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In vitro Selection of Chemically Modified DNAzymes

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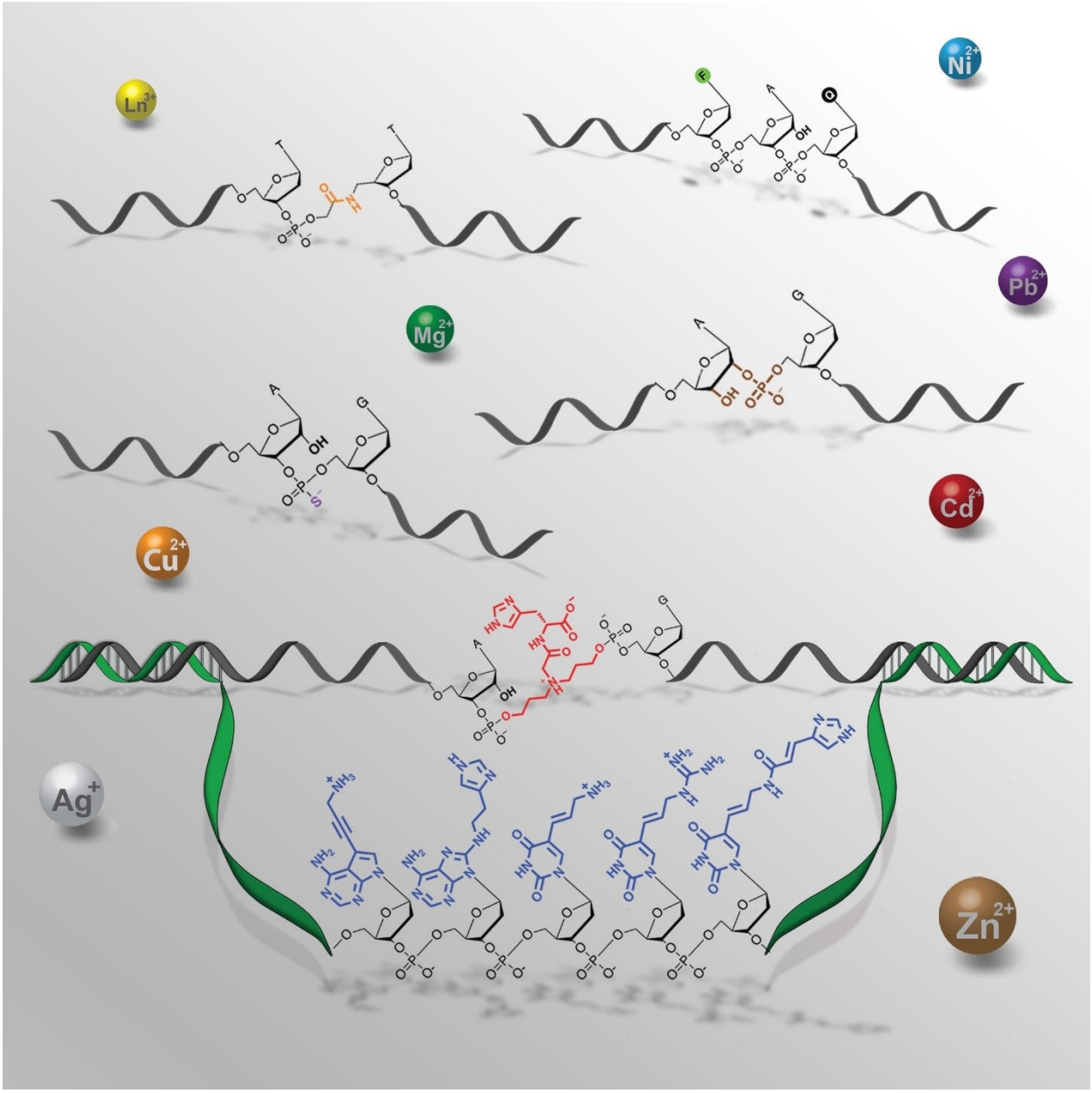

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DNAzymes are in vitro selected DNA oligonucleotides with catalytic activities. RNA cleavage is one of the most extensively studied DNAzyme reactions. To expand the chemical functionality of DNA, various chemical modifications have been made during and after selection. In this review, we summarize examples of RNA-cleaving DNAzymes and focus on those modifications introduced during in vitro selection. By incorporating various modified nucleotides via polymerase chain reaction (PCR) or primer extension, a few DNAzymes were obtained that can be specifically activated by metal ions such as Zn^{2+} and Hg^{2+}. In addition, some modifications were introduced to mimic RNase A that can cleave RNA substrates in the absence of divalent metal ions. In addition, single modifications at the fixed regions of DNA libraries, especially at the cleavage junctions, have been tested, and examples of DNAzymes with phosphorothioate and histidine-glycine modified tertiary amine were successfully obtained specific for Cu^{2+}, Cd^{2+}, Zn^{2+}, and Ni^{2+}. Labeling fluorophore/quencher pair right next to the cleavage junction was also used to obtain signaling DNAzymes for detecting various metal ions and cells. Furthermore, we reviewed work on the cleavage of 2′-5′ linked RNA and L-RNA substrates. Finally, applications of these modified DNAzymes as biosensors, RNases, and biochemical probes are briefly described with a few future research opportunities outlined at the end.

1. Introduction

With the discovery of ribozymes in the early 1980s, the search for catalytic DNA or DNAzymes started about a decade later. The first DNAzyme was reported in 1994 with RNA-cleavage activity.[1] To date, a diverse range of DNAzymes have been isolated to catalyze various chemical and biological transformations from DNA/RNA cleavage, ligation and phosphorylation to porphyrin metalation and peroxidation.[2–9] Since most natural DNA molecules are double-stranded, no catalytic activities are expected from them, and DNAzymes have so far only been isolated from in vitro selections.

With only four types of DNA bases, the chemical functionality of DNA is limited. Therefore, using modified DNA for producing better DNAzymes is a logical idea. In fact, RNA molecules possess over 50 modifications, mostly in tRNA.[10] Since DNA oligonucleotides can be chemically synthesized, the number of possible modifications is unlimited. For example, aside from the purpose of enhancing molecular recognition, synthetic modifications can also offer other functions such as fluorescence signaling that do not exist in natural RNA.[11]

In this review, we summarize the work on chemically modified DNAzymes. We only cover modifications made during in vitro selections instead of post-selection modifications (e.g. caged DNAzymes,[12,13] fluorophore labeling,[14] base modifications,[15,16] and other modifications[17–23]). Modifications made during in vitro selection are often critical for DNAzyme function, and they cannot be replaced by non-modified nucleotides. We focus on RNA-cleaving DNAzymes since they are rich in number and are most widely used for applications from biosensing[24–29] to intracellular cleavage of RNA.[30–33] This article is organized by first introducing RNA-cleaving DNAzymes in general, followed by DNAzymes containing modified nucleotides introduced during PCR or primer extension, and then modifications made in the fixed regions of selection libraries.

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2. In Vitro Selection of Non-Modified RNA-Cleaving DNAzymes

With over 20 years of development, selection of RNA-cleaving DNAzymes is now a mature process. For applications outside cells, the DNA library is typically designed to contain a single RNA linkage serving as the cleavage site, and the stability of RNA is about one-million-fold less compared to DNA.[34] Figure 1A shows a library sequence used in our lab, where the 50-nucleotide (nt) random region is positioned close to the ribo-adenine (rA) cleavage site and flanked by two putative base-paired regions. In general, a metal ion is needed to assist the RNA cleavage reaction.[26,35–39] The general RNA-cleavage mechanism is initiated by the 2′-OH group nucleophilic attacking the phosphorus center resulting in a penta-coordinated transition state.[36] Metal ions are needed to neutralize the negative charges accumulated in this transition state.[40,41] Sometimes a metal bound water can act as a general acid to donate a proton to the leaving oxygen as indicated in the crystal structure of a Pb^{2+} bound 8–17 DNAzyme.[35]

In a typical selection experiment, the library is exposed to a metal ion to achieve cleavage (Figure 1B). The cleaved sequences are fragmented into two strands with different lengths and they are separated by denaturing polyacrylamide gel electrophoresis (dPAGE). The fragment containing the random region is extracted, and two PCR steps are performed to amplify them. In PCR1, a full-length library is regenerated, which is used as the template for PCR2. In PCR2, two modified primers are used. One contains a carboxyfluorescein (FAM) label and the ribo-adenine (rA) base, and the other has an internal polymer spacer that can terminate the PCR. The resulting uneven amplicons are then separated by dPAGE to purify the positive strand containing the rA and FAM for the next round of selection.

