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Nucleic acid-cleaving catalytic DNA for sensing and therapeutics

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ABSTRACT

DNAzymes with nucleic acid-cleaving catalytic activity are increasing in versatility through concerted efforts to discover new sequences with unique functions, and they are generating excitement in the sensing community as cheap, stable, amplifiable detection elements. This review provides a comprehensive list and detailed descriptions of the DNAzymes identified to date, classified by their associated small molecule or ion needed for catalysis; of note, this classification clarifies conserved regions of various DNAzymes that are not obvious in the literature. Furthermore, we detail the breadth of functionality of these DNA sequences as well as the range of reaction conditions under which they are useful. In addition, the utility of the DNAzymes in a variety of sensing and therapeutic applications is presented, detailing both their advantages and disadvantages.

1. Introduction

The importance of enzymes as biocatalysts is indisputable, as their catalytic turnover of substrates makes life on this planet possible. Until 1982, proteins were believed to be the only biological polymer capable of catalyzing reactions, but the publication of Cech's work on self-splicing RNA, named a ribozyme by analogy to its protein cousins, changed the field forever [1]. They described an RNA self-cleaving intron that could process itself in the presence of mono- and divalent salts. Not only did this stimulate new models for the chemical makeup of early life, it opened avenues to cheaper, more highly controlled enzymatic reactions on nucleic acids. This discovery was followed a decade later with the first catalytic DNA, a sequence that could cleave an RNA substrate in the presence of only divalent cations [2]. Since the discovery of catalytic DNAs, also known as DNAzymes, a wide range of sequences have been identified.

DNAzymes have garnered attention in the biosensing and therapeutic communities, as DNA has practical advantages compared to proteins or even RNA. DNA is the least expensive of the three to manufacture [3,4], and it has the longest shelf-life as well as the best resistance to extreme conditions. Additionally, DNA can remain active under a broader range of reaction conditions, including temperature, salt concentrations, and pH [5].

While the catalytic activity of all three biopolymers requires folding of a monomer chain into a functional tertiary structure, DNA and RNA structure is more hierarchical: its secondary structure is stable independent of the tertiary arrangement [6]. This imparts nucleic acids' largest advantage, that substrate recognition can be separated from catalytic activity. This has led to an ease of computational modeling as compared to proteins, whose secondary structure elements form only as part of an overall tertiary structure that is not simple to predict.

When trying to perform brute-force evolution of catalytic biopolymers, nucleic acids' ability to be directly amplified makes DNAzymes and ribozymes simpler to screen and identify than proteins, which require more extensive manipulation to generate and then identity positive hits [7,8]. Advances in DNA synthesis make it simple to perform directed evolution [9] towards binding of a target analyte, or for any other purpose, as a large number of random sequences can be generated quickly and cheaply. There are now DNAs capable of catalytically ligating [10–12], phosphorylating [13,14], and cleaving both RNA and DNA, as well as catalyzing the disproportionation of hydrogen peroxide [15,16].

Previous reviews in this area include a global view of catalytic DNAs [17,18] as well as targeted reviews, such as those by Li and colleagues, which highlight the advances in both the discovery of DNAzymes [19] and their novel applications [20], or reviews that guide the reader through various biosensor designs that utilize DNAzymes [21]. The present review instead focuses on providing a comprehensive overview of the existing range of nucleic acid-cleaving DNAzymes so that the reader can select the one most appropriate for DNA amplification and therapeutic uses. To make this review of manageable size, modified bases [22,23] and those with functionalities outside of cleaving

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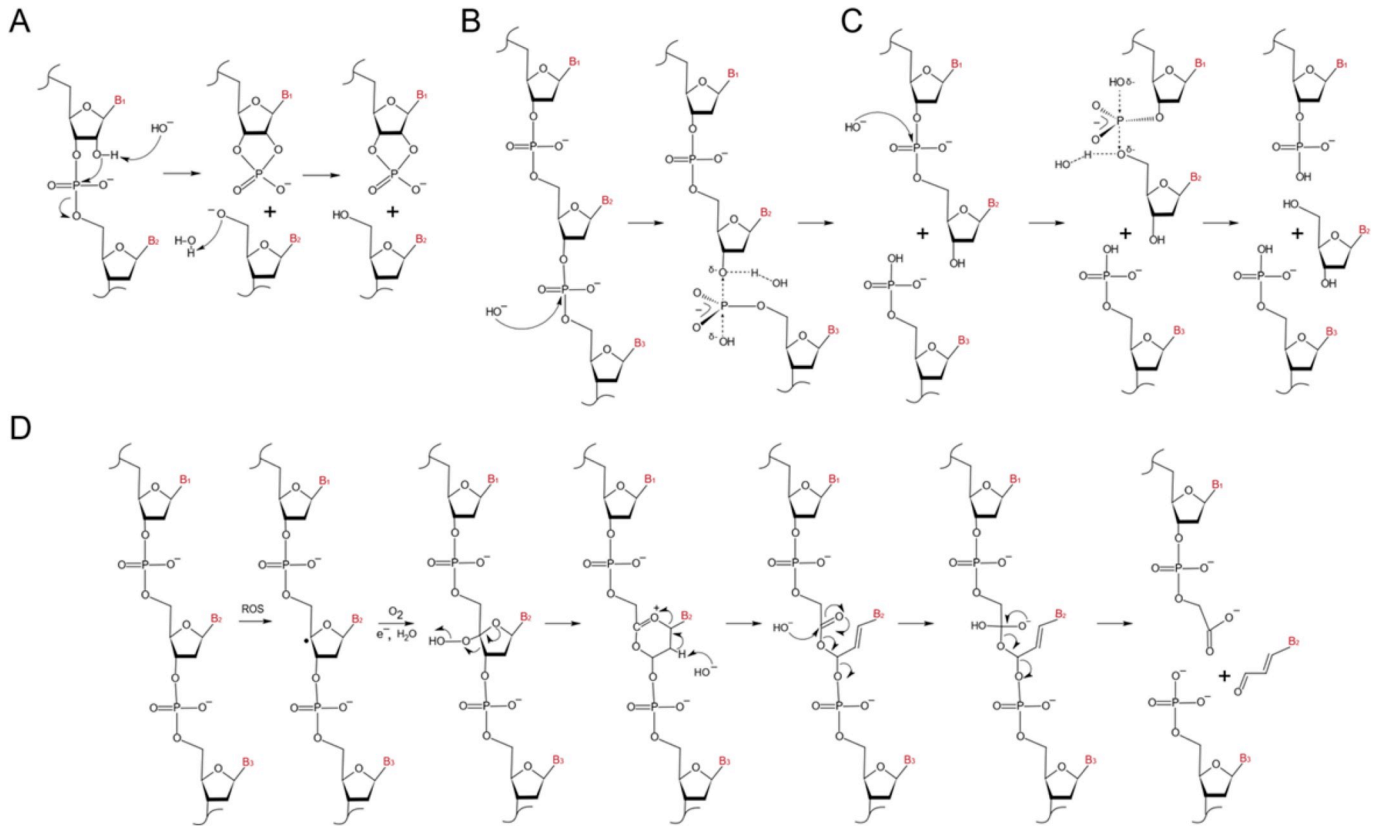

Fig. 1. Mechanisms of cleavage of the RNA backbone by DNA [26] via a 2′,3′ cyclic phosphate (A) and several proposed mechanisms of cleavage of DNA by DNA: hydrolysis to give a 5′ phosphate on the downstream product (B) [36], subsequent hydrolysis of the last phosphodiester linkage of the upstream product resulting in base excision (C) [31], and cleavage across the nucleotide resulting in a 3′ phosphoglycolate (D) are shown [32]. The mechanism for (D) is inferred from several sources, including bleomycin-induced DNA scission [33–35]. ROS, reactive oxygen species. With appropriate selection pressure, DNAzymes can also be evolved to cleave RNA via mechanism (B) [30].

