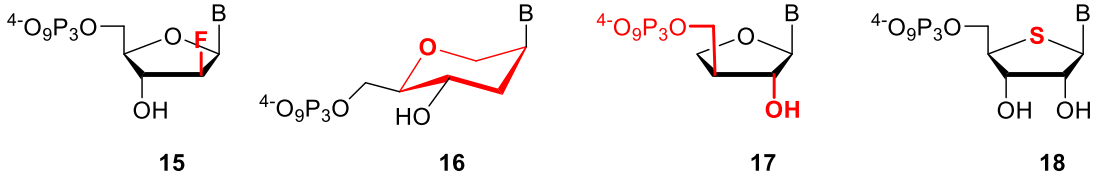

Fig. 9. Chemical structures of sugar modified dN*TPs used in SELEX.

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SELEX conversion to the L-aptamers. However, alternative, faster protocols might be appearing in the near future with the advent of polymerases capable of synthesizing L-DNA and L-RNA sequences [122,123].

Lastly, another (less drastic) modification is the 4′-thio sugar where the ring oxygen is replaced with a sulfur atom (**18** in Fig. 9). The 4′-thio-N*TPs are readily incorporated into RNA by the T7 RNA polymerase in transcription reactions and the resulting modified RNA can be reverse transcribed into DNA by Superscript™ II (RNase H^-). The compatibility of 4′-thio-N*TPs with methods of *in vitro* selection was exploited to identify an aptamer against human α-thrombin [286,287]. The 4′-thio-dN*TPs have also been synthesized and shown to be compatible with enzymatic synthesis [288], however no aptamer selection has been reported yet.

3.2.3. Phosphate modifications

Chemical alterations of the phosphate backbone are not as diverse as for the nucleobase and the sugar units. α-Phosphorothioates (Fig. 3D) are the most common phosphate modification which can be introduced into aptameric scaffolds either by the post-SELEX strategy or by the inclusion of α-thio-(d)N*TPs into the selection protocol. During the synthesis of the α-thio-(d)N*TPs two diastereomers are formed: the [Sp] and [Rp] enantiomers. Interestingly, most DNA and RNA polymerases only accept the [Sp] enantiomers as substrates and the polymerization reactions proceed with an inversion of the configuration to produce [Rp]-phosphorothioate containing oligonucleotides. Conversely, oligonucleotides containing [Rp] enantiomers are more prone to nuclease degradation than their mirror images but are more efficient at recruiting RNase H [289]. Notwithstanding, α-thio-(d)N*TPs have been included in numerous selection experiments for the isolation of aptamers. In a recent example by the group of Gorenstein, a thio-DNA aptamer against the human breast cancer cell lines MDA-MB-231 was obtained using α-thio-dA*TP instead of the natural counterpart in an *in vivo* SELEX protocol. The resulting thio-aptamer T1 displayed a remarkable affinity for the MDA-MB-231 cells (K_d = 2.5 nM) [290]. The same group recently made use of a previously selected DNA thio-aptamer recognizing the transmembrane glycoprotein CD44 to selectively deliver mesoporous silicon microparticles (SMP) to tuberculosis infected cells. The SMP can be charged with chemotherapeutic agents or antibiotics and the resulting SMP-thio-aptamer thus represents a potent carrier system [291]. This example nicely highlights the entire process from SELEX with α-thio-dA*TP to the development of a potent drug delivery system.

Besides α-thio-modified aptamers only one single example of a phosphate modified aptamer has been reported. Indeed, single α-*P*-borano-modified triphosphates (α-B-UTP and α-B-GTP) have been included in the SELEX protocol to isolate RNA aptamers capable of binding to ATP [292].

3.2.4. Aptamers with an expanded genetic alphabet

Expanding the genetic code beyond the A-T/G-C Watson-Crick canonical base pairs and the 20 amino acids of natural organisms is a long standing goal in synthetic biology [293,294]. A reprogrammation of the genetic code can lead to the formation of nucleic acids and proteins with hitherto unknown physico-chemical and structural properties. In the field of nucleic acids, most efforts have focused on the creation of synthetic nucleotides equipped with artificial nucleobases that are capable of forming a third, unnatural base pair (UBP). Most UBPs possess large aromatic moieties or nucleobases with alternate hydrogen bonding patterns (Fig. 10), although artificial metal base pairs have also been suggested as potential candidates [185,295–297].