Sometimes, counter selections are introduced to obtain DNAzymes with higher selectivity. For counter selections, the active sequences generated in the presence of competing

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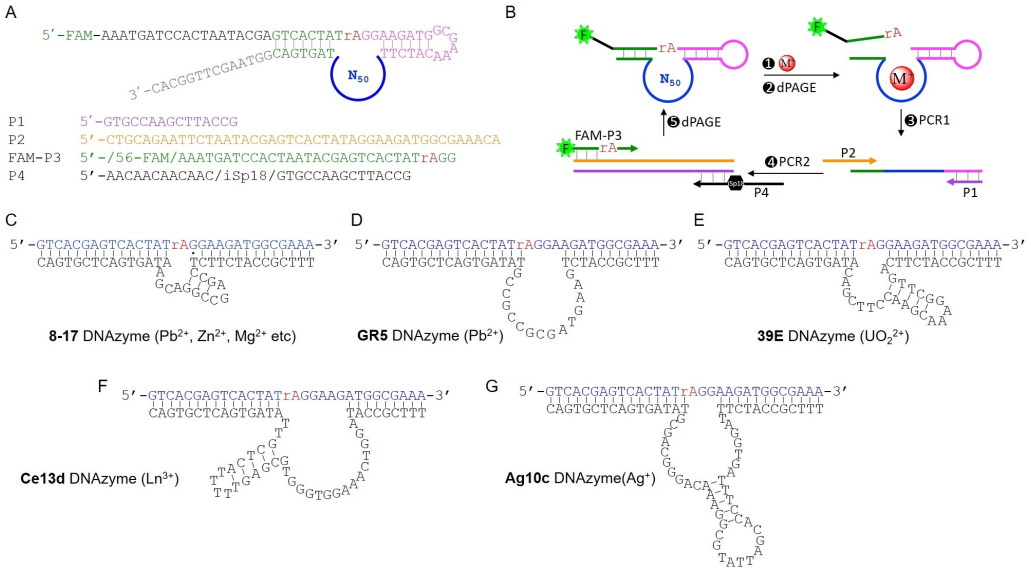

Figure 1. (A) The secondary structure of a library and primer sequences for in vitro selection of RNA-cleaving DNAzymes. (B) The steps in a typical selection process. The secondary structures of a few RNA-cleaving DNAzymes: (C) 8–17, (D) GR5, (E) 39E, (F) Ce13d, and (G) Ag10c.

molecules are discarded and the remaining uncleaved DNA strands are extracted, which are then subject to a further positive selection in the presence of target molecules. Using this method, many DNAzymes have been successfully isolated, and a few metal-specific DNAzymes are shown in Figures 1C-1G.

3. Modifications in the Random Regions of Selection Libraries.

3.1. Modified Bases for Improving Metal Binding

For selecting modified DNAzymes, the first ideas were to use modified bases. For example, to enhance Zn^{2+} binding, Joyce and coworkers[42] incorporated C5-imidazole deoxyuridine (Figure 2A) into the library by template-directed extension. The imidazole moiety is found in histidine and has good metal binding ability. To construct the library (Figure 2B), a sequence containing a 5′ biotin, and a 12-nt RNA region corresponding to the start codon region of several HIV-1 RNAs (in red) flanked by two DNA regions was hybridized with a DNA containing an N_{50} random region. After template-directed extension with the imidazole-functionalized dUTP analogue in place of dTTP, the resulting products were attached to a streptavidin column and the non-biotinylated strands were washed away. At this point, Zn^{2+} was added and the cleaved fragments were collected and amplified via PCR. After 16 rounds of selection, a DNAzyme with three catalytically essential imidazole residues was obtained (Figure 2C). This DNAzyme can cleave almost any RNA containing an AUG sequence. Under a simulated physiological buffer

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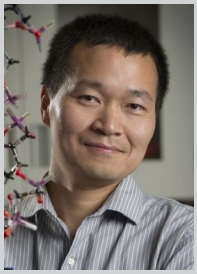

Juewen Liu received his BSc degree from the University of Science and Technology of China in 2000 and PhD degree from the University of Illinois at Urbana-Champaign in 2005. He is currently a professor of chemistry at the University of Waterloo, a University Research Chair, and a College member of the Royal Society of Canada (RSC). He is interested in DNAzymes, aptamers, biointerface science, and nanozymes. His team has discovered many new DNAzyme sequences for detecting various metals ions and developed various methods to conjugate DNA to nanomaterials.

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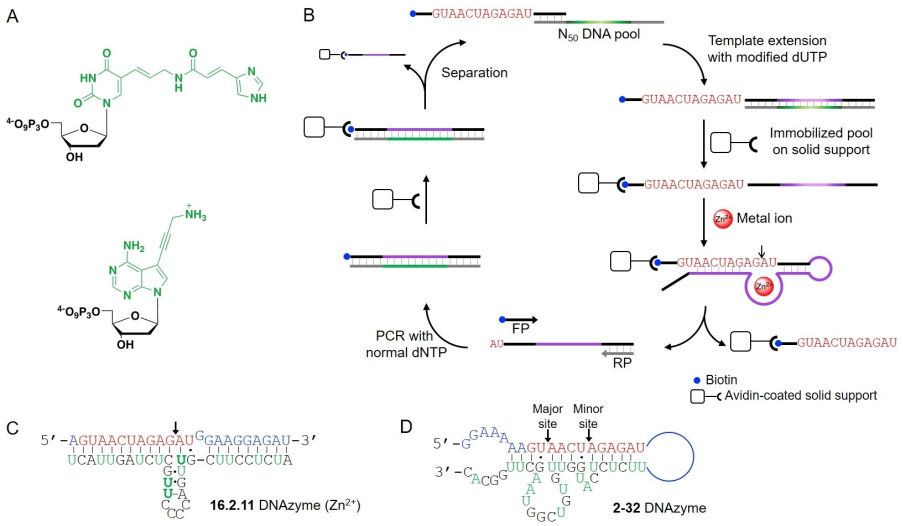

Figure 2. (A) Structures of the C5-imidazole modified dUTP and 3-(aminopropynyl)-7-deaza modified dATP. (B) The in vitro selection process of a Zn^{2+}-dependent RNA-cleaving DNAzyme containing the modified base in place of T. The secondary structure of (C) Zn^{2+}-dependent 16.2.11 DNAzyme, and (D) the 2–32 DNAzyme that cleaves two r(UA) junctions.

condition (10 μM Zn^{2+}, 1 mM Mg^{2+}, 150 mM NaCl, pH 7.5), the DNAzyme achieved a catalytic efficiency of ~10^8 $M^{-1}min^{-1}$ at 37 °C.

It is quite difficult to obtain Hg^{2+}-specific DNAzymes,[43] likely due to its weak interaction with the scissile phosphate. Using a high Hg^{2+} concentration to increase its binding would result in denaturation of DNA. The Perrin group selected a DNAzyme in the presence of Hg^{2+} using a $dA^{ime}TP$ and $dU^{aa}TP$ modified library (see Figure 3A), and a highly Hg^{2+}-specific DNAzyme named 10–13 was obtained.[44] The DNAzyme contains two domains (Figure 3F). The small stem is populated with amino/imidazole modifications, while the bigger stem-loop is rich in guanosine and cytosine. The activity required both modified bases. With 5 μM Hg^{2+}, the self-cleaving rate peaked at k_{obs} ~0.037 min^{-1} in the selection buffer (200 mM NaCl, 5 mM $MgCl_2$, and 25 mM cacodylate, pH 7.5). This DNAzyme showed no significant cleavage with other metal ions.