'standard' DNA and RNA phosphodiester bonds [24,25] have been omitted. A sampling of sensors for metals and biomolecules, as well as their application as clinical therapeutics, are provided to demonstrate the variety of applications. The DNAzymes discussed are classified first by their cleavable substrate and subsequently by their catalytic cation or small-molecule cofactor, and additionally compiled in Table A1 for ease of selection. For each DNAzyme discussed, the minimal sequence is also available in Table A2.

2. Oligonucleotide cleaving DNAzymes

The active sites of DNAzymes are more limited than those of proteins – only the sugar-phosphate backbone and four nucleobases with limited functionality are available to affect cleavage. This limited scope of activity translates to less varied reaction mechanisms; for example, there are no examples of covalent DNAzyme-substrate intermediates [17]. The mechanism of RNA cleavage by DNA is usually the same as for cleavage by small ribozymes [26], as illustrated in Fig. 1A. The 2′ hydroxyl is deprotonated as the catalytic cation stabilizes developing charge on the 3′ and 5′ oxygens, and a transesterification reaction occurs, resulting in a 2′,3′ cyclic phosphate and a 5′ hydroxyl after protonation [27]. This mechanism has proven to be beneficial for applications, as the cyclic phosphate cannot be extended until polynucleotide kinase (PNK) or another enzyme with phosphatase activity removes it, leaving a 3′ hydroxyl that a polymerase can extend [28]. The cyclic phosphate is thus a natural stopping point should extension not be desirable in an assay. Ribozymes can also cleave RNA through hydrolysis or nucleophilic attack to leave a 3′ hydroxyl and a 5′ phosphate or phosphodiester, as in the mechanisms of RNAse P or self-splicing RNA [29], and DNAzymes have also been evolved that provide the hydrolytic products [30].

DNA cleavage by DNAzymes, in contrast, often requires a more complicated cleavage mechanism due to the lack of 2′ hydroxyl on the deoxyribose. There are several proposed pathways for cleavage, with the exact mechanism dependent on the individual DNAzyme. One pathway indicates that the cleavage generates a 3′ hydroxyl and a 5′ phosphate in the presence of a base (Fig. 1B), as in RNAse P cleavage of RNA. Additionally, in some DNAzymes a second hydrolysis occurs on the other side of the new 3′ terminus to give the complete excision of a nucleoside, resulting in the two cleaved ends having 3′ and 5′ phosphates as shown in Fig. 1C [31]. Another proposed mechanism holds that the DNA is cleaved across the ribose, generating a 5′ phosphate and a 3′ phosphoglycolate that can later be converted into a 3′ phosphate (Fig. 1D) [32]. The mechanism shown is analogous to a well characterized DNA cleaving system, bleomycin-induced DNA scission [33–35].

2.1. RNA-cleaving DNAzymes

Since the 2′ hydroxyl present on RNA allows for facile cleavage, there is a larger library of RNA-cleaving DNAzymes than DNA-cleaving ones. Within the scope of RNA-cleaving DNAzymes, there are two clear subsets: DNAzymes that can cleave substrates made exclusively of RNA (full RNA) and those that can cleave chimeric RNA-DNA sequences. This distinction is important when selecting DNAzymes for different applications, as a DNAzyme that requires an RNA-DNA chimera may not be useful for therapeutic techniques, whereas it is preferred in a sensing application. The following is a showcase of DNAzymes that can cleave full RNA, single RNA bases in a DNA context, or both.

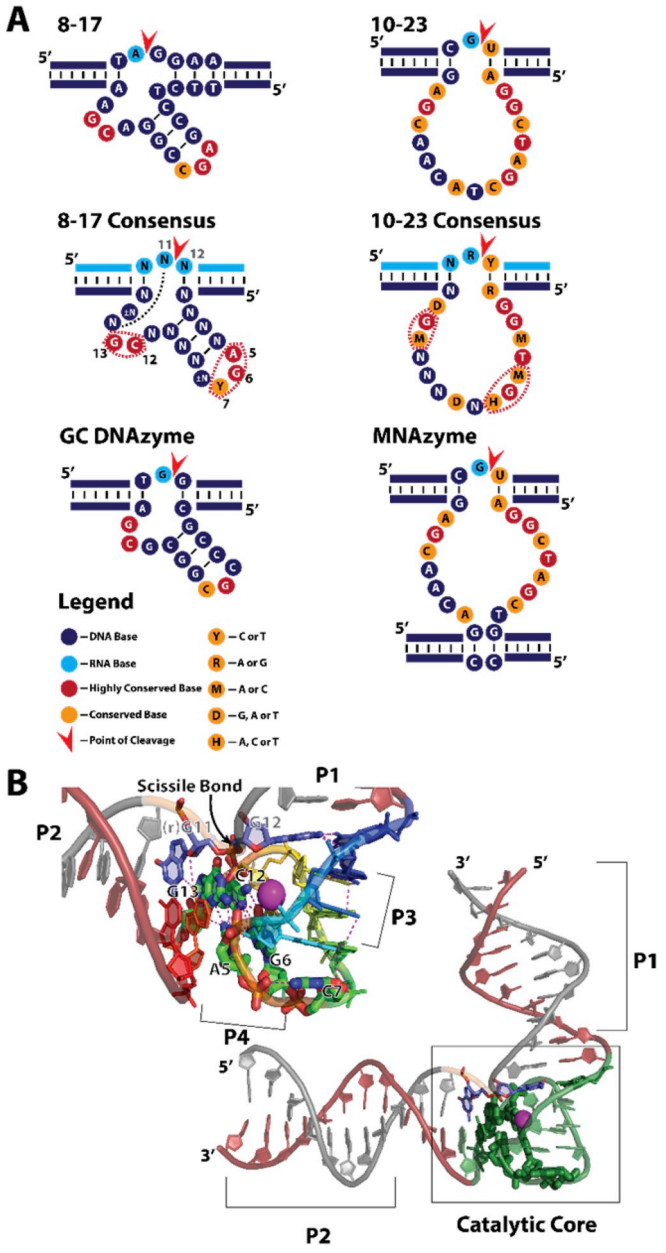

Fig. 2. Sequences and structures of full-RNA cleaving DNAzymes (A): the Pb^{2+} dependent 8–17 and its consensus sequence, along with Mg^{2+} dependent 10–23 and its consensus sequence, determined through selective mutation studies. The consensus sequences are shown binding to a full-RNA substrate to demonstrate the ability of these DNAzymes to cleave both full-RNA and chimeras. The GC DNAzyme is a Mn^{2+}-dependent analogue of 8–17 constructed of only G and C nucleotides [43], and the modified 10–23 known as an MNAzyme is constructed from four separate oligonucleotides [44]. Highly conserved bases (red) are required for catalytic activity; moderately conserved bases (orange) are believed to help specify the catalytic cation or stabilize neighboring secondary structure. The dashed outlines indicate the conserved catalytic sequence of the 8–17, and the appearance of the same pattern in the 10–23 DNAzyme. Panel B shows the crystal structure of the catalytic core of 8–17, adapted from structure 5XM8. P1 and P2 are the binding arms of the structure, with grey showing the substrate strand, and red the enzymatic strand [39]. The catlytic core (green) is highlighted in the inset, with the 5 conserved bases highlighted, as well as the bases adjacent to the scissile bond. The required RNA base is labeled (r) as it was a DNA analogue for crystalization. Currently there is no structural information available on the catalytically active conformation of 10–23. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

2.1.1. Full RNA substrate-cleaving DNAzymes

In 1994, the first reported DNAzyme could catalytically cleave a DNA:RNA chimera on the 3′ side of a riboadenine in the substrate strand in the presence of Pb^{2+} [2]. The authors suggested that this sequence could be optimized to cleave full RNA substrates instead of the chimera they used, and three years later the 8–17 and 10–23 DNAzymes were developed (Fig. 2) [37]. These two DNAzymes, named for the batch and cycle of *in vitro* selection from which they were isolated, could both cleave full RNA substrates. 8–17 was found to cleave best with Pb^{2+}, though some catalytic activity remains if Zn^{2+} or Mg^{2+} are substituted. 8–17 is also remarkable as it is a very short sequence, and later studies mapping the bases required to conserve catalytic activity identified five bases in the catalytic loop that are commonly found in many RNA-cleaving DNAzymes, highlighted in Fig. 2 with a dashed outline [38]. Crystal structures of the 8–17 DNAzyme further reinforce the necessity for these highly conserved bases, with the GC and GA conserved dinucleotides interacting via hydrogen bonds in the catalytic core [39]. It is important to note that 8–17 is one of the few DNAzymes to have its three-dimensional structure elucidated. In all cases throughout this review the secondary structure proposed by the authors of the cited papers is shown.

