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Nucleic acid enzymes based on functionalized nucleosides

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Nucleic acid-based enzymes have recently joined their proteinaceous counterparts as important biocatalysts. While RNA enzymes (ribozymes) are found in nature, deoxyribozymes or DNAzymes are man-made entities. Numerous ribozymes and DNAzymes have been identified by Darwinian selection methods to catalyze a broad array of chemical transformations. Despite these important advances, practical applications involving nucleic acid enzymes are often plagued by relatively poor pharmacokinetic properties and cellular uptake, rapid degradation by nucleases and/or by the limited chemical arsenal carried by natural DNA and RNA. In this review, the two main chemical approaches for the modification of nucleic acid-based catalysts, particularly DNAzymes, are described. These methods aim at improving the functional properties of nucleic acid enzymes by mitigating some of these shortcomings. In this context, recent developments in the post-SELEX processing of existing nucleic acid catalysts as well as efforts for the selection of DNAzymes and ribozymes with modified nucleoside triphosphates are summarized.

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Introduction

Catalysis of chemical transformations is often assumed to be mediated by small organic molecules, transition metal complexes or by proteinaceous enzymes. However, the seminal discovery of self-splicing introns and the advent of Darwinian evolution methods impressively demonstrated that nucleic acids (DNA and RNA) are also capable of promoting catalysis and this with an efficiency that can rival that of protein enzymes [1••,2]. Since the advent of the first deoxyribozyme or DNAzyme in 1994 [3], DNA molecules have been selected to catalyze a broad variety of chemical reactions (e.g. hydrolysis of ribophosphodiester linkages [1••,4•] or the Diels–Alder reaction [5]), to handle peptide substrates [6] or to recognize subtle differences in the chemical composition of their substrates [7••]. This vast chemical repertoire combined with often impressive enhancements in rate constants culminated in the completion of two recent clinical trials for the treatment of skin cancer [8] and asthma [9] based on DNAzymes. Despite these favorable features, catalytic nucleic acids still suffer from some shortcomings. While some of these limitations are inherent to their nature, others also impact different families of oligonucleotides: for instance, DNAzymes and nucleic acids in general are rather reluctant at crossing biological barriers (such as cell membranes or the blood-brain-barrier) which complicates their cellular internalization. Also, since DNAzymes consist of a native DNA backbone and are rather small oligonucleotides they are subject to efficient degradation by circulating nucleases and are prone to rapid renal filtration which massively reduces their *in vivo* residence time. Indeed, early studies on natural DNA oligonucleotides showed that unmodified nucleic acids survived less than an hour in serum [10]. In addition, decreased catalytic activities are often observed under *in vivo* conditions due to the change from rather simple buffer systems used during SELEX to very complex and crowded environments of biological systems. This decrease in catalytic activity is often exacerbated by the low physiological concentrations of M^{2+} which are highly required cofactors. Consequently, the catalytic performance of DNAzymes under *in vivo* conditions is often severely decreased and competition with antisense effects can be observed. Lastly, enzymes display a much larger chemical repertoire and higher k_{cat} values than nucleic acid-based catalysts (10–1000 s^{-1} [11] versus 1–10 min^{-1}, respectively) due to the broad functional diversity accessible to proteins compared to DNA and RNA.

This Opinion article will highlight the two main chemical strategies that have been devised to address some, or all, of these shortcomings and generally improve the properties of DNAzymes: post-SELEX modification of existing scaffolds by solid-phase synthesis and inclusion of modified nucleoside triphosphates (dN*TPs) directly in the selection protocol. Lastly, we briefly describe how these strategies have been applied to their RNA counterparts.

Chemical modification of existing scaffolds

A seemingly straightforward procedure for the enhancement of the properties of DNAzymes is the modification of existing scaffolds by solid-phase synthesis. In this post-SELEX

method, one (or multiple) nucleotide of the sequence of a DNAzyme is replaced by a suitably modified analog via its phosphoramidite building block and the activity of the resulting sequence is then compared to that of the parent DNAzyme. This method is very effective for the truncation of DNAzymes into minimal sized active sequences to facilitate chemical synthesis [12,13] as well as for the conversion of enzymes isolated by SELEX into species capable of multiple turnover (i.e. *trans* catalysts) [14]. Besides size optimization and conversion to *trans*-cleavers, the post-SELEX method also permits improvements in the resistance against nuclease degradation. This can be achieved either by modification of the global structural configuration or by addition of functional groups to existing DNAzymes. In terms of structural modifications, DNAzymes can be integrated into larger DNA nanostructures with well-defined functions and shapes [15,16]. For instance, the sides of a DNA tetrahedron were recently replaced by the sequence of DNAzyme 8–17 without significantly perturbing its catalytic activity (Figure 1b). The resulting constructs displayed a remarkable cellular uptake and biological stability both imparted by its tetrahedral nature. These combined properties conveyed an efficient gene silencing activity to the DNAzyme-tetrahedron when assayed in NIH3T3 cancer cells [17•]. As in the case of other therapeutic oligonucleotides, the biological and chemical stability of DNAzymes can also be improved by incorporating modified building blocks such as locked nucleic acids (LNA), phosphorothioates (PS), or 2'-fluoro (F) nucleotides into the biding arms of DNAzymes (and to a lesser extent their catalytic cores) [18,19].

The post-SELEX method is also beneficial for nucleic acid catalysts in other practical applications. For instance, the spatiotemporal control of the activity of DNAzymes can be achieved by photocaging specific nucleotides located either in their substrates or catalytic cores. Light irradiation of the blocked enzyme then restores the original scaffold and hence the catalytic activity [20,21•]. In a recent example, a single nucleotide of the substrate of a Zn^{2+}-dependent RNA-cleaving DNAzyme was blocked with a 2'-*O*-nitrobenzyl group and conjugated onto lanthanide-doped upconversion nanoparticles (UCNPs). After injection into zebrafish, the UCNPs converted the incident near infrared light into a localized 365 nm light emission which concomitantly removed all the photolabile blocking groups. This elegant strategy permitted a precise mapping of the Zn^{2+}-distribution in living organisms [22••].

Since nucleic acids are barely soluble in any other solvent than water, modifying the scaffold of DNAzymes with hydrophobic units can improve their catalytic activity in organic media. In a first example, Abe *et al.* demonstrated that a G-quadruplex forming DNAzyme equipped with a 5'-end polyethylene glycol (PEG) moiety was capable of oxidizing luminol in pure methanol [23•]. More recently, a high-throughput screening platform based on amphiphilic DNA-encoded libraries was developed to identify small molecule catalysts for the aldol reaction [24]. This method allowed to isolate potent catalysts and demonstrated the possibility of using DNA libraries in organic media, boding well for the conversion of existing DNAzymes into organic solvent compatible catalysts [25].