3.2. DNAzymes Independent of Divalent Metal Ions

Williams and coworkers employed the same library design as shown in Figure 2B but introduced an additional 7-amino-propynyl modified 7-deaza-dATP (Figure 2A). They conducted the selection in the absence of divalent metal ions (0.2 M NaCl, 10 mM EDTA, 50 mM Tris-HCl, pH 7.5 at 37 °C).[45] After 13 rounds of selection, a DNAzyme was identified to specifically cleave the two r(UA) linkages (Figure 2D). The site preference is likely due to decreased base stacking and/or altered hydrogen bonding around the scissile linkages.[46,47] Although the DNA-

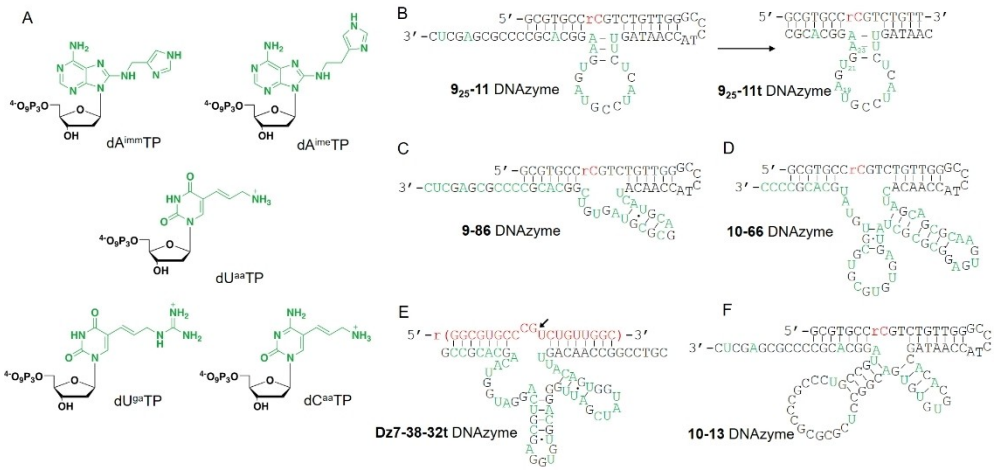

Figure 3. (A) Structures of five modified dNTP. The secondary structures of modified RNA-cleaving DNAzymes: (B) *cis*-cleaving 9_{25}-11 and *trans*-cleaving 9_{25}-11 t, (C) *cis*-cleaving 9–86, and (D) *cis*-cleaving 10–66. The secondary structure of (E) Dz7-38-32 t DNAzyme. This modified DNAzyme is capable of cleaving r(GU) junction in all RNA substrates. (F) The Hg^{2+}-dependent *cis*-cleaving 10–13 DNAzyme.

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zyme required both types of modified bases to achieve optimal cleavage, the sequences without modification still displayed some activity (7%). In fact, incorporating imidazolyl-modified dU alone had better cleavage (30%) than only using the amino-modified dA (10%). Under optimized conditions, the DNAzyme can cleave both sites with a similar rate (0.06 min^{-1} versus 0.07 min^{-1}).[45]

RNase A is a cationic (p*I* = 9.3) protein containing 124 amino acids,[48] and it specific cleaves phosphodiester linkages in the 3′-direction from cytidine or uridine. The mechanism starts with an initial transesterification to form the 2′,3′-cyclic phosphate intermediate followed by a hydrolytic cleavage to yield corresponding 3′-nucleoside phosphate. The His12, His119, and Lys41 located at the positively charged active site are critical for catalysis. The imidazole on His12 acts as a general base to deprotonate 2′-OH group in the RNA substrate to facilitate the internal nucleophilic attack on the phosphorus atom. At the same time, the imidazole on His119 acts as a general acid to protonate the 5′ oxygen leaving group. Meanwhile, the cationic amine on Lys41 provides electrostatic stabilization to neutralize the excess negative charge accumulated in the transition state.[49] With this mechanism, RNase A achieves metal-independent catalysis.

The Perrin group reasoned that introducing the functional groups in RNase A may obtain metal-independent DNAzymes, which may overcome the problem of low free intracellular Mg^{2+} concentration. Two modified nucleotide triphosphates (dA^{ime}TP and dU^{aa}TP in Figure 3A) were first used to obtain a DNAzyme in a buffer without divalent metal ions (200 mM NaCl, 1 mM EDTA, 50 mM sodium cacodylate, pH 7.4) at 24 °C.[50] They followed the approach described in Figure 2B except the Sequenase Version 2.0 DNA Polymerase was used to accommodate the two modified bases during the library construction. Although the resulting DNAzyme 9_{25}-11 (Figure 3B) only displayed up to 100-fold enhancement of catalytic activity over the unmodified DNAzyme (k_{cat} 0.0015 min^{-1}), both the cationic amines and imidazoles were necessary and acted in harmony to cleave the RNA. Later, the group truncated it into a *trans*-cleaving DNAzyme named 9_{25}-11 t with 31 bases to achieve multiple turnovers. 9_{25}-11 t only showed a k_{cat} of ~0.04 min^{-1} and a catalytic efficiency 5.3×10^5 $M^{-1}min^{-1}$.[51] Its 18-nt catalytic domain contained 4 imidazole and 6 allylamino modified nucleotides. The bell-shape pH-rate profile with two pK_a values (7.6 and 8.4) was consistent with the general acid/base catalysis by a pair of imidazole side chains. The imidazole on dA_{23} functioned as a general base to deprotonate 2′-OH, and the cationic amine in dU21 played a similar role to Lsy41 in RNase A.[49]

To enhance the catalytic activity, the Perrin group included a third modified base (dU^{ga}, Figure 3A). Since introducing dA^{ime} destabilized DNA duplex, while dU^{ga} and dC^{aa} increased duplex stability,[52] by incorporating imidazole, guanidine, and amine groups (Figure 3A), overall more stable DNA structures were allowed. The gain-of-function may offset the loss in sequence diversity due to dA^{ime} being unable to be incorporated more than three in a row.[53] The selection resulted in the 9–86 DNAzyme (Figure 3C) showing a rate constant of 0.13 min^{-1} at 37 °C.[54] Subsequent characterizations also highlighted the importance of these three modified bases. A critical guanidinium cation was suggested to increase DNAzyme stability, which was particularly important since no divalent metal ions were involved.

By incorporating the same three modified bases in another selection and applying a more stringent condition, a *cis*-cleaving DNAzyme named 10–66 (Figure 3D) with a rate constant of 0.63 min^{-1} at 37 °C was identified.[55] Interestingly, it still displayed a moderate rate of 0.1 min^{-1} even in the low millimolar monovalent metal ions (1 mM EDTA, 5 mM Na_2HPO_4, pH 7.4). The *trans*-cleaving 10–66 t exhibited a catalytic efficiency of 6×10^5 $M^{-1}min^{-1}$, comparable to some metal-dependent DNAzymes.[55] The effect of the linker tethering the imidazole was also tested by a new selection, although no better DNAzymes were identified.[56]

While M^{2+}-independent cleavage was demonstrated, these DNAzymes cannot function with all-RNA substrates. The Perrin group recently obtained the Dz7-38-32 t DNAzyme from a library containing not only three modified bases (dA^{ime}TP, dU^{ga}TP, and dC^{aa}TP) but also a segment with 17 ribonucleotides as the target substrate.[57] Dz7-38-32 t (Figure 3E) cleaved the all-RNA substrate at the r(GU) junction with k_{cat} 1.06 min^{-1} and a catalytic efficiency of 7.7×10^5 $M^{-1}min^{-1}$ in physiological condition (150 mM K^+, 0.5 mM Mg^{2+}, and 50 mM cacodylate, pH 7.45) at 30 °C. In fact, this k_{cat} value is 3–4 orders of magnitude higher than 10–23 DNAzymes under the low Mg^{2+} condition. The DNAzyme tolerated most metals except for Pb^{2+} or Hg^{2+}. Moreover, its bell-shape pH profile suggested two functional groups with near neutral pK_a's acting as general base and acid respectively for catalysis resembling the RNase A mechanism.

3.3. Modifications for improving resistance to nuclease degradation

In 2015, the Holliger group reported DNAzymes containing four sugar-modified nucleotides (Figure 4A).[58] In each modification, the canonical ribofuranose sugar of DNA and RNA was replaced by either a five-or six-membered ring analogue (2′-fluoroarabino nucleic acids, FANA; arabino nucleic acids, ANA; hexitol nucleic acids, HNA; and cyclohexene nucleic acids, CeNA). The intention was to produce catalytic XNAs (X for xenobiotic) that could enhance nuclease resistance and enable chemical reactions not accessible to its natural counterparts. In vitro selection of XNAzymes was made possible thanks to the engineered polymerases (Table 1).[59] Each XNA required a different DNA-

Table 1. Polymerases used for enzymatic XNA synthesis, and reverse transcription.