The 10–23 DNAzyme was found in parallel with 8–17, but in this case cleaving in the presence of Mg^{2+}. Like 8–17, 10–23 is capable of cleaving both full RNA and chimeric substrates [40], but it has a preference for rA followed by a pyrimidine (C/U/T) at the cleavage site [37]. The sequences of 8–17 and 10–23 have been analyzed through mutational studies, as well as abasic nucleotide substitutions, to isolate the bases necessary for catalysis, which allows us to identify both the 8–17 pattern within 10–23, [40] and the additional six conserved bases in 10–23 that appear to be necessary for Mg^{2+} preference [41]. 10–23 has one of the fastest rates of cleavage of any RNA-cleaving DNAzyme while using a commonly-used cation, and thus 10–23 has risen to prominence in bioanalytical spheres [42].

Additionally, Multi-component DNAzymes, or MNAzymes, have been derived from the 10–23 sequence [45]. These MNAzymes contain an interrupted 10–23 catalytic loop upon which a second set of binding arms are added, so that the loop can be correctly reconstructed only if a fourth DNA bridges across the gap and closes the loop. This allows for added specificity, as all four DNAs need to be present for the reaction to occur, but it does increase the complexity of the structural assembly. Polylysine-*g*-dextran has been shown to increase the cleavage activity of MNAzymes when included in the reaction buffer, potentially facilitating the assembly of the active complex through volume exclusion [44].

The 8–17 structure is also found in several other published DNAzymes, though not all of them exhibit the same substrate specificity. The GC DNAzyme (Fig. 2) is an example that can cleave full RNA substrates, albeit very slowly relative to the parent 8–17, in the presence of Mn^{2+}. It is about 10-fold more active on chimeric substrates [43]. Additionally, several other DNAzymes that contain the 8–17 conserved sequence show no affinity for full RNA substrates, cleaving only chimeras [46–48]. These will be discussed in a subsequent section.

There are also several DNAzymes that do not have the 8–17 sequence that are capable of cleaving full RNA substrates, although they have a higher affinity for chimeric substrates. In 2008 the Li lab published the results of a screen for DNAzymes that cleave specifically at chimeric pyrimidine-pyrimidine junctions r(C/U)|(C/T) sequences. These junctions were specifically chosen because 8–17 and its sequence analogues exhibit much slower cleavage kinetics with these target dinucleotides. Three of their hits, named S4, S9 and S21 (Fig. 3), also showed some level of catalytic activity on the corresponding full RNA substrates [49]. Cleavage by all of these sequences exhibits a strong dependence on the presence of Mn^{2+}, and S4 also requires K^+ or Na^+ for full activity.

2.1.2. Classification of RNA-DNA chimera-cleaving DNAzymes

The largest category of DNAzymes are those which cleave RNA-DNA

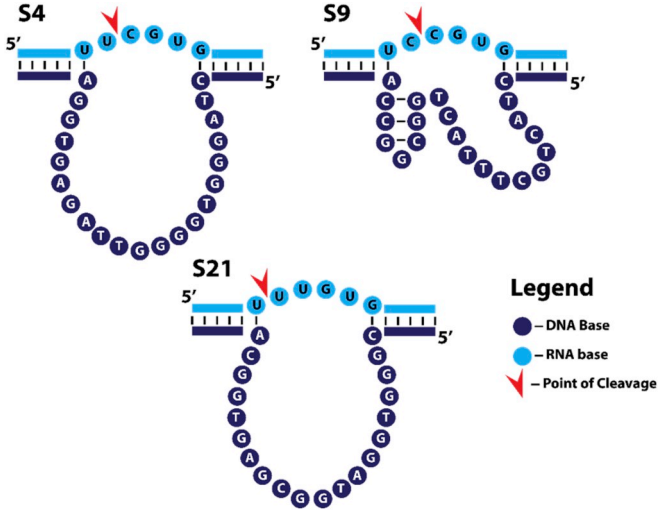

Fig. 3. Sequences and structures of the full RNA substrate cleaving DNAzymes that do not belong to the 8–17 family: S4, S9 and S21. All require Mn^{2+}, and all cleave chimeric substrates better than they do the full RNA. The GUG sequence in each of the substrate strands is a remnant of the primers used to generate the SELEX pool, so it is not known if the sequence is strictly required for DNAzyme activity.

chimeras. This is perhaps due to practical considerations: the chimeras are much easier to work with than full RNA, especially when immobilized onto a surface as a cleavage target as is often done during SELEX to evolve cleaving moieties. Chimeras are additionally chosen more often as they are likely to be more chemically stable than full RNA in biological media, while maintaining the chemical reactivity of RNA's 2′ hydroxyl. Also, in principle chimeras are cleaved more specifically, since a full-RNA substrate for an RNA-cleaving enzyme has a potential non-specific cleavage site at every phosphate. This has been of particular interest to the low-resource point-of-care community, for which reagent storage in ambient conditions is extremely important. Below, we describe this family of DNAzymes, classifying them in terms of metal ion or other ligand dependence to enable informed choices of which DNAzymes could fit into an assay's workflow.

2.1.3. DNAzymes dependent on the common divalent ions Mg^{2+} and Mn^{2+}

Fig. 4 illustrates several chimera-cleaving DNAzymes. As with the DNAzymes capable of cleaving full RNA sequences, many require divalent cations. E6, one of the chimera-cleaving DNAzymes that contain 8-17's conserved bases, cleaves in the presence of Mg^{2+}, with Na^{+} accelerating cleavage (the rate increases linearly with Na^{+} concentration above 0.1 M) [47]. CT10.3.29, a DNAzyme designed to preferentially cleave the rC-T junction that 8–17 cannot, cleaves in the presence of Mg^{2+} and Mn^{2+} [50]. While it is not the fastest pyrimidine-pyrimidine cleaving DNAzyme, it is of particular interest due to the investigators' ability to alter only the base opposite the 5′ cleavage site dinucleotide of the sequence, shown in orange in Fig. 4, in order to control the DNAzyme's preference for the cleavage dinucleotide. The M5 variant of CT10.3.29 shown in Fig. 4 with a T as its 39th base prefers r(U/A)-(A/T) as well as rG-T cleavage sites, while the M4 variant with a G as the 39th base prefers r(U/C)-(A/T). Clearly the sequence preference is not dictated solely by Watson-Crick pairing at the junction, and A or C at position 39 gives much less active DNAzymes. S15, a DNAzyme found in the same SELEX screen as the full-RNA DNAzymes shown in Fig. 3, also cleaves pyrimidine-pyrimidine chimeras in the presence of both Mn^{2+} and Mg^{2+} [49].