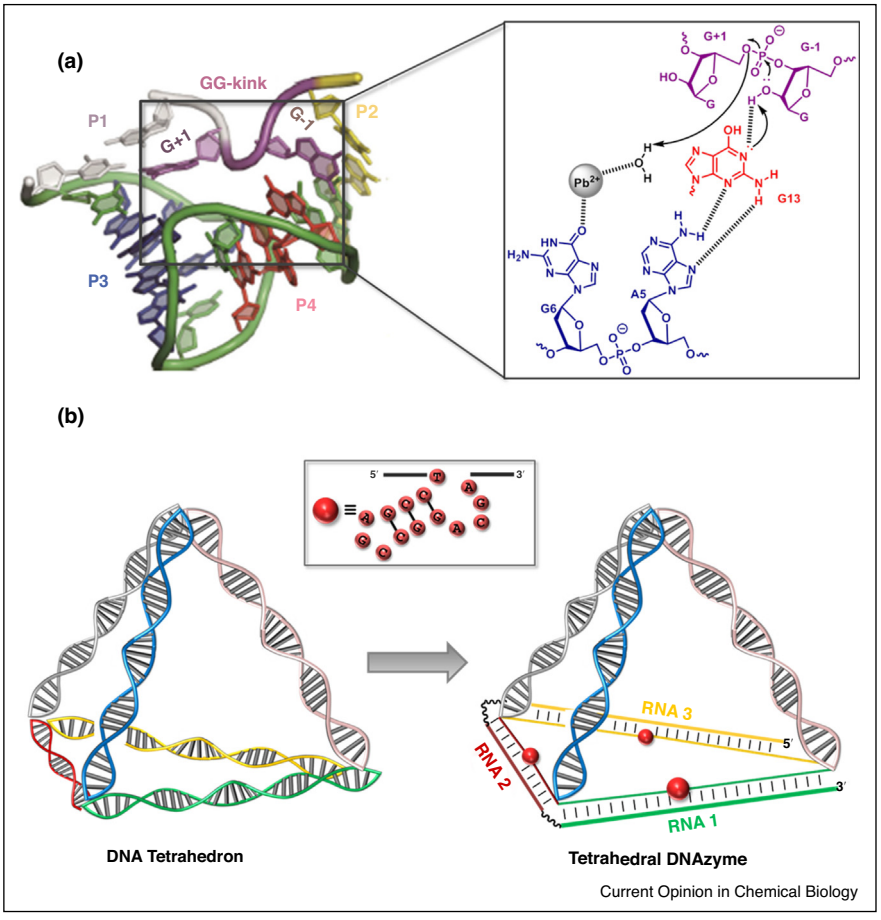
Lastly, the introduction of heavy atoms or paramagnetic labels into the scaffold of DNAzymes could facilitate their structural characterization by X-ray crystallography [26] or electron paramagnetic resonance (EPR) spectroscopy, respectively. In this context, the introduction of selenium containing nucleotides by solid-phase synthesis has advanced as a popular strategy to alleviate the phase problem encountered in nucleic acid crystallography [27] and has already permitted the elucidation of the X-ray structure of a Diels–Alder ribozyme [28••]. Similarly, various phosphoramidite building blocks exist for the labelling of RNA [29] and DNA [30] oligonucleotides with persistent radicals (e.g. nitroxides) by solid-phase synthesis. This approach has been applied for the investigation of the mechanism and folding of ribozymes [31,32] and aptamers [33•] but not for DNAzymes. Recently, a method combining the incorporation of modified triphosphates and solid-phase synthesis has been developed for the position-selective labeling of RNA [34••] which could accelerate and facilitate the introduction of labels into the scaffold of nucleic acid catalysts.

In contrast, appendage of chemical functionalities on the nucleotides composing the catalytic core only led to moderate improvements (up to ~40-fold [35]) in observed rate constants (k_{obs}) and did not substantially alleviate the strong M^{2+}-dependence of DNAzymes [36]. These modest improvements in terms of rate constants and M^{2+}-dependence might be helped in the future by the recent elucidation of crystal structures of DNAzymes (Figure 1a) [37••,38••] which will guide the introduction of functional groups to specific locations and concomitantly reduce the massive effort involved in these structure-activity relationship (SAR) studies.

Chemical diversity in SELEX

In addition to solid-phase synthesis, additional functional groups can be introduced into nucleic acids via the enzymatic polymerization of dN*TPs. Unlike solid-phase synthesis, this approach only requires the dN*TPs to be accepted as substrates by natural or engineered polymerases. The choice of the location of the modification and to a certain extent, the nature of the chemical groups is dictated in part by structural investigations [39] but largely by empirical rules. Modifications affixed at the C5-position of pyrimidines and *N*7 of 7-deazapurines are usually well-tolerated by DNA polymerases. In contrast, alteration of the sugar moiety is more delicate since this often leads to poor substrate recognition of the