XNA	Synthesis (DNA→XNA)	Reverse transcription (DNA→XNA)
FANA	D4K	RT-521L
ANA	D4K	RT-521L
HNA	6G12 I521L	RT-521L
CeNA	6G12	RT-521L

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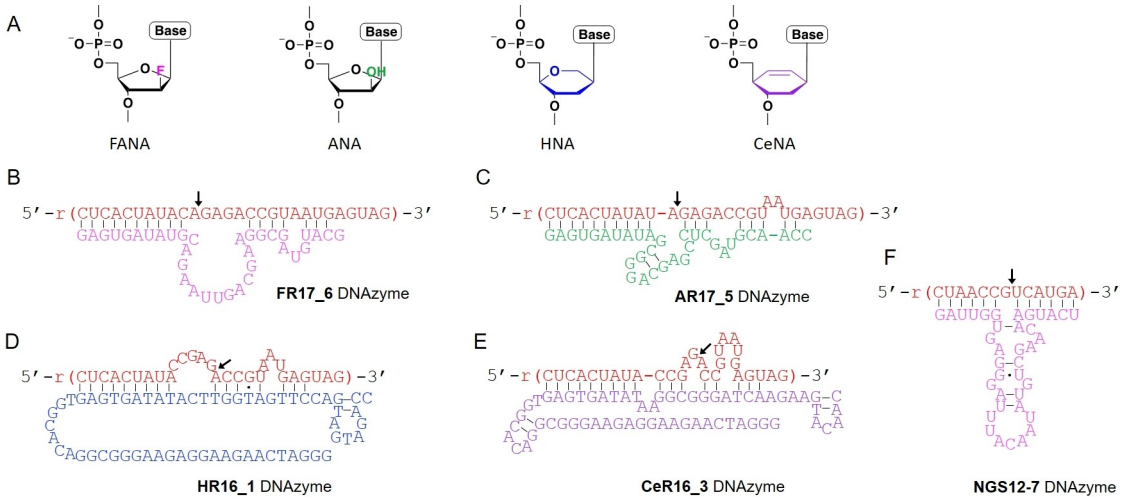

Figure 4. (A) Structures of four xenobiotic nucleic acids (XNAs). The chemical modifications are highlighted in color. The secondary structures of the RNA-cleaving XNAzymes: (B) FR17_6, (C) AR17_5, (D) HR16_1, (E) CeR16-3, and (F) NGS12-7. The color of the enzyme strands corresponds to the XNAs incorporated in the selections.

dependent XNA polymerase. Since direct amplification of XNA was not yet possible, an XNA reverse transcription (RT) step was needed to generate the complementary DNA for selected sequences. The RT521 L was chosen because it also has RNA-dependent DNA polymerase activity.

Four different RNA-cleaving XNAzymes were reported. For the FANA incorporated selection, the FR17_6 FANAzyme (Figure 4B) exhibited a comparable rate (k_{obs} 0.058 min^{-1}) to analogous ribozymes and DNAzymes. For AR17_5 ANAzyme (Figure 4C), its sequence shares 12 of the 14 core residues of the 8–17 DNAzyme, although a direct conversion of the 8–17 sequence to ANA resulted in no activity. In addition, the AR17_5 exhibited a significant slower rate (k_{obs} 0.0012 min^{-1}) in the presence of 50 mM Mg^{2+} at pH 8.5. For the HR16_1 HNAzyme (Figure 4D) and CeR16-3 CeNAzyme (Figure 4E), the catalytic rates were even slower under the same condition.[58] In general, these XNAzymes exhibited slower catalytic rates than most known DNAzymes, and none of them functioned with the Michaelis-Menten kinetics.

Later, Wang *et al.* isolated an efficient RNA-cleaving FANAzyme from an unbiased N_{25} library. Unlike the previous examples, a naturally occurring DNA polymerase (Tgo DNA polymerase) was used for constructing the FANA library and a reverse transcriptase (Bst DNA polymerase, large fragment) was used for copying the FANA templates back to DNA.[60] The most active species, NGS12-7 (Figure 4F) cleaved the RNA substrate at a specific GU junction with a k_{cat} ~0.2 min^{-1} and K_M ~600 nM in the presence of 25 mM Mg^{2+} at pH 8.5. The values were comparable to those for many ribozymes and DNAzymes. This was the first example of an XNAzyme exhibiting the Michaelis-Menten kinetic profile. The NGS12-7 also showed activity in the presence of Ca^{2+} or Mn^{2+}, although the rates were at least 3-fold slower. In addition, the rate constant with the RNA/DNA chimeric substrate was 7-fold faster than the all-RNA substrate, suggesting that the helicity of a B-form FANA-DNA hybridization provided a more favorable geometry for in-line nucleophilic attack of the nearby phosphodiester bond. In a follow-up study, Wang *et al.* compared the NGS12-7 and 10–23 DNAzymes using a chimeric substrate. The steady-state kinetics showed that NGS12-7 had better catalytic efficiency (k_{cat}/K_M ~1×10^6 $M^{-1}min^{-1}$) than 10–23 (k_{cat}/K_M ~3.5×10^5 $M^{-1}min^{-1}$) in 25 mM Mg^{2+} (200 mM NaCl, pH 8.5).[61] Moreover, NGS12-7 had superior catalytic activity than known DNAzymes for cleaving chimeric substrates under simulated physiological conditions (150 mM NaCl, 1 mM $MgCl_2$, pH 7.5).

3.4. Limitations of Using Modified Nucleotides

The above work using modified nucleotides, although quite powerful, suffered from a few drawbacks. First, the amplification step required more expensive engineered polymerases to incorporate the modified nucleotides. In addition, low efficiency and fidelity of those engineered polymerases were of concern.[62] Thus, less stringent selection conditions sometimes had to be used so that sufficient sequences were left for amplification. In the case of HNA and CeNA, their higher duplex stability also effected the efficiency of RT. While many base modifications are tolerated by commercial DNA polymerases, sugar modifications can be even more problematic and a trial-and-error process is needed.[63,64] In some cases, PCR needs to be replaced by primer extension reactions. Second, each of the above DNAzymes contained multiple modified nucleotides making the synthesis difficult. In addition, prediction of XNA secondary structures requires additional software and expertise not available to many researchers.[65] Finally, since most of the modifications are not yet commercially available, none of the DNAzymes were actually used by others beyond the labs of creation.

One way to solve these problems is to place modifications in the fixed region, while the random region is still made of the normal nucleotides. Since the most influencing modifications

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should be near the cleavage junction for RNA-cleaving DNAzymes, nearly all the literature examples with such fixed-site modifications are on the two nucleotides forming the cleavage junction. In the next section, we review this strategy.

4. Modifications introduced in the fixed region of the selection libraries.

4.1. Phosphorothioate Modifications

Phosphorothioate (PS) modification is commonly used for studying metal binding in ribozymes and DNAzymes.[66–68] One of the non-bridging oxygen atoms in the cleavage junction is replaced by sulfur (Figure 5A), and this modification can change the metal binding preference from hard metals such as Mg^{2+} to softer metals such as Mn^{2+} and Cd^{2+}. We used this method and found that the PS-modified Ce13d DNAzyme became active with all the thiophilic metals including Hg^{2+}, Cd^{2+}, Pb^{2+} and Cu^{2+},[69] whereas before the modification, the normal Ce13d works mainly with trivalent lanthanide ions.[70]

This work led us to embed a PS modification at the scissile phosphate in the library to perform new selections. Since the PS was in the primer of PCR, the normal PCR protocol and reagents were used. Using Cd^{2+} and Cu^{2+} as target metal ions, we obtained the Cd16 (Figure 5C) and Cu10 (Figure 5D) DNAzymes, respectively. Although both DNAzymes showed simple secondary structures with very small catalytic loops, they exhibited high activity. Before this work, no one successfully used these two metals to obtain efficient RNA-cleaving DNAzymes. Therefore, metal/phosphate binding is critical for the reaction.

Incorporating a single PS generated a pair of diastereomers (Figure 5B). In fact, the two DNAzymes had their R_p substrates cleaved 100-fold and 37-fold faster than the S_p substrates, respectively, suggesting highly specific metal binding instead of simple electrostatic interactions. Furthermore, both DNAzymes displayed remarkable selectivity for their target metal ions (Figure 5E). Compared to base modification, the cost of PS DNA synthesis is also low.

4.2. Modification of Other Metal Ligands

The scissile phosphate is critical for metal binding. With a natural phosphate, many hard metal ions such as Mg^{2+}, Ca^{2+}, Na^{+}, and lanthanides can activate the DNAzymes. When a PS modification is introduced, softer metals such as Cu^{2+} and Cd^{2+} can be used. However, many first-row transition metals are borderline, and it has been hard to obtain selective DNAzymes for them. In fact, most of them can activate the 8–17 DNAzyme,[73] and selections performed with these transitions metals often resulted in or were expected to result in the 8–17 motif.[73–75] Based on our work, using the PS modification could not result in new DNAzymes either (unpublished results).