2.1.4. DNAzymes dependent on uncommon metal ions Ag^{+}, Hg^{2+}, Cu^{2+}, Co^{2+}, and UO_2^{2+}

Many RNA-cleaving DNAzymes have been selected for use with

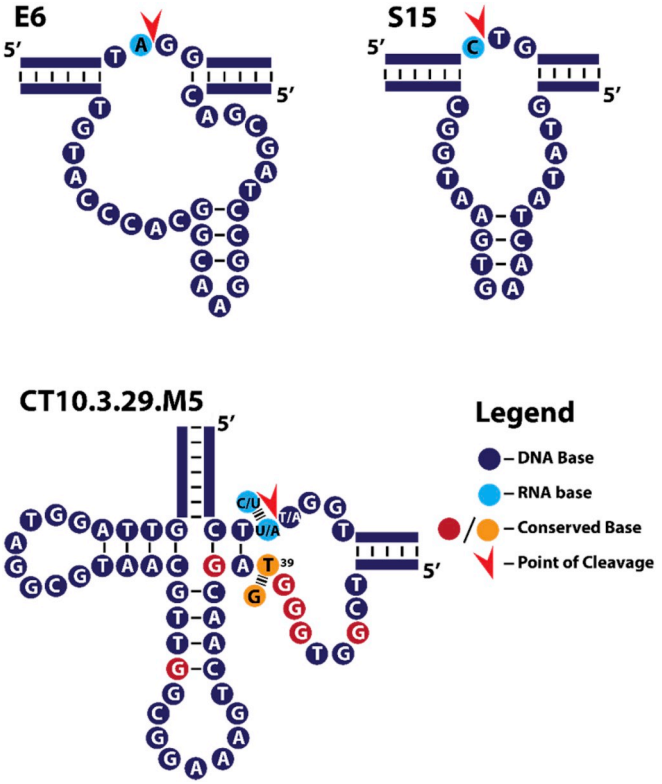

Fig. 4. Shown are the sequences and structures of the magnesium- and manganese-dependent chimera-cleaving DNAzymes E6, [47] CT10.3.29M5 [50] and S15 [49]. Conserved bases for CT10.3.29 M5 were identified by comparing all hits within a selection pool for consensus. Changing the orange base (discussed as base 39 in the text) alters the sequence preference for the RNA base (light blue) at the cleavage site. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

cations that are not ubiquitously found in buffers. Such DNAzymes, for example Ag10c and its derivatives, which cleave in the presence of Ag^{+} [51,52], can be used as sensors to detect the ions in solution, and demand for field tests of contaminated water samples have led to efforts to develop DNAzymes for most heavy metals. Additionally, these DNAzymes (shown in Fig. 5) have been screened to be as selective for their cation as possible; whereas Ag10c has slight catalytic activity in the presence of Hg^{2+}, $E_{Hg}0T$ [53] is much more sensitive to the mercuric ion. $E_{Hg}0T$ was derived from mutations to DNAzyme 39, which cleaves in the presence of UO_2^{2+} [54]. Despite being the first lead-dependent DNAzyme discovered, 8–17 is not very selective for lead, so a search for a more specific DNAzyme led to the isolation of GR-5, which is responsive only to Pb^{2+} [55]. While there are DNA-cleaving DNAzymes that use copper as a cofactor, there are currently no identified Cu^{2+}-dependent RNA-cleaving sequences. There is, however, the PSCu10 DNAzyme, which contains a phosphorothioate bond to the 3′ of the RNA base, allowing for self-cleavage specifically in the presence of the thiophilic Cu^{2+} ion [56]. DEC22-18 is a DNAzyme that cleaves in the presence of Co^{2+}, though it can also use either Ni^{2+} or Mn^{2+} [57]. DEC22-18 is also of interest because the substrate strand can be cleaved between an internal fluorophore and quencher pair, allowing for fluorescence signaling for Co^{2+} allowing for the DNAzyme itself to perform analogously to a TaqMan probe, generating signal from the cleavage directly. This cleavage through a fluorogenic substrate can also been seen in several other DNAzymes, namely the Mg^{2+} cleaving MgZ [58], Mg^{2+}, Mn^{2+}, Co^{2+}, or Ni^{2+} dependent 5J-A28, [59] and the previously mentioned GR5 [55].

Achieving selectivity for one single metal ion is a common challenge in the development of metal-dependent DNAzymes, as can be seen from

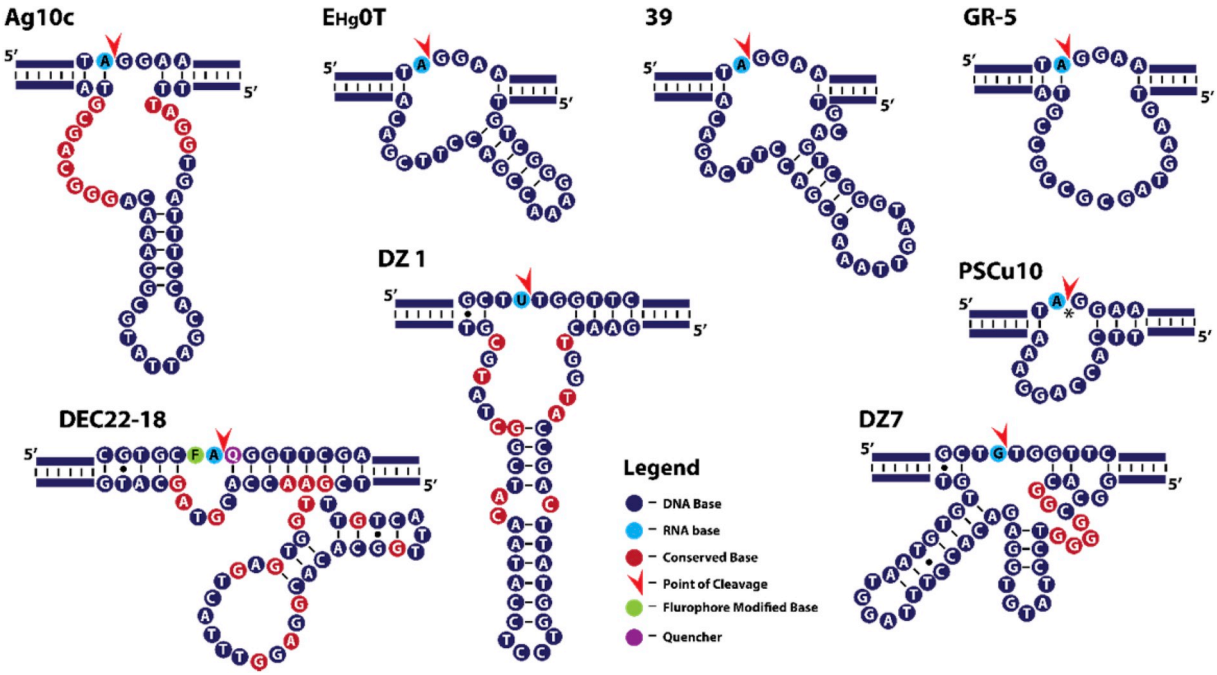

Fig. 5. Sequences and structures of transition metal dependent chimeric substrate cleaving DNAzymes: the Ag^{+} dependent Ag10c [51], Hg^{2+} dependent $E_{Hg}0T$ [53], UO_2^{2+} dependent 39, [54] Pb^{2+} dependent GR-5, [55] and Co^{2+} dependent DEC22-18 [57]. F indicates the internal fluorophore fluorescein-dT and Q is the internal quencher DABCYL-dTPSCu10 is a DNAzyme with a phosphorothioate linkage at the cleavage site (indicated by an asterisk) that confers dependence on Cu^{2+} for self-cleavage [56]. DZ1 and DZ7 catalysis can be assisted by a range of metal ions (Mn, Ni, Co, Zn, Pb, Cd)$^{2+}$ [60]. Conserved bases are identified by comparing all hits within a selection pool. The conserved bases in Ag10c are based on a reselection experiment that provided a smaller variant [52].

the lack of specificity for the catalytic ion exemplified by 5J-A28 or DZ1, the latter of which can catalyze cleavage in the presence of Mn^{2+}, Ni^{2+}, Co^{2+}, Zn^{2+}, Pb^{2+} or Cd^{2+} [60]. The catalytic activity of the DNAzyme varies with the different metal ions, as with the other DNAzymes identified in the same screen, but in all cases they are responsive to more than one ion, which could easily confound test results.