Figure 1



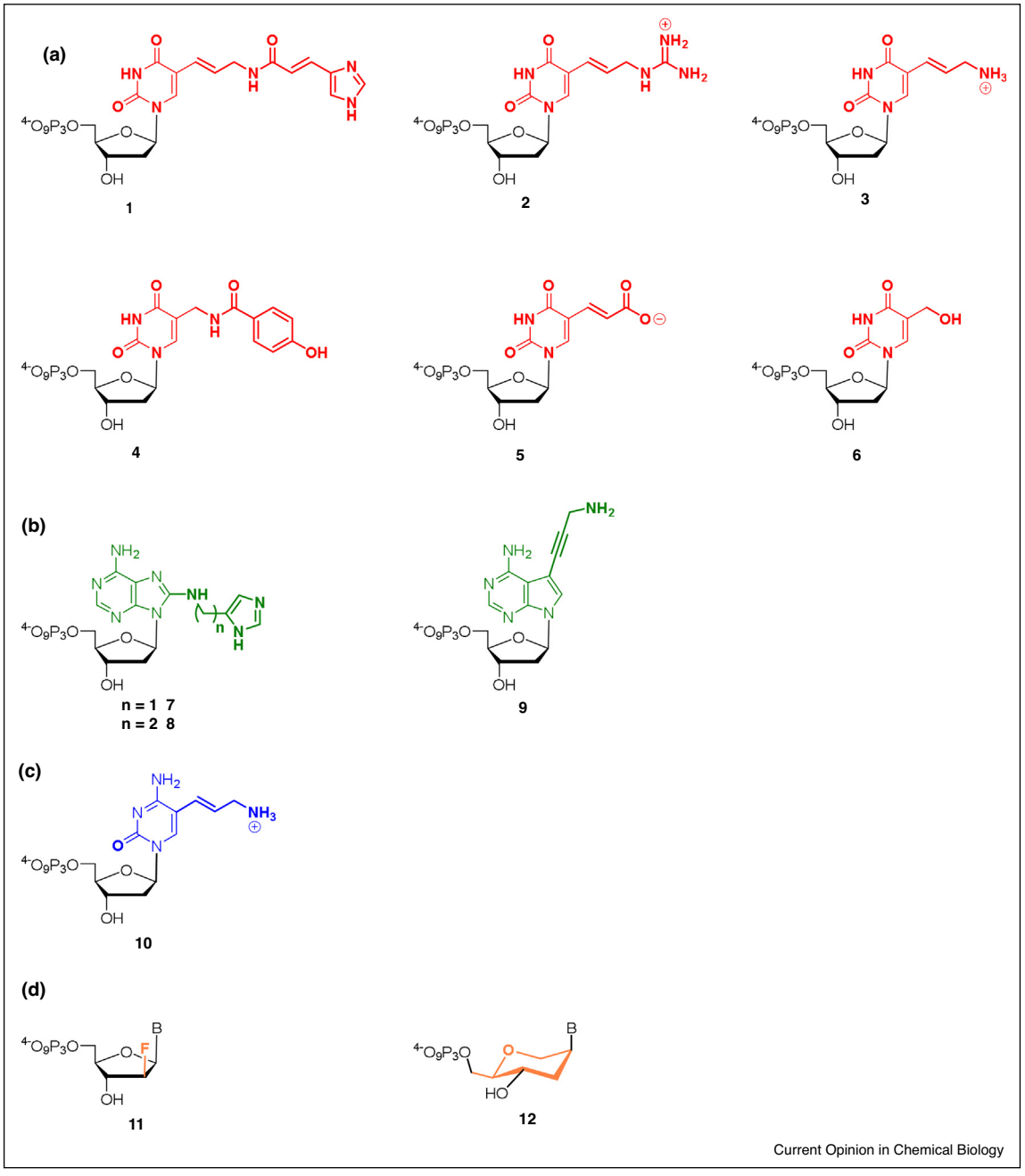
(a) Close-up view of the catalytic core of the RNA-cleaving DNAzyme 8–17 and schematic representation of the putative chemical mechanism deduced from the structural analysis. In this proposed mechanism, the *N*1 atom of the critically conserved residue G13 (in red) forms a hydrogen bond with the 2'−OH of rG-1 (in violet) located on the substrate thus acting as general base to increase the nucleophilicity of this group in the subsequent in-line attack on the phosphate moiety. G13 also forms a hydrogen bond with A5 (in blue) forming a non-canonical dG13:dA5 base pair. The Pb^{2+} cofactor is coordinated to both the O6 atom of G6 and a water molecule. The water molecule acts as general acid by donating a proton to the O5' atom of the leaving group. The sequences used in the crystallization of DNAzyme 8–17 were 5'-TGTAACGCAC**TGCCAGCGGCTCGAA**ATCTCTCTCGT for the catalyst and 5'-ACGAGAGAGAT*GGG*GGTGCGTTACA for the substrate (catalytic core residues are shown in bold and kink site RNA nucleotides in italic). Adapted from Ref. [38••]. **(b)** Schematic representation of the construction of tetrahedral DNAzymes. Three edges of a DNA tetrahedron were converted to single stranded oligonucleotides that contained the 8–17 catalytic motif (shown in the insert) and designed to target different regions of EGFP mRNA. The resulting construct was shown to hydrolyze three different RNA substrates connected together by spacers as well as mRNA in cells [17•].

corresponding nucleotides by polymerases [40]. Modifications brought at the level of the C2' and *O*4' positions have been shown to be processed by polymerases especially by specifically engineered polymerases [41–43]. Chemical alterations to the backbone are less common and essentially rely on phosphorothioate and phosphonomethyl modifications [44•].

The polymerization of dN*TPs has been used for a number of applications including the labelling of DNA and RNA with fluorophores [45,46••], the construction of fully modified genes both *in vitro* and *in vivo* [47] and the enzymatic immobilization of oligonucleotides and functional nucleic acids on solid supports [48,49]. Modified triphosphates also represent a convenient vector for the introduction of chemical diversity in selection experiments. Substitution of one or more canonical nucleotides with the corresponding dN*TP in primer extension reactions or PCR leads to the creation of modified randomized libraries of oligonucleotides that are directly amenable to SELEX. This procedure will avoid the uncertain SAR studies involved in the post-SELEX method and directly generate DNAzymes that display the desired modification pattern. A first example was reported by Santoro and Joyce who used a dU*TP equipped with an imidazole moiety (**1** in Figure 2) instead of its natural counterpart dTTP to select a Zn^{2+}-dependent RNA-cleaving DNAzyme. A synthetic, multiple turnover, version of the

resulting DNAzyme 16.2–11 was shown to indiscriminately hydrolyze two different 18-mer and 19-mer all-RNA substrates with an impressive catalytic efficiency (Table 1) [50]. The imidazole moieties of the three conserved modified nucleotides coordinate the Zn^{2+} cofactor which participates in catalysis, presumably through the activation of a 2'−OH group located on the substrate. Inspired by this seminal work, and recognizing the general acid–base mechanism adopted by the protein RNase A, the Perrin laboratory devised a selection protocol where two modified nucleotides replaced their natural counterparts with the intent of creating a synthetic mimic of the active site of the ribonuclease but in an oligonucleotide context. Application of this selection protocol with nucleotides **3** and **8** (Figure 2) resulted in the identification of the M^2

Figure 2



Chemical structures of all the dN*TPs that have been used in selection experiments for the isolation of modified DNAzymes. **(a)–(c)** nucleobase modifications; **(d)** sugar modified dN*TPs.

Table 1

Summary of the specifications of all reported DNAzymes obtained by including dN*TPs in selection experiments