We suspected that a nitrogen-based ligand might be useful for binding the first-row transition metal ions. To solve this problem, we collaborated with the Sleiman group and introduced a nitrogen-rich histidine-glycine modified tertiary amine ligand (Figure 6A). This ligand contained multiple metal binding sites, and it is possible to form more complex metal binding structures. We then used Zn^{2+} and Ni^{2+} as target metals for two

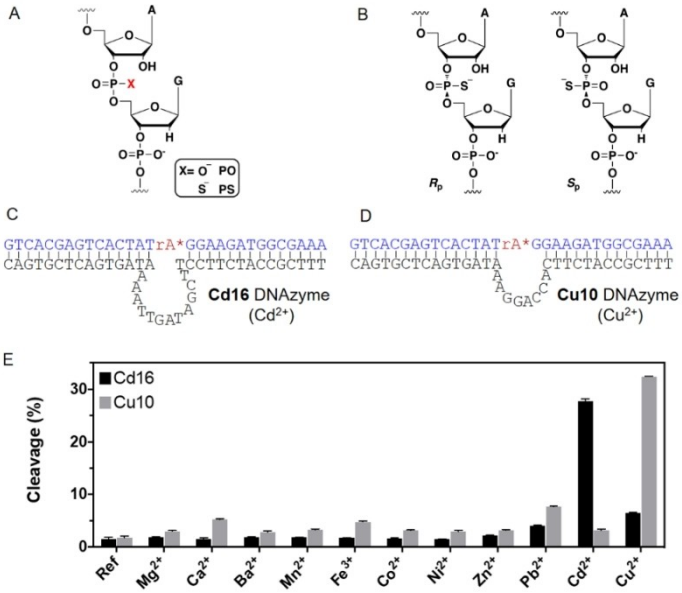

Figure 5. (A) Structure of the normal phosphodiester linkage (PO), and the PS modification. (B) The structures of the two PS diastereomers. The secondary structures of the two PS-modified (denoted as an asterisk) RNA-cleaving DNAzymes: (C) Cd16, and (D) Cu10. (E) Cleavage percentage of the unseparated PS substrate with the Cd16 and Cu10 DNAzymes in the presence of 10 μM various metals with the exception of 1 mM Mg^{2+}, Ca^{2+}, and Ba^{2+}. The 15 min reactions were run in 25 mM NaCl, 50 mM MES buffer (pH 6.0) at 25 °C. Figures were replotted with permission from ref. [71] (Copyright © 2016, American Chemical Society) and ref. [72] (Copyright © 2015, Oxford University Press).

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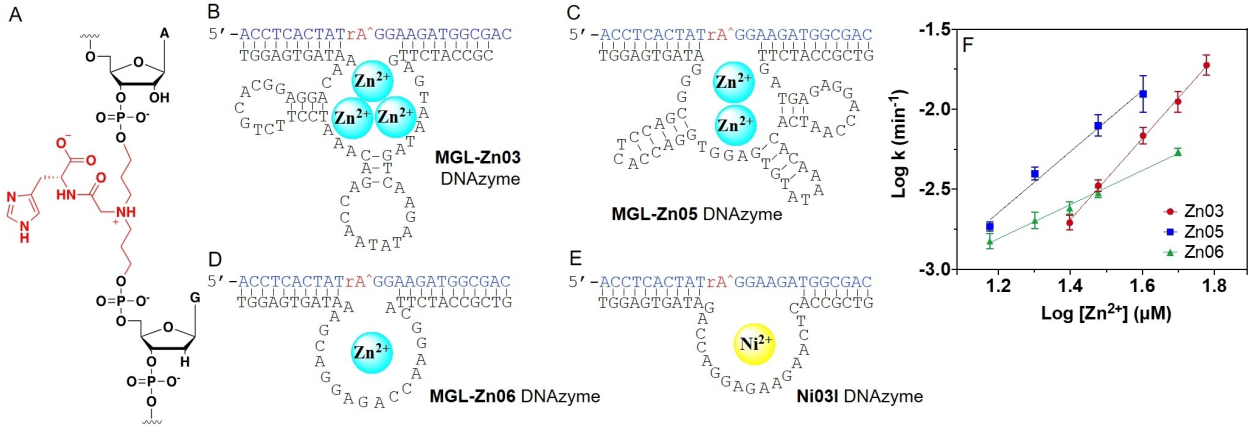

Figure 6. (A) The structure of the glycyl-histidine functionalized tertiary amine modification at the cleavage junction (named rA G). The secondary structures of the selected DNAzymes hybridized with the modified substrate: (B) MGL–Zn03 binds with three Zn^{2+} ions, (C) MGL–Zn05 binds with two Zn^{2+} ions, (D) MGL–Zn06 binds with one Zn^{2+} ion, and (E) MGL–Ni03 I can bind one Ni^{2+} or two Co^{2+} ions. (F) Double log plots of rate constant of each DNAzyme versus [Zn^{2+}]. The linear fitting showed a slope of 2.6, 1.9, and 1.0 for Zn03, Zn05, and Zn06, respectively. Figure was adapted with permission from ref. [76] (Copyright © 2019, Wiley-VCH).

separate selections. Using Zn^{2+}, we obtained a series of DNAzymes and they can bind one, two and three Zn^{2+} ions, respectively (Figure 6B–D).[76] Using a concentration-rate double log plot, the slope represented the number of Zn^{2+} ions (Figure 6F). Interestingly, the more Zn^{2+} binding, the better selectivity. The selectivity for Zn^{2+} over Co^{2+} for them reached from around 20, 1000 to 5000-fold. The MGL–Zn03 DNAzyme represents the most selective DNAzymes for Zn^{2+}, with the Zn^{2+} activity even higher than Pb^{2+}.

Similarly, a Ni^{2+}-dependent DNAzyme was also obtained using the same library. The truncated structure formed a simple catalytic loop with 17 nucleotides rich in purine (Figure 6E).[77] The enzyme was only active in the presence of Ni^{2+} or Co^{2+}. It is interesting to note that many other functional nucleic acids can hardly distinguish Ni^{2+} or Co^{2+}, such as the NiCo riboswitch.[78]

4.3. Change of Cleavage Site Dinucleotide Junctions

For most selections of RNA-cleavage DNAzymes, a purine-purine cleavage junction was used, such as rA–G. Strictly speaking, the change of the cleavage junction is not a modification, since all the nucleotides are still natural. We briefly discuss them since such changes have a strong influence on DNAzyme activity. The 10–23 DNAzyme can cleave any purine-pyrimidine junctions. However, the activity of r(AC) and r (GC) was greatly reduced.[30,79,80] For the 8–17 and 17E DNAzymes, they can cleave any rNG junction (rN stands for all four standard ribonucleotides).[30,73] If a DNAzyme can accept more junctions, it is more versatile in cleaving RNA. Cruz et al. conducted 16 parallel selections to cover all possible combinations of dinucleotide junctions in a DNA/RNA chimeric substrate.[74] Initially, five representative DNAzyme variants together could cleave 14 out of the 16 dinucleotide junctions in the presence of Mg^{2+} and Mn^{2+}.[74] Upon a comprehensive mutation study, the 8–17 motif can actually cleave all 16 combinations with at least 1000-fold over the background, although cleaving pyrimidine-pyrimidine junctions remained slow.[81]

To find an efficient DNAzyme that can cleave pyrimidine-pyrimidine junctions, Schlosser et al. conducted four separate selections (with rCC, rUC, rCT, and rUT junctions). Four best DNAzymes showed cleavage rates range from 0.04 to 0.15 min^{-1}. These were 1–3 orders of magnitude faster than the best DNAzymes reported previously,[82] although were still 10–100 fold slower than the DNAzymes that cleave purine-purine junctions.[74,81] A reselection was performed based on sequences from the rCT pool,[74] and the obtained CT10-3.29 DNAzyme with a more complex structure had a rate constant of 0.3–1.4 min^{-1}.[83] Recently, Wang et al. reported a new small Mg^{2+}-dependent DNAzyme 10–12opt that favorably cleaved the r (UN) junctions where N can be any ribonucleotides.[84] The 10–12opt can only efficiently cleave all-RNA substrates.