Besides the creation of semi-synthetic organisms capable of sustaining UBP replication *in vivo* [298,299] and of participating in the site-specific incorporation of a modified amino acid into a protein [300], UBPs have an important and direct implication in the development of chemically modified aptamers. In this context, the **Ds** nucleotide bears a relatively large thiophene moiety capable of excluding the canonical bases while the small **Px** base pair fits well in the **Ds** partner and its nitro group prevents mispairing with dA [301]. The **Ds**-**Px** base pair is truly orthogonal to the natural nucleotides and is amplified by commercial polymerases in PCR with a high fidelity -close to that of the canonical nucleotides [302]. In addition, Hirao et al. have devised a selection protocol coined ExSELEX (genetic alphabet expansion for systematic evolution of ligands by exponential enrichment) to generate aptamers with an expanded genetic code [41,303]. In the ExSELEX protocol, **Ds** nucleotides are introduced into DNA libraries at specific, predetermined locations by solid-phase synthesis. The **Ds**-containing DNA libraries can then be incubated with the target and the recovered sequences are PCR amplified with the six nucleoside triphosphates (*i.e.* the 4 natural and **Ds** and **Px**). A barcode system included in the PCR primer regions flanking the randomized library acts as a positioning system to accurately determine the location of the **Ds** residues after sequencing of the enriched libraries [51,302]. ExSELEX experiments yielded highly potent aptamers against numerous targets including the $VEGF_{165}$ and interferon-γ. These aptamers display binding affinities in the low pM range, which is rarely obtained in selections with natural nucleic acids. Recently, a **Ds**-randomized library has been employed in an ExSELEX experiment for the identification of a **Ds**-modified aptamer against vWF. In this version of ExSELEX, the identification of the location of the **Ds** nucleotides proceeded first by a replacement PCR of the enriched population where the modified nucleotides were substituted either with natural dAs or dTs. Following next-generation sequencing of the PCR amplicons, all the different sequences were isolated by probe hybridization-capture and subjected to a modified Sanger sequencing method. This version of the ExSELEX yielded an anti-vWF aptamer with a K_d value of 75 pM. Moreover, the half-life in human serum as well as the thermostability of the resulting **Ds**-modified aptamer could be further improved by including an additional, remarkably stable mini-hairpin motif at the 3′-end of the sequence. This modification did not alter the binding affinity (actually the K_d value was even slightly improved to 61 pM), highlighting the usefulness of this strategy for the stabilization of aptamers [304]. Thus, the introduction of the **Ds**-**Px** pair combined with a post-SELEX stabilization of sequences with mini-hairpin motifs is a highly efficient method for the generation of highly potent aptamers.

The **dP**-**dZ** system consists of two finely designed and tuned aromatic nucleobases displaying an alternate puAAD-pyDDA (*i.e.* purine {acceptor-acceptor-donor} and pyrimidine {donor-donor-acceptor}) hydrogen-bonding pattern. The complementary nucleotides **dZ** and **dP** form a base pair that is more stable than a canonical dG-dC pair and discriminate for mismatches with an efficiency comparable to that of the natural base pairs [305,306]. These properties explain why this AEGIS (artificially expanded genetic information system) can efficiently

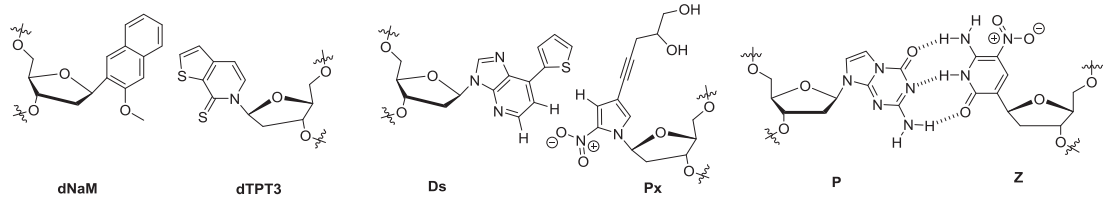

Fig. 10. Examples of nucleotides with modified nucleobases that have been used as unnatural base pair systems.