DNAzyme	dN*TP(s)	Reaction catalyzed	Substrate	Single turnover (k_{obs})	Multiple turnover (k_{cat}/K_m)	Cofactor(s)	References
16.2–11	**1**	RNA hydrolysis	All-RNA	$1.5\,min^{-1}$	$\sim 10^8\,M^{-1}\,min^{-1}$	Zn^{2+} and Mg^{2+}	[50]
11–17PheO	**4**	RNA hydrolysis	Single rC	$0.20\,min^{-1}$	n.a.	Ca^{2+}, Mg^{2+}, Mn^{2+} or Zn^{2+}	[65]
10–13	**3** and **8**	RNA hydrolysis	Single rC	$0.04\,min^{-1}$	n.a.	Hg^{2+}	[66]
	1 and **9**	RNA hydrolysis	All-RNA	0.06 and $0.07\,min^{-1}$	n.a.	None	[53]
9_{25}-11	**3** and **8**	RNA hydrolysis	Single rC	$0.20\,min^{-1}$ [a]	$5.3 \times 10^5\,min^{-1}\,M^{-1}$ [a]	None	[13,51,52]
9-86	**2**, **8**, and **10**	RNA hydrolysis	Single rC	$0.13\,min^{-1}$ [a]	n.a.	None	[55]
10–66	**2**, **8**, and **10**	RNA hydrolysis	Single rC	$0.50\,min^{-1}$ [a]	$5 \times 10^5\,min^{-1}\,M^{-1}$	None	[14]
12–91	**2**, **8**, and **10**	RNA hydrolysis	All-RNA	$0.06\,min^{-1}$	n.a.	None	[56]
7-38-32	**2**, **8**, and **10**	RNA hydrolysis	All-RNA	0.06 and $4.9\,min^{-1}$	$8.2 \times 10^4\,min^{-1}\,M^{-1}$	None	[57••]
20–49	**2**, **7**, and **10**	RNA hydrolysis	Single rC	$3.1 \times 10^{-3}\,min^{-1}$	n.a.	None	[58]
AmideAm1	**3**	Amide-bond hydrolysis	Single aliphatic amide bond	$10^{-3}\,min^{-1}$	n.a.	Zn^{2+}/Mg^{2+} or Zn^{2+}/Mn^{2+}	[61••]
FR17_6	FANA **11**	RNA hydrolysis	All-RNA	$0.06\,min^{-1}$	Multiple turnover	Mg^{2+}	[63]
F2R17_1	FANA **11**	RNA ligation	5'ppp-RNA and 3'-OH-RNA	$2 \times 10^{-4}\,min^{-1}$	n.a.	Mn^{2+} or Mg^{2+}	[63]
FplmR4_2	FANA **11**	FANA ligation	3'-phosphorylimidazolide FANA and 5'-OH-FANA	$0.04\,min^{-1}$	n.a.	Zn^{2+}	[63]
NGS12-7	FANA **11**	RNA hydrolysis	All-RNA	$0.027\,min^{-1}$	$2.9 \times 10^5\,min^{-1}\,M^{-1}$	Ca^{2+}, Mn^{2+}, or Mg^{2+}	[64•]
NGS12-7	FANA **11**	DNA hydrolysis	All-DNA	$0.19\,min^{-1}$	Multiple turnover	Mg^{2+}	[64•]

[a] Determined at 24°C.

$^{+}$-independent RNA-cleaving DNAzyme 9_{25}-11 [13,51]. The presence of the amine and imidazole moieties on the scaffold of 9_{25}-11 was found to be strictly required to support global folding as well as a general acid-base catalytic mechanism [51]. This finely tuned enzyme led to a 10^3-fold improvement in rate constant when compared to M^{2+}-independent, unmodified self-cleaving DNAzyme species [52]. Following a similar protocol, Sidorov *et al.* isolated another DNAzyme capable of hydrolyzing RNA in the absence of M^{2+} cofactors [53]. Unlike 9_{25}-11, this DNAzyme was obtained with nucleotides **1** and **9** which allowed for sampling of larger sequence and chemical spaces than with nucleotide **8** which is not well polymerized. In addition, while 9_{25}-11 only catalyzes the hydrolysis of substrates containing single ribonucleotides at an optimum temperature of 13°C, this DNAzyme readily cleaved all-RNA substrates at 37°C with only slightly lower first order rate constants albeit at two distinct cleavage sites. Collectively, these two catalysts demonstrated that additional imidazole and amine groups massively aided functionality deprived DNA to promote RNA hydrolysis in the absence of M^{2+} cofactors. In contrast, both catalysts presented a number of shortcomings and a partial solution to these was obtained by including a third modified nucleotide in the selection protocol to accompany triphosphates **8** and **10**. This additional nucleotide **2** bears a guanidium group which was hypothesized to offer electrostatic stabilization of the negatively charged phosphate backbone of DNA through its high pK_a value as well as cooperation in the hydrolysis of phosphodiester linkages [54]. A first selection experiment with these dN*TPs led to the identification of DNAzyme 9–86 which promoted the hydrolysis of a single ribophosphodiester bond and functions with first-order rate constants comparable to that of 9_{25}-11 but at higher optimal temperatures (37°C) [55]. An increase in the length of the randomized sequence from N_{20} to N_{40} led to the selection of DNAzyme 10–66 which self-cleaved a substrate containing a single embedded ribocytosine with an impressive rate constant and even functioned in a minimal buffer system that normally does not sustain catalytic activity by any unmodified nucleic acid species [14]. Even though 10–66 could be converted into a *trans*-cleaving species, both DNAzymes were very reluctant at recognizing and hydrolyzing all-RNA substrates which is an important prerequisite for potential gene silencing applications. Recognizing that the B-helical nature of a sequence containing a single ribonucleotide might prevent the recognition of a more A-helical-like structure of an all-RNA substrate, a selection experiment with an oligonucleotide containing 12 consecutive RNA nucleotides permitted identification of DNAzyme 12–91 (Table 1). While 12–91 displayed an appreciable catalytic efficiency for the hydrolysis of an all-RNA substrate, comparable to the DNAzyme identified by Sidorov *et al.*, this enzyme displayed even higher catalytic activity with a substrate that contained a single

embedded RNA nucleotide [56]. Lastly, a solution to M^{2+}-independent all-RNA cleavage with multiple turnover kinetics was found through the identification of DNAzyme 7-38-32 [57••]. This catalytic DNA molecule was obtained by selection with a larger sequence space (12–91 was obtained with an N_{20} randomized library) and nucleotides **2**, **8**, and **10**. DNAzyme 7-38-32 cleaved the 19-nt long RNA substrate at a specific site and displayed a catalytic efficiency (i.e. k_{cat}/K_M) comparable to that of 10–66 and 9_{25}-11. Collectively, all these DNAzymes obtained by SELEX with base-modified dN*TPs clearly demonstrate the potential and the usefulness of this method in the context of RNA hydrolysis. However, a number of unanswered questions still remain: 1) all these DNAzymes benefit from protein-like functional groups connected to the nucleobase via rather short and rigid linker arms. The exact length and nature of this linker arm is still matter of debate [58] and more exotic non-protein like functionalities should be explored in selection experiments to further increase the catalytic potency (especially in terms of k_{cat} values) [59]; 2) as for unmodified DNAzymes, no general rules have been established for the choice and the nature of the length of the randomized library [60]; 3) there might be an excess of functional groups due to the nature of the enzymatic preparation of the modified libraries: for instance the catalytic core of 7-38-32t contains 12 guanidinium, 10 imidazole and 6 amine groups while RNase A only employs two histidines and one amine for catalysis.

So far, most base-modified catalysts identified by SELEX were designed to hydrolyze RNA substrates. A notable exception was recently reported by Zhou *et al.* who included three different dU*TPs (**3**, **5**, and **6**) in selection experiments to identify amide-hydrolyzing DNAzymes—a reaction that is notoriously difficult to achieve by canonical nucleic acids. The isolated modified DNAzymes hydrolyzed an aliphatic amide bond with first rate constants of ~0.2 $hour^{-1}$ and with appreciable yields (up to 60%) [61••].