The cleavage junction can also contain modified nucleotides. For the Ce13d DNAzyme, we have replaced the rA by a 2-aminopurine (a fluorescent adenine analog), which allowed highly sensitive detection of Na^{+} ions.[14,85,86] Sometimes, other types of modifications such as hypoxanthine were also tested for probing specific groups.[87,88] However, direct selections using them has yet to be demonstrated.

4.4. Fluorophore/Quencher Modifications

Aside from enhancing activity and affect metal binding, modifications have also been made for signaling. Since many DNAzymes showed excellent metal specificity, they have been used for developing metal biosensors.[26] Typically, a fluorophore and a quencher were respectively labeled on the ends of the DNAzyme (Figure 7A), resulting in quenched fluorescence. Cleavage of the substrate and release of the fluorophore bearing fragment can produce a fluorescence signal.[24,89] Although such post-modifications of the selected DNAzyme are

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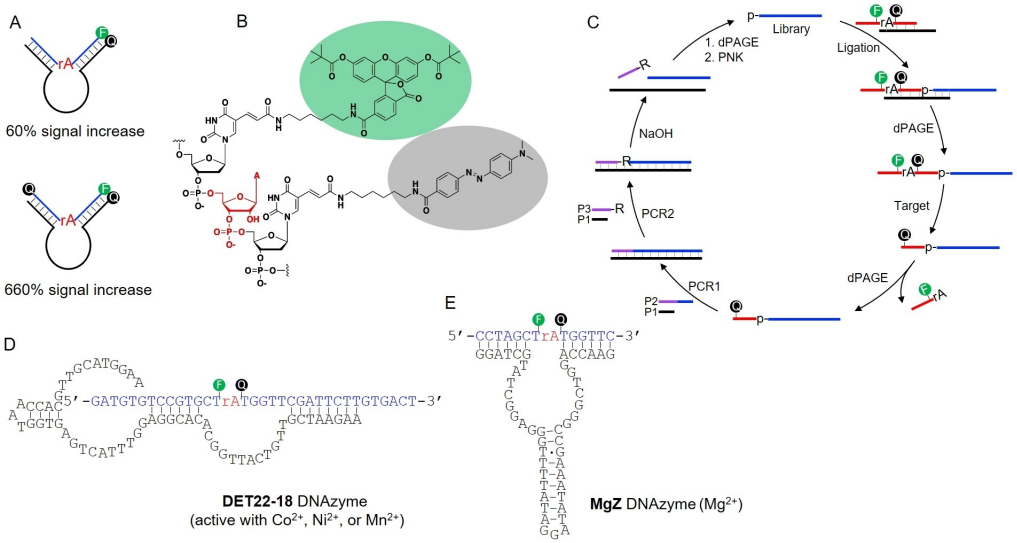

Figure 7. (A) DNAzyme-based fluorescence sensor designs with one or two quenchers. (B) The structure of a fluorescein-dT (green) and dabcyl-dT quencher (grey) modified cleavage junction. (C) Illustration of the selection scheme, and each selection cycle consists of multiple steps including ligation, cleavage reaction, PCR, dPAGE and phosphorylation. The secondary structures of *trans*-cleaving (D) DET22-18, and (E) MgZ DNAzymes.

common,[89,90] incomplete hybridization may increase the background.[91] Although adding more quenchers can help, the cost of the synthesis is also higher (Figure 7A).[90,92] In addition, signal generation requires not only the cleavage reaction but also the release of the cleavage product, which can also delay signal generation.

To overcome these problems, the Li lab labeled a fluorophore and a quencher right on either side of the cleavage junction (Figure 7B). The short distance between the fluorophore and quencher resulted in efficient fluorescence quenching. It also minimized false positive signals because the fluorescence was only generated when the RNA linkage was cleaved (e.g. melting of the DNAzyme complex would not produce a signal). In theory, the design could show ~70-fold fluorescence enhancement upon cleavage.[9] This in vitro selection strategy is quite technically demanding and requires more steps than typical selections (Figure 7C). Using this method, the Li group isolated some interesting metal and pH-dependent DNAzymes.[11,93] For example, the DET22-18 DNAzyme shown in Figure 7D is the second fastest DNAzyme reported reaching a k_{cat} ~7 min^{-1} at 23 °C in 5 mM $MgCl_2$ and 50 mM HEPES, pH 6.8 containing 10 mM $CoCl_2$.[9]

Using the same method coupled with hyper-mutagenic PCR protocol, Li et al. obtained five DNAzymes with catalytic activities covering a wide pH range (pH 3–8) via five parallel selections. Although the selection buffer contained a mixture of metal ions (Mn^{2+}, Co^{2+}, Cd^{2+}, and Ni^{2+}), each DNAzyme showed different metal specificity dependent on the selection pH. The fluorescence enhancement was ~3-fold better than the end-to-end labeling. Chiuman and Li used Mg^{2+} as a cofactor to obtain an RNA-cleaving DNAzyme named MgZ (Figure 7E).[94] This DNAzyme with a three-way junction secondary structure displayed a rate of 1 min^{-1} and 26-fold fluorescence enhancement upon cleavage.

The Li lab further isolated a series bacterial sensing DNAzymes using this technology,[95] in particular, the use of crude extracellular matrix (CEM) as the selection target. CEM is a complex mixture and its composition differs in various bacterial strains. Their RFD-EC1 DNAzyme was specific for an unidentified protein (MW between 30k and 50k Da) in the CEM from *E.coli*.[96,97] Importantly, it displayed no activity with the CEM of other Gram-negative or Gram-positive bacteria.[96] Under optimal conditions, RFD-EC1 can detect 10^3 cells.[98] This DNAzyme was engineered into a simple and inexpensive litmus test,[99] and printable paper-based sensors.[100] The same group reported another RNA-cleaving DNAzyme, RFD-CD1, which only targeted a particular strain of *C. difficile*, a gram-positive bacterium.[101] RFD-CD1 was activated by a truncated version of a transcription factor (TcdC) unique to the specific strain of *C. difficile*. Similar methods were also used to obtain DNAzymes for H. pylori, a pathogen linked to gastric carcinoma,[102] klebsiella pneumoniae,[103] and breast cancer.[104]

4.5. Cleaving 2′-5′ Linked RNA

Although nucleic acids are predominately linked by 3′-5′ phosphodiester bonds, 2′-5′-linked RNA also exists in nature and has been studied in biological, medicinal, and prebiotic research (Figure 8A).[105–108] So far, the DNAzymes mentioned above were selected to cleave 3′-5′ RNA linkage. The Silverman group previously reported a RNA-ligating DNAzyme that showed a low activity in cleaving 2′-5′ RNA (0.005 min^{-1}).[109,110]

To obtain efficient 2′-5′ RNA-cleaving DNAzymes, Ordoukhanian and Joyce introduced this linkage in the selection library and obtained the 2′:15-2 DNAzyme (Figure 8B).[111] This enzyme exhibited an optimal k_{cat} of ~0.01 min^{-1} at 37 °C in 50 mM EPPS buffer (pH 7.5) containing 150 mM NaCl and 25 mM Mg^{2+}.

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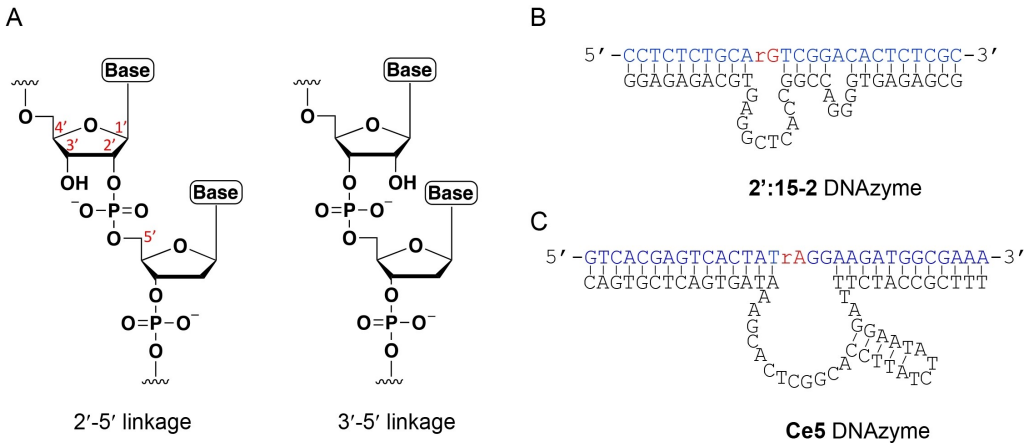

Figure 8. (A) Structures of 2′-5′ and 3′-5′ linked RNA. The secondary structures of (B) the 2′:15-2 DNAzyme cleaving the 2′-5′ rGT linkage in the presence of Mg^{2+}, and (C) the Ce5 DNAzyme cleaving the 2′-5′ rAG linkage in the presence of Ce^{3+}.