2.1.5. DNAzymes dependent only on monovalent ions

Rarer than the divalent cation dependent DNAzymes are those requiring monovalent cations only (see Fig. 6). That is not to say they are not available, as the DNAzyme G3 cleaves a riboadenosine on its substrate strand in the presence of either Na^{+} or NH_4^{+} [61]. Additionally, EtNa, named after its two catalytic cofactors, cleaves in the presence of Na^{+}, with ethanol accelerating its activity when added up to 72% v/v [48]. EtNa can also cleave in the presence of isopropanol, methanol or DMSO. NaA43 is another Na^{+} cleaving DNAzyme, which contains the conserved 8–17 bases [62]. It was believed to bind exclusively sodium for some time, until the discovery of Ce13d, a Ce^{3+} dependent DNAzyme that was later found to contain the same catalytic core sequence as NaA43. Subsequent investigations demonstrated that NaA43 requires Na^{+} and can be accelerated by Ce^{3+}, while Ce13d requires Ce^{3+} and can be accelerated by Na^{+} [46].

2.1.6. DNAzymes with activity at low pH

With the exceptions of EtNa and Ag10C, which have both been found to cleave at a pH of 6.0, and 39, which cleaves at 5.5, every DNAzyme described so far cleaves in the pH range 7.0–7.5. This lack of variability in the pH motivated the directed evolution of DNAzymes that specifically allow for a broader range of assay conditions, shown in Fig. 7. DZ15WS and DZ27WS self-cleave only in a pH 4.0–4.5 sodium citrate buffer, and have been shown not to require divalent ions [63]. In fact, divalent cations reduce cleavage activity, with Cd^{2+} completely inhibiting cleavage of the DNAzymes. An additional study by the Li lab selecting for a pH-specific cleavage led to the identification of DNAzymes that cleave only at pH values of 3.0, 3.8, 4.8 and 6.0, with the DNAzymes correspondingly named pH3DZ1, pH4DZ1, pH5DZ1 and pH6DZ1 [64]. These DNAzymes do not exhibit specificity towards one cation, though all of them showed cleavage in the presence of Mn^{2+} and Na^{+}. Interestingly pH3DZ1, pH4DZ1 and pH5DZ1 have no proposed secondary structures; presumably they adopt pH-dependent secondary and/or tertiary structure that is not captured by structure prediction programs like NuPack [65] and UNAfold [66] that assume canonical ionization states for the bases — we verified that these programs do not predict any stable secondary structures.

2.2. DNA-cleaving DNAzymes

DNAzymes capable of cleaving at a DNA nucleotide are significantly rarer than those that employ a 3′-OH. These DNAzymes can be classified according to the divalent cation used to enable catalytic activity.

The first subclass was discovered in 1996 by the Breaker group when performing in vitro screening for DNAzymes active with Cu^{2+} [68]. This subclass is named Pistol-Like DNAzymes due to the characteristic shape of the drawing of the secondary structure, shown in Fig. 8. These DNAzymes cleave themselves in the presence of micromolar Cu^{2+} [69], with some sequences having enhanced activity in the presence of both Cu^{2+} and hydrogen peroxide [68] or ascorbic acid [32]. The cleavage site for these DNAzymes is located in a Watson-Crick helix on the ‘barrel’ of the ‘pistol,’ and cleavage is possible with any barrel sequence as long as Watson-Crick base pairing is maintained.

F8-X is a DNAzyme with a copper cofactor but it is structurally unrelated to the Pistol-Like DNAzyme family [31]. F8-X is particularly useful in applications, as the cleavage site is between two variable binding arms, allowing for a copper-mediated DNAzyme that has no sequence restrictions. Just like the Pistol-Like DNAzyme, its activity can be enhanced by the presence of hydrogen peroxide, as well as DTT or Mn^{2+}.

Another subclass of DNA-cleaving DNAzymes was identified in 2009 by the Silverman lab [72]. This DNAzyme, called 10MD5, was found to cleave in the presence of either Mn^{2+} and Zn^{2+}, though either Cd^{2+} or

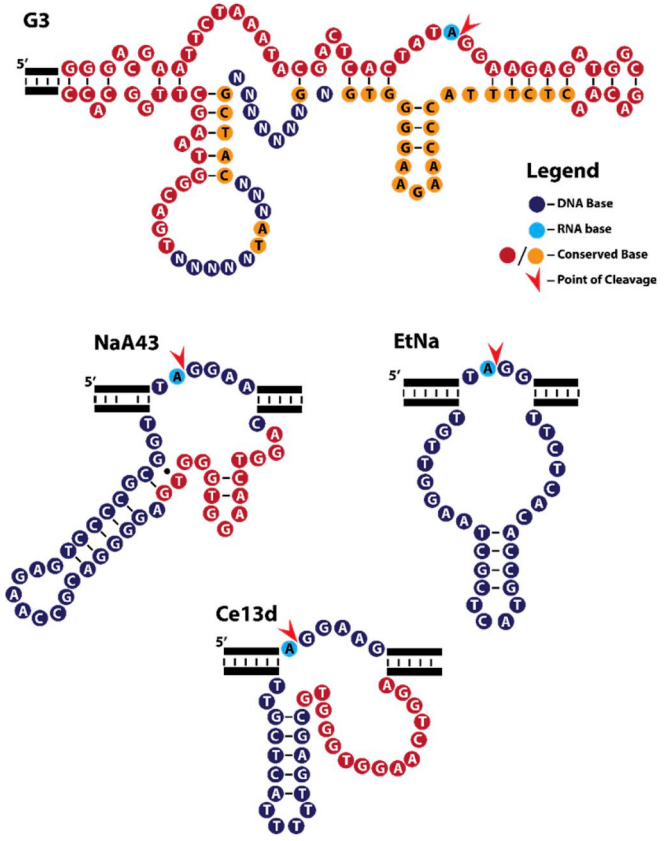

Fig. 6. Sequences and structures of the monovalent ion chimeric substrate DNAzymes: the Na^+ or NH_4^+ dependent G3, [61] Na^+ dependent NaA43 [62] and EtNa [48], and Ce13d [46], a variant of NaA43 that prefers $Ce3^+$ over Na^+. There is a strong but essentially accidental sequence homology to the substrates of the previously listed DNAzymes: TrAGGAA is in these sequences due to the selection conditions used in these various publications – it is the substrate sequence of 8–17. Due to the field's understanding that 8–17 is an active DNAzyme dependent on divalent transition metal ions, most DNAzymes that were reselected for altered ion specificity originated from the 8–17 sequence.

Ca^{2+} can be substituted for Mn^{2+} with some decrease in catalytic activity. 10MD5 is active only in a very narrow pH range around 7.5. The cleavage site in this class of DNAzymes is located in a Watson-Crick helix and requires the ATGˆT sequence to cleave, but it will tolerate any sequence to the 3′ side of the cleavage site as long as Watson-Crick base pairing is maintained. 9NL27, a variant of 10MD5 that was identified when reselecting the sequence to broaden its pH tolerance, varies by only 5 bases in the enzymatic loop, shown in green in Fig. 8 [70]. 9NL27 is notable as it requires only Zn^{2+} for catalysis, and selective mutation of the 5 bases that differ between 10MD5 and 9NL27 showed that only the T16 to A and G19 to C changes are required for exclusive Zn^{2+}-dependent catalysis. The orange bases in the substrate, however are necessary to achieve cleavage activated by Zn^{2+}, further limiting the range of DNA analytes that can be cleaved [73].