As is the case for aptamers, DNAzymes obtained with sugar-modified dN*TPs included in SELEX are scarce due to the rather poor substrate tolerance of these nucleotide analogs by polymerases [62]. In a first example, engineered versions of the TgoT polymerase were used for the synthesis Xenobiotic Nucleic Acid (XNA) libraries and their conversion to wild-type DNA [42]. These mutant polymerases allowed for identification of various XNAzymes, particularly with 2'-fluoroarabino nucleic acid (FANA **11**), with ribonuclease and ligase activities albeit with modest first order rate constants when compared to that observed for base-modified or wild-type catalysts and at rather high Mg^{2+} concentrations [63]. More recently, an efficient RNA-cleaving FANAzyme was isolated using only naturally occurring DNA polymerases for the construction of the FANA library (Tgo DNA polymerase) and for its transcription back into wild-type DNA (wild-type Bst DNA polymerase, large fragment). The most active species (and the most abundant sequence) NGS12-7 displayed an appreciable catalytic efficiency for the hydrolysis of all-RNA substrates with Michaelis–Menten kinetics and required the presence of 20 mM Mg^{2+} for activity—comparable to what wild-type DNAzymes such as 10–23 or 8–17 necessitate [1••]. Interestingly, FANAzyme NGS12-7 was more proficient at hydrolyzing phosphodiester bonds with DNA than with RNA substrates (~7-fold improvement in rate constant) [64•]. These selection experiments have led to the identification of DNAzymes displaying similar biochemical properties to wild-type catalysts but with the advantages displayed by sugar-modified nucleotides such as improved nuclease resistance.

Modified ribozymes

As mentioned in the introduction, the post-SELEX method as well as the inclusion of modified triphosphates in selection experiments can be used to ameliorate the general properties of ribozymes. However, examples of chemically modified ribozymes, either synthetic constructs obtained by SELEX or naturally occurring systems, are far less abundant than for DNAzymes. Early examples involving the modification of the hammerhead ribozyme by solid-phase synthesis strived to identify nuclease resistant analogs [67] or catalytically more competent species [68]. These early examples were then rejoined by other synthetic analogs displaying a triazole-based backbone motif substituting the natural phosphate group [69].

An important improvement of the scope of ribozymes was achieved by the Eaton laboratory by using a pyridylmethyl-substituted U*TP to identify a ribozyme capable of catalyzing the Diels–Alder cycloaddition [70]. More recently, an ingenious method, combining RNA polymerase-mediated incorporation of modified ribonucleotides and ligation, was devised to introduce fluorescent residues into the structural motif of the minimal hammerhead ribozyme. The resulting modified transcripts were then used to probe the folding and mechanism of this important biocatalyst [46••].

In contrast, it is very likely that new modified analogs of ribozymes will emerge in the near future. Indeed, these RNA-based catalysts have been characterized comparatively well by structural studies which gave insight into their folding and catalytic mechanisms [71]. This important knowledge might then be exploited to drive chemical modification by solid-phase synthesis of existing ribozymes towards the rapid development of more stable or catalytically more competent species. In addition, various polymerases have been engineered to transcribe [40,72,73], even under PCR-like conditions [74•], a broader range of modified nucleotides. These mutant

polymerases, combined with post-transcriptional labelling methods [75], will allow the diversification of the chemical space explored in selection experiments.

Conclusions and future perspectives

Since their first advent, DNAzymes have rapidly progressed into an important class of biocatalysts. The chemical methods described herein have addressed certain shortcomings met with catalysts based on canonical DNA and have also expanded the scope of DNAzymes to reactions that are difficult to achieve without the aid of modified scaffolds (e.g. amide bond hydrolysis). However, additional chemical methods will be required to further improve the performance of DNAzymes in the future. For instance, strategies such as the inclusion of pK_a motifs [76••] or mini-hairpin structures [77] that have been developed for aptamers could be transferred to DNAzymes and ribozymes to further consolidate their intricate three-dimensional structures. Moreover, the selection of modified DNAzymes could benefit from new SELEX protocols developed to either circumvent the need for a polymerase-based construction of libraries [78•,79•] or one-step selection methods that will facilitate and accelerate the identification of new catalysts [80]. The selection-based approach could also benefit from the inclusion of additional (more) exotic functional groups on nucleoside triphosphates [59] or alternative backbone modifications [44•]. These, and other emerging methods, combined with the development of new chemistries and engineered polymerases will certainly contribute to a further improvement and fine-tuning of the properties of DNA enzymes and ribozymes in order to allow development of not only novel therapeutic agents, but also biosensing devices, imaging agents, and tools for the modification of proteins based on nucleic acids catalysts [81,82].

Conflict of interest statement

Nothing declared.

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References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. •• Santoro SW, Joyce GF: **A general purpose RNA-cleaving DNA enzyme**. *Proc Natl Acad Sci U S A* 1997, **94**:4262-4266.
Describes the selection of the most active DNAzymes (8–17 and 10–23) to date.

2. Roth A, Weinberg Z, Chen AGY, Kim PB, Ames TD, Breaker RR: **A widespread self-cleaving ribozyme class is revealed by bioinformatics**. *Nat Chem Biol* 2014, **10**:56-60.

3. Breaker RR, Joyce GF: **A DNA enzyme that cleaves RNA**. *Chem Biol* 1994, **1**:223-229.

4. • Mei SHJ, Liu ZJ, Brennan JD, Li YF: **An efficient RNA-cleaving DNA enzyme that synchronizes catalysis with fluorescence signaling**. *J Am Chem Soc* 2003, **125**:412-420.
In this report, the selection of the second most active DNAzyme (after 10–23 and 8–17) is described.

5. Chandra M, Silverman SK: **DNA and RNA can be equally efficient catalysts for carbon-carbon bond formation**. *J Am Chem Soc* 2008, **130**:2936-2937.

6. Chandrasekar J, Silverman SK: **Catalytic DNA with phosphatase activity**. *Proc Natl Acad Sci U S A* 2013, **110**:5315-5320.

7. •• Sednev MV, Mykhailiuk V, Choudhury P, Halang J, Sloan KE, Bohnsack MT, Höbartner C: **N6-methyladenosine-sensitive RNA-cleaving deoxyribozymes**. *Angew Chem Int Ed* 2018, **57**:15117-15121.
First report of DNAzymes capable of distinguishing RNA substrates containing the natural base modification N6-methyladenosine.

8. Cho EA, Moloney FJ, Cai H, Au-Yeung A, China C, Scolyer RA, Yosufi B, Raftery MJ, Deng JZ, Morton SW *et al.*: **Safety and tolerability of an intratumorally injected DNAzyme, Dz13, in patients with nodular basal-cell carcinoma: a phase 1 first-in-human trial (DISCOVER)**. *Lancet* 2013, **381**:1835-1843.

9. Krug N, Hohlfeld JM, Kirsten AM, Kornmann O, Beeh KM, Kappeler D, Korn S, Ignatenko S, Timmer W, Rogon C *et al.*: **Allergen-induced asthmatic responses modified by a GATA3-specific DNAzyme**. *N Engl J Med* 2015, **372**:1987-1995.

10. Shaw JP, Kent K, Bird J, Fishback J, Froehler B: **Modified deoxyoligonucleotides stable to exonuclease degradation in serum**. *Nucleic Acids Res* 1991, **19**:747-750.