Although its catalytic efficiency of ~10^8 $M^{-1}min^{-1}$ was on par with some of the reported 3′-5′ RNA-cleaving DNAzymes, the catalytic rate was at least 10-fold slower than typical DNAzymes.[111] Later, the Liu group selected the Ce5 DNAzyme (Figure 8C) that cleaved the 2′-5′ using Ce^{3+} reaching a rate of 0.16 min^{-1} (10 μM Ce^{3+} in 25 mM KCl and 50 mM MES, pH 6).[112] This rate was similar to the Ce13d DNAzyme cleaving the 2′-5′ substrate under similar conditions.

4.6. Cleaving L-RNA

Another interesting modification is to generate enantiomers. Natural nucleic acids contain D-ribose and D-deoxyribose. By replacing them with the L-counterparts, the resulting nucleic acids are referred to as L–DNA or L-RNA (Figure 9A). This synthetic mirror image of natural biomolecules is resistant to nuclease degradation. The mirror forms of catalytic nucleic acids are called spiegelzymes (or L–DNAzyme). Retaining the catalytic functionality was demonstrated in two speigelzymes. The 10–23 and GR5L-enzymes can cleave their corresponding L-substrates in the presence of Mg^{2+} and Pb^{2+}, respectively.[113,114] In fact, the D-enzymes can only pair up and cleave the D-substrate, and vice versa.

Although these spiegelzymes were directly engineered from the previously selected DNAzymes, direction selections are also possible. Back in 2002, the Joyce group first isolated an L-RNA cleaving DNAzyme (L: 15–30). However, the reported catalytic rate was only 0.001 min^{-1}.[111] Recently, Tram and colleagues selected an L-RNA-cleaving DNAzyme named LRD-BT1 (Figure 9B). The L-modification was limited to the single moiety at the cleavage junction to minimize the substrate binding incompatibility between the L- and D- isomers. In the presence of Mg^{2+} and Mn^{2+}, LRD-BT1 achieved a k_{cat} of 2.6 min^{-1} and a K_M of 280 nM, and the activity was specifically for the LrG junction.[115] Structural analysis indicated the four nucleotides in the loop of the hairpin formed a kissing loop with the four nucleotides right next to the LrG cleavage site. As long as these two segments were complementary to each other, the sequences were changeable. The kissing loop might bring the catalytically important nucleotides in the stem-loop region closer to the cleavage site.

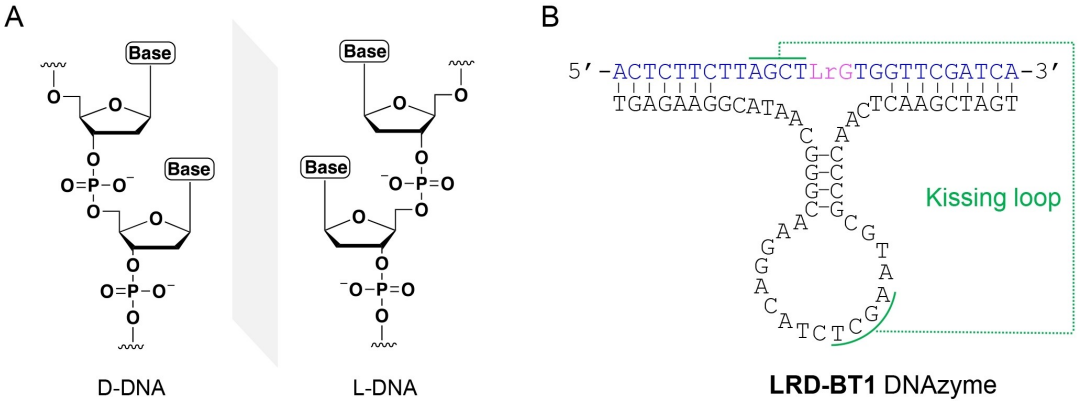

Figure 9. (A) Illustration of natural occurring DNA (D-DNA) and its synthetic mirror image form (L–DNA). (B) The secondary structure of an L-RNA cleaving DNAzyme named LRD-BT1. LrG denotes for L-riboguanosine.

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4.7. Cleaving the Amide Bond

Although the scope of this article is on RNA-cleaving DNAzymes, one particular DNAzyme for amide bond cleavage is worth mentioning here, since it is difficult to be achieved by canonical nucleic acids. The Silverman group reported several aliphatic amide-hydrolyzing DNAzymes via a carefully designed selection strategy.[116] For each selection, the thymine residues in the N_{40} random region were replaced with one of the modified bases containing a protein-like functional group, such as primary amino, carboxyl, or primary hydroxyl (Figure 10A). The designated cleavage site was modified with a simple aliphatic amide bond anchored between two DNA pieces (Figure 10B). To ensure that only amide cleavage products were selected, a key step was to add an amino capture strand (blue) and chemically ligate by the EDC chemistry (Figure 10B, Step 2). Many different DNAzymes were obtained with k_{obs} values ranged from 0.1 to 0.4 h^{-1}.

5. Applications of Modified DNAzymes.

The applications of conventional DNAzymes are also available to these modified DNAzymes, but the modifications also enabled some unique applications. Here, a few representative applications are briefly discussed.

5.1. Biosensors

Most of the modified DNAzymes were intended for sensing. We show two examples here. One is for the detection of Cd^{2+} using the Cd16 DNAzyme. The sensor design is shown in Figure 11A with a fluorophore-labeled on one end of the substrate strand and a quencher on the corresponding end of the enzyme. Cleavage produced a fluorescence enhancement specifically depended on Cd^{2+} concentration (Figure 11B), while other metal ions showed no response (Figure 11C). The sensor achieved a detection limit of 1.1 nM Cd^{2+} in buffer, and 1.6 nM Cd^{2+} in spiked rice sample extracts.[72]

Another interesting example is the detection of CEM produced by bacteria. The Li lab selected a series of signaling DNAzymes. Since a fluorophore and a quencher were already labelled right next to the cleavage site during the selection, the resulting DNAzymes were directly used as sensors (Figure 11D). The RFD-EC1 exhibited fluorescence enhancement with an increasing concentration of the CEM produced by *E. coli* K12,[96] a nonpathogenic strain as a model bacterium (Figure 11E).[98] This real-time sensing method can detect down to 10^4 bacterial cells. By culturing the cells for 8 h, a single colony forming unit can be detected. No fluorescence signals were detected with other Gram-negative bacterial pathogens, indicating high specificity (Figure 11F).[96]

5.2. Intracellular RNA Cleavage

One of the early motivations of DNAzymes was for cleavage of intracellular RNA. However, the activity of DNAzymes is low inside cells due to low intracellular metal concentration (e.g. free Mg^{2+} concentration below 2 mM). We reviewed above Perrin's work for mimicking RNase A to bypass the need for metal ions.[50,57,117,118] In their latest development, the Dz7-38-2 exhibited a k_{cat} of 0.27 min^{-1} and K_m of 3.3 μM ($k_{cat}/K_M \sim 10^5$ M^{-1} min^{-1}) under simulated physiological conditions (0.5 mM Mg^{2+}, 150 mM KCl, pH 7.5) at 37 °C.[57] Compare to unmodified DNAzymes (8–17 and 10–23), this k_{cat} was 3–4 orders of

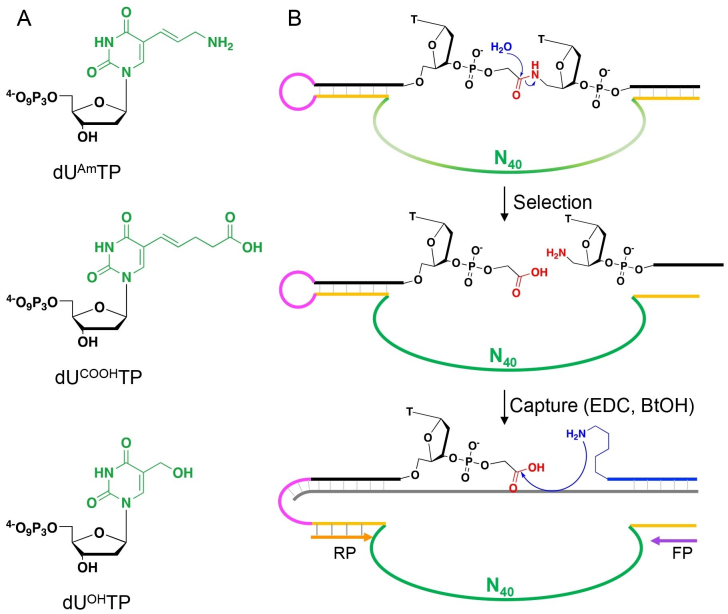

Figure 10. (A) Structures of $dU^{Am}TP$, $dU^{COOH}TP$, and $dU^{OH}TP$ used in aliphatic amide bond cleaving DNAzyme selections. (B) Key steps of the in vitro selection, where the DNA-catalyzed hydrolysis is followed by the capture of the revealed carboxylic acid using a 5′-amino oligonucleotide.