In 2013, the Breaker lab published two more DNA-cleaving DNAzymes dependent on Zn^{2+} [71]. In the selection subgroup they named Class I, they found a highly conserved sequence in the loop that correlated to catalytic activity. Notably, the cleavage site was located in the conserved sequence of that loop. This Class I DNAzyme is currently the fastest DNA-cleaving DNAzyme known, capable of nearly 90% cleavage in 5 min. The Class II sequences had a similar structure and cation dependence, but with a cleavage site immediately adjacent to the conserved sequence.

2.3. Allosteric DNAzymes

On occasion, a biosensor strategy needs to be more complex than a single cleavage step. DNAzymes can be used to perform stimulus-responsive steps just like their protein counterparts. Moreover, those multiple steps can be designed to mimic the if-then nature of allosteric enzymes. This has been done by incorporating the strictly conserved sequences of two cleaving DNAzymes into the same active loop, as shown by the DRc dual-activity enzyme, which combines the PLDz DNAzyme that cleaves in the presence of Cu^{2+} and ascorbic acid, modified with the conserved bases of 8–17's catalytic core. The DRc DNAzyme, shown in Fig. 9, is allosteric in that a switch in the cleavage mechanism and the specificity for DNA vs. RNA cleavage depends on the availability of a chimeric substrate [74]. An example of a more traditionally allosteric DNAzyme is the UO_2^{2+}-dependent 39 DNAzyme, whose cleavage activity additionally requires the binding of

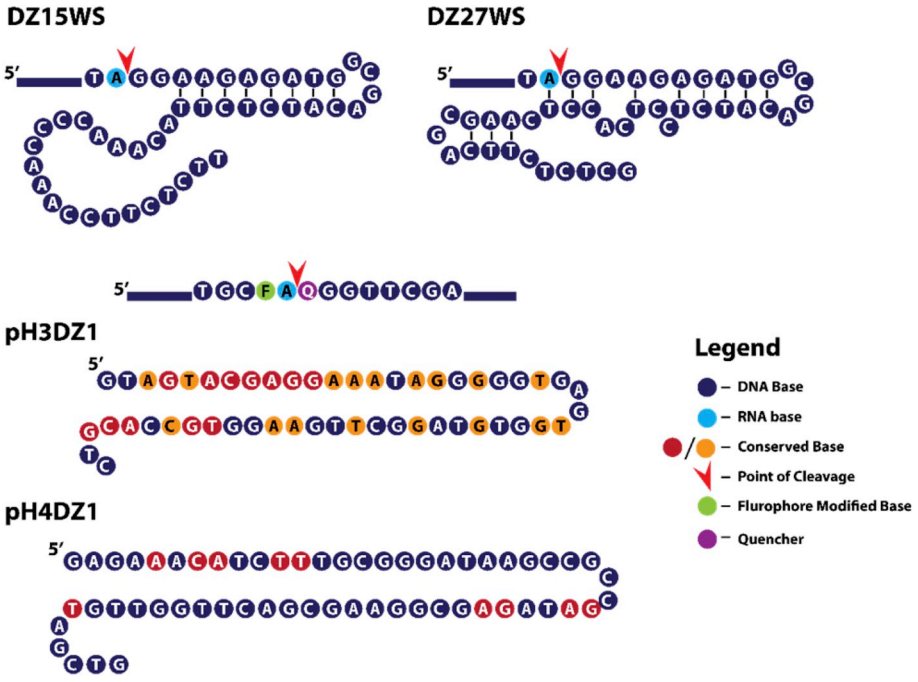

Fig. 7. Sequences and structures of DNAzymes capable of cleavage in low pH conditions: pH 4 responsive DZ15W5, [63] pH 4.5 responsive DZ27WS [63], pH 3 responsive pH3DZ1 [67] and pH4 responsive pH4DZ1 [67]. Conserved bases are identified by comparing all positive hits within a selection pool for consensus. pH5DZ1 and pH6DZ1 are not shown; all sequences are available in Table A1.

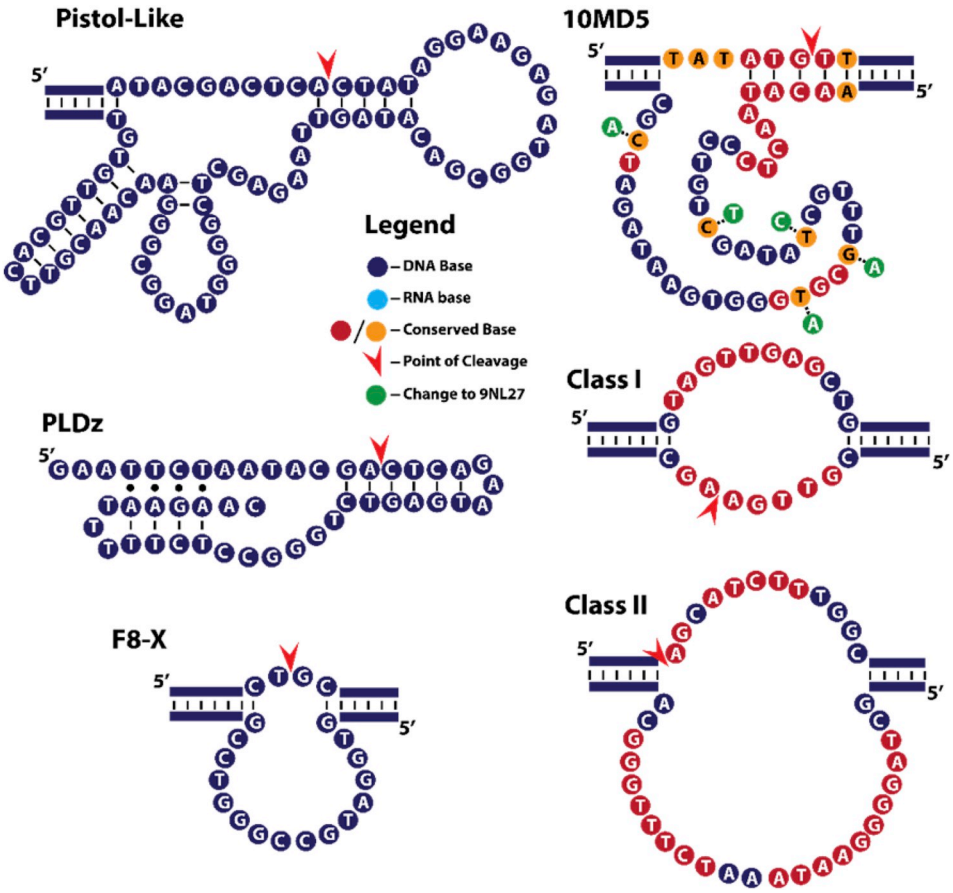

Fig. 8. Sequences and structures of DNA-cleaving DNAzymes: the Cu^{2+} mediated Pistol-Like DNAzyme [68], PLDz [32], and F8-X [31], as well as the Mn^{2+} and Zn^{2+} dependent 10MD5 [70] and Zn^{2+} dependent Class I and II [71]. The bases required to change 10MD5 to 9NL27 are shown in green [70]. Conserved bases are identified by comparing all positive hits within a selection pool for consensus. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Hg^{2+} in the stem-loop to compensate for T-T mismatches that had been introduced in the catalytic core, generating a dual-sensor DNAzyme that reports on the simultaneous presence of uranium and mercury [53].

Colorimetric detection can also be introduced into the DNAzyme sequence, as in the case of the Dual DNAzyme, a DNAzyme derived from the Pistol-Like sequence with the addition of a G-quadruplex sequence. Upon cleavage of the DNAzyme and formation of the quadruplex, hemin can intercalate and initiate the generation of a color change in solution [76]. The same concept has also been utilized when detecting a nucleic acid analyte, as in the case of a MNAzyme- and G-quadruplex-containing system that generates a color change upon formation of the MNAzyme complex [75].