11. Davidi D, Noor E, Liebermeister W, Bar-Even A, Flamholz A, Tummler K, Barenholz U, Goldenfeld M, Shlomi T, Milo R: **Global characterization of in vivo enzyme catalytic rates and their correspondence to in vitro k(cat) measurements**. *Proc Natl Acad Sci U S A* 2016, **113**:3401-3406.

12. Torabi S-F, Wu P, McGhee CE, Chen L, Hwang K, Zheng N, Cheng J, Lu Y: **In vitro selection of a sodium-specific DNAzyme and its application in intracellular sensing**. *Proc Natl Acad Sci U S A* 2015, **112**:5903-5908.

13. Lermer L, Roupioz Y, Ting R, Perrin DM: **Toward an RNaseA mimic: a DNAzyme with imidazoles and cationic amines**. *J Am Chem Soc* 2002, **124**:9960-9961.

14. Hollenstein M, Hipolito CJ, Lam CH, Perrin DM: **A DNAzyme with three protein-like functional groups: enhancing catalytic efficiency of M^{2+}-independent RNA cleavage**. *ChemBioChem* 2009, **10**:1988-1992.

15. Shimron S, Magen N, Elbaz J, Willner I: **pH-programmable DNAzyme nanostructures**. *Chem Commun* 2011, **47**:8787-8789.

16. Yang XL, Tang YA, Mason SD, Chen JB, Li F: **Enzyme-powered three-dimensional DNA nanomachine for DNA walking, payload release, and biosensing**. *ACS Nano* 2016, **10**:2324-2330.

17. • Thai HBD, Levi-Acobas F, Yum SY, Jang G, Hollenstein M, Ahn DR: **Tetrahedral DNAzymes for enhanced intracellular gene-silencing activity**. *Chem Commun* 2018, **54**:9410-9413.
This article describes the incorporation of the sequence of DNAzyme 8–17 into a DNA tetrahedron in order to improve the cell penetration, nuclease resistance, and overall gene silencing activity.

18. Schubert S, Gül DC, Grunert H-P, Zeichhardt H, Erdmann VA, Kurreck J: **RNA cleaving '10-23' DNAzymes with enhanced stability and activity**. *Nucleic Acids Res* 2003, **31**:5982-5992.

19. Chakrayarthy M, Aung-Htut MT, Le BT, Veedu RN: **Novel chemically-modified DNAzyme targeting integrin alpha-4 RNA transcript as a potential molecule to reduce inflammation in multiple sclerosis**. *Sci Rep* 2017, **7**:8.

20. Hwang K, Wu PW, Kim T, Lei L, Tian SL, Wang YX, Lu Y: **Photocaged DNAzymes as a general method for sensing metal ions in living cells**. *Angew Chem Int Ed* 2014, **53**:13798-13802.

21. • Kamiya Y, Arimura Y, Ooi H, Kato K, Liang XG, Asanuma H: **Development of visible-light-responsive RNA scissors based on a 10-23 DNAzyme**. *ChemBioChem* 2018, **19**:1305-1311.
In this article, azobenzene units were incorporated into the catalytic core of DNAzyme 10–23 which allowed for the reversible, light-dependent photoswitching of protein expression.

22. •• Yang Z, Loh KY, Chu Y-T, Feng R, Satyavolu NSR, Xiong M, Nakamata Huynh SM, Hwang K, Li L, Xing H *et al.*: **Optical control of metal ion probes in cells and zebrafish using highly selective DNAzymes conjugated to upconversion nanoparticles**. *J Am Chem Soc* 2018, **140**:17656-17665.
This article describes a nanoprobe based on a photocaged-DNAzyme for the real-time monitoring of Zn^{2+} in zebrafish from the embryonic to the larval stage.

23. • Abe H, Abe N, Shibata A, Ito K, Tanaka Y, Ito M, Saneyoshi H, Shuto S, Ito Y: **Structure formation and catalytic activity of DNA dissolved in organic solvents**. *Angew Chem Int Ed* 2012, **51**:6475-6479.
First report of a DNAzyme equipped with a PEG group that maintains strong catalytic activity in an organic solvent.

24. Hook KD, Chambers JT, Hili R: **A platform for high-throughput screening of DNA-encoded catalyst libraries in organic solvents**. *Chem Sci* 2017, **8**:7072-7076.

25. Yu TM, Zhou WH, Liu JW: **Ultrasensitive DNAzyme-based Ca2+ detection boosted by ethanol and a solvent-compatible scaffold for aptazyme design**. *ChemBioChem* 2018, **19**:31-36.

26. Lin LN, Sheng J, Huang Z: **Nucleic acid X-ray crystallography via direct selenium derivatization**. *Chem Soc Rev* 2011, **40**:4591-4602.

27. Carrasco N, Buzin Y, Tyson E, Halpert E, Huang Z: **Selenium derivatization and crystallization of DNA and RNA oligonucleotides for X-ray crystallography using multiple anomalous dispersion**. *Nucleic Acids Res* 2004, **32**:1638-1646.

28. •• Serganov A, Keiper S, Malinina L, Tereshko V, Skripkin E, Hobartner C, Polonskaia A, Phan AT, Wombacher R, Micura R *et al.*: **Structural basis for Diels-Alder ribozyme-catalyzed carbon-carbon bond formation**. *Nat Struct Mol Biol* 2005, **12**:218-224.
This article presents the first crystal structure of a ribozyme catalyzing a carbon–carbon bond forming reaction. This structure reveals that the ribozyme forms a catalytic core that permits to fit only one of the product enantiomers.

29. Weinrich T, Jaumann EA, Scheffer U, Prisner TF, Gobel MW: **A cytidine phosphoramidite with protected nitroxide spin label: synthesis of a full-length TAR RNA and investigation by in-line probing and EPR spectroscopy**. *Chem Eur J* 2018, **24**:6202-6207.

30. Granz M, Erlenbach N, Spindler P, Gophane DB, Stelzl LS, Sigurdsson ST, Prisner TF: **Dynamics of nucleic acids at room temperature revealed by pulsed EPR spectroscopy**. *Angew Chem Int Ed* 2018, **57**:10540-10543.

31. Kim NK, Murali A, DeRose VJ: **Separate metal requirements for loop interactions and catalysis in the extended hammerhead ribozyme**. *J Am Chem Soc* 2005, **127**:14134-14135.

32. Edwards TE, Sigurdsson ST: **EPR spectroscopic analysis of U7 hammerhead ribozyme dynamics during metal ion induced folding**. *Biochemistry* 2005, **44**:12870-12878.

33. • Hetzke T, Vogel M, Gophane DB, Weigand JE, Suess B, Sigurdsson ST, Prisner TF: **Influence of Mg2+ on the conformational flexibility of a tetracycline aptamer**. *RNA* 2019, **25**:158-167.
In this article, nitroxide-modified nucleotides were incorporated in the tetracycline binding RNA aptamer to evaluate its Mg^{2+}-dependent conformational flexibility.