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be incorporated into DNA by polymerases and replicated by PCR without loss of information through numerous cycles [307]. The larger sequence space created by the addition of the **dZ** and **dP** nucleotides into DNA was also explored in SELEX for the identification of various GACT**ZP** aptamers [308]. In this AEGIS-SELEX protocol, the determination of the position of the modified nucleotides within the sequences is achieved by an alternate sequencing protocol: the enriched population stemming from the selection experiment is PCR amplified in the presence of the four natural dNTPs and **dP**TP as lone modification. All the **dZ** in the population will direct the incorporation of **dP** nucleotides; the absence of **dZ**TP will trigger the misincorporation of natural dT and dC opposite all the **dP** nucleotides in the sequences [309]. In an alternative protocol, **dZ** can be converted into dC and dT while **dP** will be mutated into dA and dG. The exact location of the modified nucleotides can then be inferred from the deep sequencing data of the products stemming from these replacement PCR protocols. Application of AEGIS-SELEX combined with this alternative sequencing method led to the isolation of modified aptamers targeted against MDA-MB-231 breast cancer cells [308], HepG2 liver cancer cells [310], the biomarker glypican 3 [311], and the protective antigen from *Bacillus anthracis* [312]. All these aptamers display K_d values in the low nM range and are extremely stable to nuclease degradation.

The advantage of the GACT**ZP** AEGIS over other existing systems is that the **dZ** and **dP** nucleotides are readily accepted by commercially available polymerases (even though a variant of the Taq polymerase obtained by CST displayed a higher efficiency in incorporating **dZ**TP opposite **dP** [313]), they are not prone to epimerization and oxidation, and are mainly found in a single tautomeric form [314].

4. Conclusions and perspectives

The chemical modification of the scaffold of aptamers is an attractive solution to remediate the shortcomings of these functional nucleic acids. As highlighted in this review, modification of aptamers can be achieved either by supplementing random libraries with functional groups directly in the SELEX protocol or by post-SELEX solid-phase chemical synthesis. The post-SELEX strategy is particularly efficient for the introduction of phosphorothioate units to improve the resistance against nuclease digestion as well as for the incorporation of chemical entities aiming at reducing the rapid renal filtration. However, the introduction of larger modifications often causes small structural changes which in turn perturb the binding efficiency of aptamers. The post-SELEX optimization thus often requires tedious and uncertain SAR studies to identify mutants displaying an acceptable binding capacity. On the other hand, when functionalized libraries are included in the SELEX protocol, the resulting aptamers do not have to undergo SAR studies and by the same token can be equipped with chemical entities that favor interactions with their intended targets. Obviously, aptamers obtained by SELEX with (d)N*TPs can also be further modified by post-SELEX optimization as nicely exemplified by the only commercialized aptamer-based drug pegaptanib. Novel synthetic approaches for the gram-scale preparation of (d)N*TPs coupled with methods for the discovery of engineered polymerases will further increase the chemical landscapes amenable to selection experiments and allow the discovery of more potent modified aptamers decorated with exotic functional groups. Furthermore, chemical modification can be complemented by other methods such as *in silico* approaches including deep learning [315] or new SELEX protocols [246,280,316,317] in order to accelerate the identification of modified aptamers. Therefore, chemistry plays a central role in the development of next-generation modified aptamers with enhanced properties for the development of potent drug delivery systems, therapeutic agents, and point-of-care diagnostic platforms. Progress in nucleic acid chemistry will certainly help bridging the gap between aptamers and antibodies [12,230,318] and provide aptamers with the right impetus to accelerate clinical and commercial applications [14].

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