3. Emerging sensing applications for DNA-Catalyzed cleavage

The simplicity and stability of DNAzymes has led to their implementation in a variety of assays, primarily for sensing or therapeutic purposes. These applications are driven by the thermal and buffer stability of the DNAzyme that allows for their substitution for enzymes or other fragile components in bioassays. The cleavage event is additionally dependent on both hybridization-mediated structure and the metal cofactor, generating two potential analytes for each DNAzyme to detect. The incorporation of modified bases such as fluorescent probes and quenchers, as in the case of the MgZ DNAzyme discussed previously, allows for the DNAzymes to act as single-step, real-time readouts that do not require complicated instrumentation to interpret.

As described in the allostery section above, the DNAzymes can be designed with additional DNA functionalities, thus incorporating two different cleaving sequences, mixing both cleaving and other functional DNAzyme properties, or the addition of an aptamer-binding sequence to detect a small molecule target. These additional functions can allow for two-step assays to be performed in sequence, expanding the range of processes a single DNA sequence can perform; for instance, an aptamer can require binding to the target molecule to expose the self-cleaving DNAzyme sequence. DNAzymes can also feed into enzymatic assays, such as amplification strategies like polymerase chain reaction (PCR) for greater sensitivity, or horseradish peroxidase (HRP) for rapid signal generation.

In the following we present a showcase of the wide range of applications for DNAzymes in sensing. While the literature in this area is rapidly expanding, we have selected publications that highlight the advantages of DNAzymes and their potential for translation to applications that benefit from use at the point of sample collection.

3.1. Ion sensors

Heavy metals pose serious threats to human and environmental health, and as such many techniques have been developed to detect them. Due to the low permissible concentrations of lead (15 ppb) and mercury (2 ppb), the EPA suggests using Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES), a technique that is excellent for detecting trace quantities in a mixed sample, but entirely too expensive and immobile for field use [77]. At the other extreme there are several affordable tests that are commercially available for home use, but these tests cannot differentiate which metals they are detecting. In between these two extremes lie electrochemical sensors, such as the portable anodic stripping voltammetry (ASV) sensor that can detect Hg^{2+}, Pb^{2+}, and Cu^{2+} in a clean sample [78] or the carbon nanotube enhanced ASV assay capable of detecting trace quantities of lead and cadmium in processed water samples [79]. Most applicable to on-site testing is a surface-enhanced Raman spectroscopy (SERS) sensor capable of detecting metal ions in a complex organic mixture, removing the need to fully purify a sample before analysis [80]. These techniques,

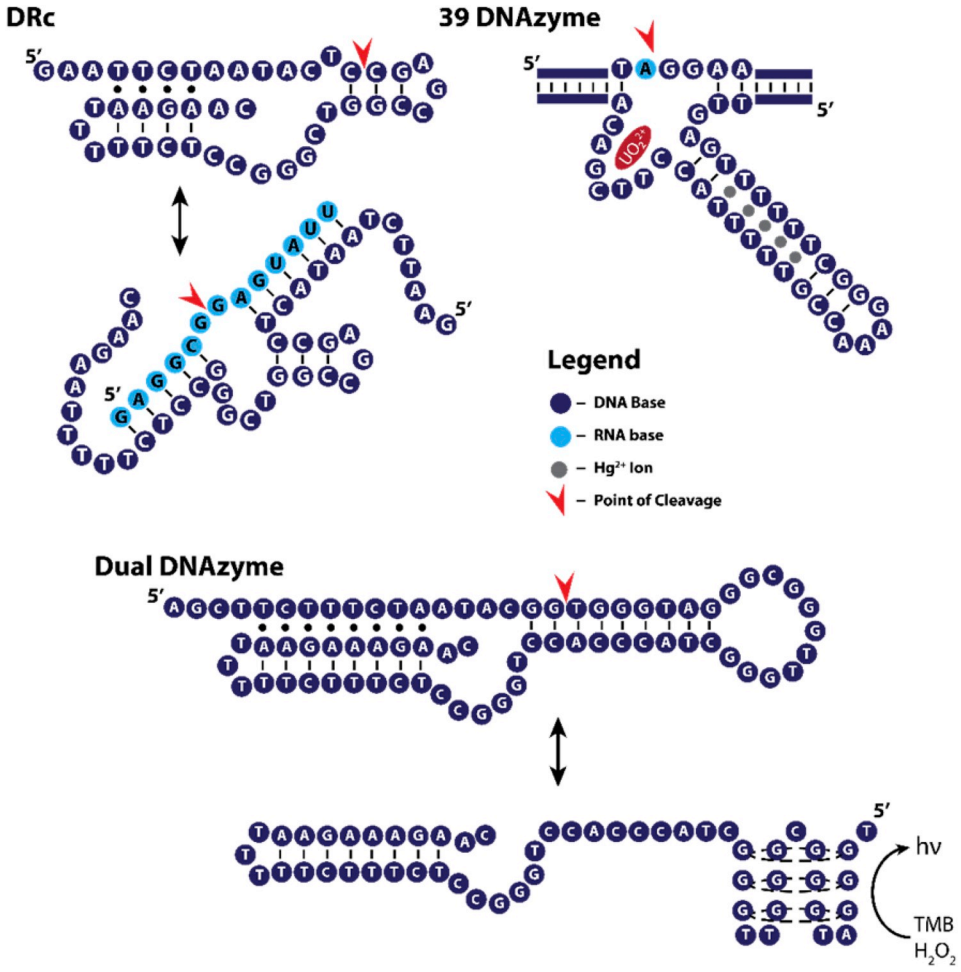

Fig. 9. Sequences and structure representations of several allosteric DNAzymes. The 8–17 and Pistol-Like DNAzyme hybrid, DRc, that can cleave through either mechanisms depending on the available analyte [74]. The Dual DNAzyme undergoes cleavage by the Pistol-Like mechanism that then allows for refolding to form a G-quadruplex for subsequent colorimetric signal generation through TMB oxidation [75]. Instead of introducing another DNAzyme's functionality, the addition of T-T mismatches to the DNAzyme 39 sequence led to a requirement for Hg^{2+} (grey circle) for cleavage, generating a DNAzyme that can simultaneously detect the presence of two analytes: mercury and uranium [53].

while accurate, are complex and expensive.

DNAzymes, with their cheap components and highly specific catalytic properties, provide a logical alternative for metal ion sensors, especially for field work. Ag10c, the DNAzyme dependent on silver, was designed with the intent to detect Ag^{+} contamination in lake water [51]. The $E_{Hg}0T$ DNAzymes were screened specifically for selectivity for only Hg^{2+} ions, with the aim of providing a sensitive mechanism for detecting trace mercury in water samples [53]. A straightforward fluorescence readout is easily obtained in real time due to the incorporation of a fluorophore and quencher on the substrate strand. Cleavage in the presence of Hg^{2+} separates the pair and generates a visible fluorescent signal. GR-5 was discovered as an optimization of the lead-catalyzed but promiscuous 8–17 for on-site detection of Pb^{2+} [55]. Similarly, PLDz DNAzymes were utilized as copper sensors to determine pollution levels in samples ranging from bottled water to sewage; a schematic of this assay is shown in Fig. 10 [81]. In all of these cases, the DNAzyme provides for a simpler mechanism of detection that can replace inherently complicated existing metal ion tests and allow for a cheaper, more portable assay.

The metal ions that enable DNAzyme cleavage can also be quantified in more complex environments. 17E, a variant of the DNAzyme 39 that can cleave in the presence of Ca^{2+} and Mg^{2+}, was screened in the presence of serum so that it would be functional in a cell [82]. This capability to detect the metal concentration in an non-invasive manner allows for real-time tracking of these analytes in a live cell. NaA43, a sodium-dependent DNAzyme, has been utilized as a Na^{+} tracker in cells using a photo-caging technique that blocks the catalytic 2′ hydroxyl from degradation in the cell until brief exposure to 365 nm light [62]. This photolabile group allows for a delayed start to the imaging, generating a technique that can be initiated by an external stimuli at any time following the initial setup.