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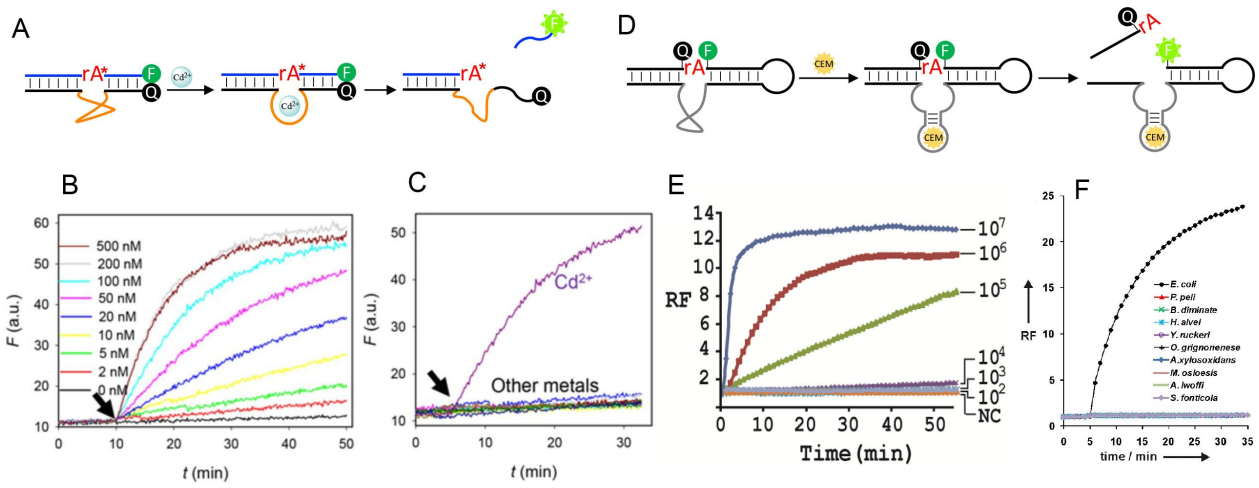

Figure 11. (A) Schematic design of the fluorescent DNAzyme sensor for Cd^{2+} detection. The asterisk denotes for the PS modification. Fluorescence kinetics of the Cd16 sensor containing a PS modification in the presence of (B) various Cd^{2+} concentrations and (C) 100 nM of various metals. The arrowhead indicated the point of metal addition. Figures were adapted from ref. [72] (D) A fluorogenic DNAzyme sensor for CEM. Fluorescence kinetics of RFD-EC1 in the presence of (E) CEM prepared from 10^2–10^7 *E. coli* cells, and (F) CEM from various Gram-negative and bacteria. Figures were adapted from ref. [98] and ref. [96] (Copyright © 2011, Wiley-VCH).

magnitude higher. However, the efficacy of this DNAzyme has yet to be demonstrated by intracellular work.

5.3. Reaction Probes

Many reactions can produce a mixture of 3′-5′ and 2′-5′ linked RNA. Since they have the same mass, their identification has to rely on biochemical methods. Typically, RNase T2 digestion,[119] or alkaline hydrolysis was used to probe the existence of 2′-5′ RNA linkage.[109] The latter method is more accessible, but the reaction rate is very slow, only ~0.043 h^{-1} at 37 °C requiring a few days of incubation.[120] On the other hand, the Ce5 DNAzyme exhibited a cleavage rate of 0.16 min^{-1} at room temperature.[112] Its 220-fold faster rate makes Ce5 useful for probing the 2′-5′ RNA linkage.

When a PS-modified RNA is mixed with a strongly thiophilic metal such as Tl^{3+}, three products are generated: the cleavage product, the desulfurized (3′-5′ linked) uncleaved product, and the isomerized product (2′-5′ linked and also desulfurized) uncleaved product (Figure 12A).[120] Under our reaction condition, only a small fraction (~7%) was cleaved while reaming 93% substrate were desulfurized and isomerized (Figure 12B).[120] We collected the uncleaved products. Once hybridized with the 17E DNAzyme, only the 3′-5′ linked substrate was cleaved in the presence of 10 mM Mg^{2+}. On the other hand, only the 2′-5′ linkage was cleaved with the Ce5 DNAzyme and 10 μM Ce^{3+}.[112] Thus, this simple method allowed quick identification of the reaction products.

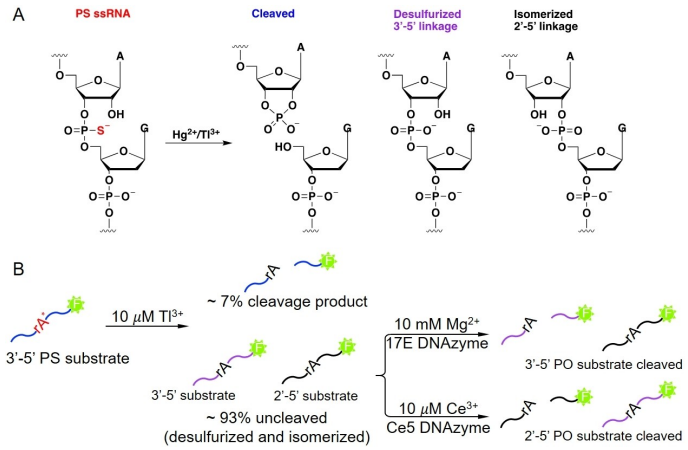

Figure 12. (A) Structures of the three products from a PS-RNA substrate reacting with Hg^{2+} or Tl^{3+}. (B) Experimental design of probing the cleavage, desulfurization, and isomerization reaction products. The uncleaved substrate after Tl^{3+} treatment was isolated and reacted with the 17E or Ce5 DNAzyme for quantification and characterization.

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6. Conclusions and Future Perspectives

In summary, we reviewed the in vitro selection of chemically modified RNA-cleaving DNAzymes. Taking advantage of solid phase DNA synthesis, PCR and primer extension reactions and synthetic chemistry, versatile modifications have been incorporated in DNAzymes for various purposes from enhanced metal binding, nuclease resistance to fluorescence signaling. Given the power of organic synthesis, the potential of combining the rational design of modified nucleic acids and in vitro selection is enormous.

Given the progress, we believe a few future developments can benefit this field. First, given the infinite possibilities of synthetic chemistry, we expect more types of modifications to be introduced for specific recognition of various targets. We have seen examples in the isolation of metal-specific DNAzymes, and it would be interesting to expand to small molecule recognition. Second, another type of modification is the 6-letter alphabet DNA developed by Benner and coworkers. In addition to the A, T, C, G, they have synthesized a few more Watson-Crick type of base pairs by shuffling the position of the hydrogen bond donors and acceptors. This can introduce new chemical functionalities and this technology is quite powerful for selecting better aptamers.[121,122] Its applications in DNAzymes has yet to be demonstrated. Third, to make the selected DNAzymes more widely used, they need to be commercially available. The PS modification is a cost-effective example, but most other modifications are not. This gap needs to be filled in order to see a broader impact of these DNAzymes. Finally, we have focused on examples of RNA-cleaving DNAzymes, while modifications on other types of DNAzymes are less explored, especially modifications introduced during the selection.[2,123] Given the diverse range of DNAzyme reactions, by rationally design modification strategies to match the reactions, we may also see improvements in other types of DNAzymes.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: aptamers · biosensors · DNAzymes · deoxyribozymes · SELEX

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