34. •• Liu Y, Holmstrom E, Yu P, Tan KM, Zuo XB, Nesbitt DJ, Sousa R, Stagno JR, Wang YX: **Incorporation of isotopic, fluorescent, and heavy-atom-modified nucleotides into RNAs by position-selective labeling of RNA**. *Nat Protoc* 2018, **13**:987-1005.
This article describes the detailed protocol for a new, pause-restart method for the site-specific introduction of labels into long (>60 nucleotides) RNA sequences. The processive elongation phase of the T7 RNA polymerase is stalled by adding only limited amounts of the triphosphates.

35. Liu Y, Li ZW, Liu GF, Wang Q, Chen W, Zhang D, Cheng MS, Zheng ZB, Liu KL, He JL: **Breaking the conservation of guanine residues in the catalytic loop of 10-23 DNAzyme by position-specific nucleobase modifications for rate enhancement**. *Chem Commun* 2013, **49**:5037-5039.

36. Lam CH, Perrin DM: **Introduction of guanidinium-modified deoxyuridine into the substrate binding regions of DNAzyme 10–23 to enhance target affinity: implications for DNAzyme design**. *Bioorg Med Chem Lett* 2010, **20**:5119-5122.

37. •• Ponce-Salvatierra A, Wawrzyniak-Turek K, Steuerwald U, Höbartner C, Pena V: **Crystal structure of a DNA catalyst**. *Nature* 2016, **529**:231-234.
This article reports the first crystal structure of a DNAzyme.

38. •• Liu HH, Yu X, Chen YQ, Zhang J, Wu BX, Zheng LN, Haruehanroengra P, Wang R, Li SH, Lin JZ *et al.*: **Crystal structure of an RNA-cleaving DNAzyme**. *Nat Commun* 2017, **8**:10.
This work describes the first crystal structure of an RNA-cleaving DNAzyme and reveals the complex tertiary structure adopted by the backbone to form a highly compact active site.

39. Kropp HM, Durr SL, Peter C, Diederichs K, Marx A: **Snapshots of a modified nucleotide moving through the confines of a DNA polymerase**. *Proc Natl Acad Sci U S A* 2018, **115**:9992-9997.

40. Houlihan G, Arangundy-Franklin S, Holliger P: **Exploring the chemistry of genetic information storage and propagation through polymerase engineering**. *Acc Chem Res* 2017, **50**:1079-1087.

41. Larsen AC, Dunn MR, Hatch A, Sau SP, Youngbull C, Chaput JC: **A general strategy for expanding polymerase function by droplet microfluidics**. *Nat Commun* 2016, **7**:9.

42. Pinheiro VB, Taylor AI, Cozens C, Abramov M, Renders M, Zhang S, Chaput JC, Wengel J, Peak-Chew S, McLaughlin SH *et al.*: **Synthetic genetic polymers capable of heredity and evolution**. *Science* 2012, **336**:341-344.

43. Chen TJ, Hongdilokkul N, Liu ZX, Adhikary R, Tsuen SS, Romesberg FE: **Evolution of thermophilic DNA polymerases for the recognition and amplification of C2'-modified DNA**. *Nat Chem* 2016, **8**:557-563.

44. • Liu C, Cozens C, Jaziri F, Rozenski J, Marechal A, Dumbre S, Pezo V, Marliere P, Pinheiro VB, Groaz E *et al.*: **Phosphonomethyl oligonucleotides as backbone-modified artificial genetic polymers**. *J Am Chem Soc* 2018, **140**:6690-6699.
First report of a synthetic genetic polymer modified both at the level of the sugar and the phosphate units to be compatible with the identification of functional nucleic acids and fully bioorthogonal to natural nucleic acids.

45. Guixens-Gallardo P, Zawada Z, Matyasovsky J, Dziuba D, Pohl R, Kraus T, Hocek M: **Brightly fluorescent 2'-deoxyribonucleoside triphosphates bearing methylated bodipy fluorophore for in cellulo incorporation to DNA, imaging, and flow cytometry**. *Bioconjug Chem* 2018, **29**:3906-3912.

46. •• Li Y, Fin A, McCoy L, Tor Y: **Polymerase-mediated site-specific incorporation of a synthetic fluorescent isomorphic G surrogate into RNA**. *Angew Chem Int Ed* 2017, **56**:1303-1307.
This article describes the enzymatic incorporation of single, fluorescent, ribonucleotide analogs at specific positions of the hammerhead ribozyme for structural and mechanistic investigations.

47. Eremeeva E, Abramov M, Margamuljana L, Rozenski J, Pezo V, Marliere P, Herdewijn P: **Chemical morphing of DNA containing four noncanonical bases**. *Angew Chem Int Ed* 2016, **55**:7515-7519.

48. Röthlisberger P, Levi-Acobas F, Sarac I, Baron B, England P, Marliere P, Herdewijn P, Hollenstein M: **Facile immobilization of DNA using an enzymatic his-tag mimic**. *Chem Commun* 2017, **53**:13031-13034.

49. Jakubovska J, Tauraite D, Meskys R: **A versatile method for the UVA-induced cross-linking of acetophenone-or benzophenone-functionalized DNA**. *Sci Rep* 2018, **8**:10.

50. Santoro SW, Joyce GF, Sakthivel K, Gramatikova S, Barbas CF: **RNA cleavage by a DNA enzyme with extended chemical functionality**. *J Am Chem Soc* 2000, **122**:2433-2439.

51. Perrin DM, Garestier T, Hélène C: **Bridging the gap between proteins and nucleic acids: a metal-independent RNase A mimic with two protein-like functionalities**. *J Am Chem Soc* 2001, **123**:1556-1563.

52. Ting R, Thomas JM, Lermer L, Perrin DM: **Substrate specificity and kinetic framework of a DNAzyme with an expanded chemical repertoire: a putative RNaseA mimic that catalyzes RNA hydrolysis independent of a divalent metal cation**. *Nucleic Acids Res* 2004, **32**:6660-6672.

53. Sidorov AV, Grasby JA, Williams DM: **Sequence-specific cleavage of RNA in the absence of divalent metal ions by a DNAzyme incorporating imidazolyl and amino functionalities**. *Nucleic Acids Res* 2004, **32**:1591-1601.

54. Salvio R: **The guanidinium unit in the catalysis of phosphoryl transfer reactions: from molecular spacers to nanostructured supports**. *Chem Eur J* 2015, **21**:10960-10971.

55. Hollenstein M, Hipolito CJ, Lam CH, Perrin DM: **A self-cleaving DNA enzyme modified with amines, guanidines and imidazoles operates independently of divalent metal cations (M^{2+})**. *Nucleic Acids Res* 2009, **37**:1638-1649.

56. Hollenstein M, Hipolito CJ, Lam CH, Perrin DM: **Toward the combinatorial selection of chemically modified DNAzyme RNase A mimics active against all-RNA substrates**. *ACS Comb Sci* 2013, **15**:174-182.

57. •• Wang YJ, Liu EK, Lam CH, Perrin DM: **A densely modified M2+-independent DNAzyme that cleaves RNA efficiently with multiple catalytic turnover**. *Chem Sci* 2018, **9**:1813-1821.
First example of an efficient modified DNAzyme capable of hydrolyzing all-RNA substrates with Michaelis–Menten kinetics in the absence of M^{2+} cofactors.

58. Hipolito CJ, Hollenstein M, Lam CH, Perrin DM: **Protein-inspired modified DNAzymes: dramatic effects of shortening side-chain length of 8-imidazolyl modified deoxyadenosines in selecting RNaseA mimicking DNAzymes**. *Org Biomol Chem* 2011, **9**:2266-2273.

59. Hollenstein M: **Synthesis of deoxynucleoside triphosphates that include proline, urea, or sulfonamide groups and their polymerase incorporation into DNA**. *Chem Eur J* 2012, **18**:13320-13330.

60. Velez TE, Singh J, Xiao Y, Allen EC, Wong OY, Chandra M, Kwon SC, Silverman SK: **Systematic evaluation of the dependence of deoxyribozyme catalysis on random region length**. *ACS Comb Sci* 2012, **14**:680-687.

61. •• Zhou C, Avins JL, Klauser PC, Brandsen BM, Lee Y, Silverman SK: **DNA-catalyzed amide hydrolysis**. *J Am Chem Soc* 2016, **138**:2106-2109.
Describes the first nucleic acids catalysts capable of hydrolyzing aliphatic amide bonds.

62. Röthlisberger P, Hollenstein M: **Aptamer chemistry**. *Adv Drug Deliv Rev* 2018, **134**:3-21.

63. Taylor AI, Pinheiro VB, Smola MJ, Morgunov AS, Peak-Chew S, Cozens C, Weeks KM, Herdewijn P, Holliger P: **Catalysts from synthetic genetic polymers**. *Nature* 2015, **518**:427-430.

64. • Wang Y, Ngor AK, Nikoomanzar A, Chaput JC: **Evolution of a general RNA-cleaving FANA enzyme**. *Nat Commun* 2018, **9**:5067.
Describes the isolation of a sugar (FANA) modified DNAzyme capable of hydrolyzing both RNA and DNA substrates with efficient multiple turnover kinetics.

65. Lam CH, Hipolito CJ, Hollenstein M, Perrin DM: **A divalent metal-dependent self-cleaving DNAzyme with a tyrosine side chain**. *Org Biomol Chem* 2011, **9**:6949-6954.

66. Hollenstein M, Hipolito C, Lam C, Dietrich D, Perrin DM: **A highly selective DNAzyme sensor for mercuric ions**. *Angew Chem Int Ed* 2008, **47**:4346-4350.

67. Beigelman L, McSwiggen JA, Draper KG, Gonzalez C, Jensen K, Karpeisky AM, Modak AS, Matulicadamic J, Direnzo AB, Haeberli P *et al.*: **Chemical modification of hammerhead ribozymes – catalytic activity and nuclease resistance**. *J Biol Chem* 1995, **270**:25702-25708.

68. Burgin AB, Gonzalez C, MatulicAdamic J, Karpeisky AM, Usman N, McSwiggen JA, Beigelman L: **Chemically modified hammerhead ribozymes with improved catalytic rates**. *Biochemistry* 1996, **35**:14090-14097.

69. El-Sagheer AH, Brown T: **New strategy for the synthesis of chemically modified RNA constructs exemplified by hairpin and hammerhead ribozymes**. *Proc Natl Acad Sci U S A* 2010, **107**:15329-15334.

70. Tarasow TM, Tarasow SL, Eaton BE: **RNA-catalysed carbon-carbon bond formation**. *Nature* 1997, **389**:54-57.

71. Ren AM, Micura R, Patel DJ: **Structure-based mechanistic insights into catalysis by small self-cleaving ribozymes**. *Curr Opin Chem Biol* 2017, **41**:71-83.

72. Siegmund V, Santner T, Micura R, Marx A: **Screening mutant libraries of T7 RNA polymerase for candidates with increased acceptance of 2'-modified nucleotides**. *Chem Commun* 2012, **48**:9870-9872.

73. Pech A, Achenbach J, Jahnz M, Schulzchen S, Jarosch F, Bordusa F, Klussmann S: **A thermostable D-polymerase for mirror-image PCR**. *Nucleic Acids Res* 2017, **45**:3997-4005.

74. • Chen TJ, Romesberg FE: **Polymerase chain transcription: exponential synthesis of RNA and modified RNA**. *J Am Chem Soc* 2017, **139**:9949-9954.
A mutant DNA polymerase is shown to be capable of transcribing RNA and 2'-fluoro-modified RNA and of synthesizing RNA–DNA mixed sequences under PCR conditions.

75. Walunj MB, Tanpure AA, Srivatsan SG: **Post-transcriptional labeling by using Suzuki-Miyaura cross-coupling generates functional RNA probes**. *Nucleic Acids Res* 2018, **46**:10.

76. •• Verdonck L, Buyst D, de Vries A-M, Gheerardijn V, Madder A, Martins JC: **Tethered imidazole mediated duplex stabilization and its potential for aptamer stabilization**. *Nucleic Acids Res* 2018, **46**:11671-11686.
This article describes a method for the general stabilization of duplexes via the introduction of an imidazolium-based pK_a-motif.

77. Matsunaga K, Kimoto M, Hirao I: **High-affinity DNA aptamer generation targeting von Willebrand factor A1-domain by genetic alphabet expansion for systematic evolution of ligands by exponential enrichment using two types of libraries composed of five different bases**. *J Am Chem Soc* 2017, **139**:324-334.

78. • Kong DH, Yeung W, Hili R: **In vitro selection of diversely functionalized aptamers**. *J Am Chem Soc* 2017, **139**:13977-13980.
This article describes the use of the T4 DNA ligase to construct modified libraries and their use in SELEX for the identification of a potent anti-thrombin aptamer.

79. • Chen Z, Lichtor PA, Berliner AP, Chen JC, Liu DR: **Evolution of sequence-defined highly functionalized nucleic acid polymers**. *Nat Chem* 2018, **10**:420-427.
Describes the general use of ligase-built libraries for the identification of high-binding affinity aptamers against protein targets.

80. Le ATH, Krylova SM, Kanoatov M, Desai S, Krylov SN: **Ideal-filter capillary Electrophoresis (IFCE) to facilitate one-step selection of aptamers**. *Angew Chem Int Ed* 2019, **58**:2739-2743 http://dx.doi.org/10.1002/anie.20181297.

81. Morrison D, Rothenbroker M, Li Y: **DNAzymes: selected for applications**. *Small Methods* 2018, **2**:1700319.

82. Silverman SK: **Catalyic DNA: scope, applications, and biochemistry of deoxyribozymes**. *Trends Biochem Sci* 2016, **41**:595-609.