3.2. Biosensors

Conventional methods for the detection of biological analytes have so far been primarily either immunoassays such as the Enzyme-Linked ImmunoSorbent Assay (ELISA) for protein and small molecule biomarkers [83–85] or nucleic acid amplification techniques like the polymerase chain reaction (PCR) for nucleic acid sequence biomarkers [86,87]. ELISAs have numerous complicated manual steps. PCR generally requires multiple manual steps for preparation, and the reaction requires thermocycling, which has a significant energy and complexity requirement. These two techniques, while sensitive and specific, are difficult to use in low resource settings. While there have been some efforts to miniaturize thermocyclers so that they can be brought into the field [88] and to simplify ELISAs so that they can be performed by minimally trained staff [89], they are still relatively complicated assays to perform away from the central lab.

DNAzymes can be incorporated into ELISAs to replace fragile enzymes, as in the assay shown in Fig. 11. Here analyte detection has been performed through an antibody sandwich assay in which the analyte bound to a capture antibody is recognized by a detection antibody that is conjugated to PbS nanoparticles. The immobilized PbS particles in the well generate Pb^{2+} ions upon HNO_3 treatment, and transfer of the solution to a chamber containing an electrode bearing an 8–17 DNAzyme. Cleavage of an 8–17 substrate with a ferrocene tag on the end allows the newly flexible complementary DNA to bring the tag closer to the electrode surface and generate an electrical current [90]. This

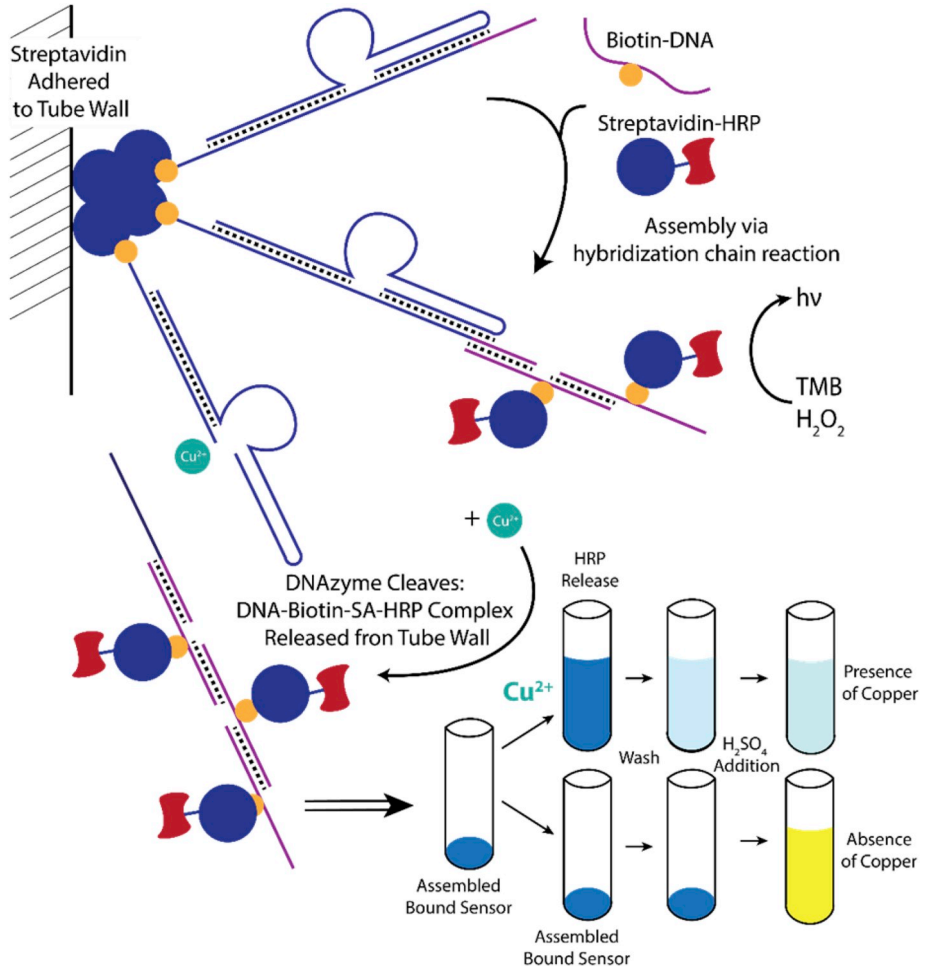

Fig. 10. Cu^{2+} sensing by utilization of an immobilized Pistol-Like DNAzyme to cleave HRP-conjugated streptavidin-biotin concatemers. In the presence of Cu^{2+}, the DNAzyme cleaves a segment that is hybridized to a DNA concatemer with HRP bound via biotin and streptavidin. Thus, the presence of Cu^{2+} is identified by the lack of an HRP-driven color change [81]. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

technique has also been used to separate the fluorophore from its quencher [91], yielding a fluorescent signal that is significantly less instrument-intensive to analyze.

DNAzymes can also be used to detect specific cell types, as in the case of the DNAzymes RFD-CD1 and RFD-EC1, which can specifically cleave in only the presence of *C. difficile* and *E. coli* [92]. These DNAzymes contain the fluorophore and quencher motif seen in other DNAzymes, which was subsequently utilized in a paper-based printed *E. coli* sensor, allowing for the rapid and cheap production of these portable sensors [93]. Another sensor for breast cancer diagnosis has also utilized this technique, with the AAI2-5 DNAzyme capable of detecting the presence of a cytoplasmic protein from the MDA-MB-231 cell line, with a similar fluorophore-quencher signal generation.

The DNAzyme itself can also be used as the analyte, as in the case of MNAzymes. The target nucleotide sequence bridges across the MNAzyme arms, stabilizing the active DNAzyme enzyme conformation, which can then cleave a separate substrate strand [45]. This construct allows both for a lower background signal in the sensors and the ability to use the same expensive fluorophore and quencher-labeled substrate strand in a different assay through simply changing the sequences of the two catalytic halves. This cost-cutting measure means that altering the target sequence entails synthesizing just three unmodified DNA sequences, with all the costly components retained and repurposed.

Additionally a DNAzyme can be used to generate an amplification cascade to increase the output of the initial detection signal, as in the case of the EC1 DNAzyme cleaving one of two concatenated DNA circles in the presence of *E. coli* and initiating rolling circle amplification (Fig. 12) [94]. The same concept can be seen in another scheme from the Liu group that utilizes the MgZ DNAzyme to generate primers for rolling circle amplification upon binding of a complementary target sequence [95]. In both strategies, the DNAzyme generates an overhang that phi 29 DNA polymerase can shorten through its 3′→5′ exonuclease activity, allowing for subsequent amplification. This allows for a single binding event to generate many-fold its signal, thus lowering the limit of detection of the assay.

3.3. Therapeutics

With the arrival of Onpratto [96], Alnylam Pharmaceuticals' RNAi drug that was recently approved by the FDA, DNA and RNA therapeutics have finally hit the mainstream. CRISPR and other RNA-targeted cleavage techniques have also burst onto the global stage, allowing for more permanent inactivation of target genes. CRISPR [97], as well as zinc finger nucleases [98], are both bulky mechanisms that need to be introduced into the host through a complicated series of steps. RNAi, despite being a smaller system, activates an immune response in normal mammalian cells that blunts or even negates its effect in many cases [99]. DNAzymes, on the other hand, can be effective mechanisms for gene regulation in vivo, while being simpler to introduce into the host system. Additionally, they are very cost-effective and easy to modify should mutations arise. The 10–23 DNAzyme dominates the therapeutics space, as it is capable of cleaving full-RNA

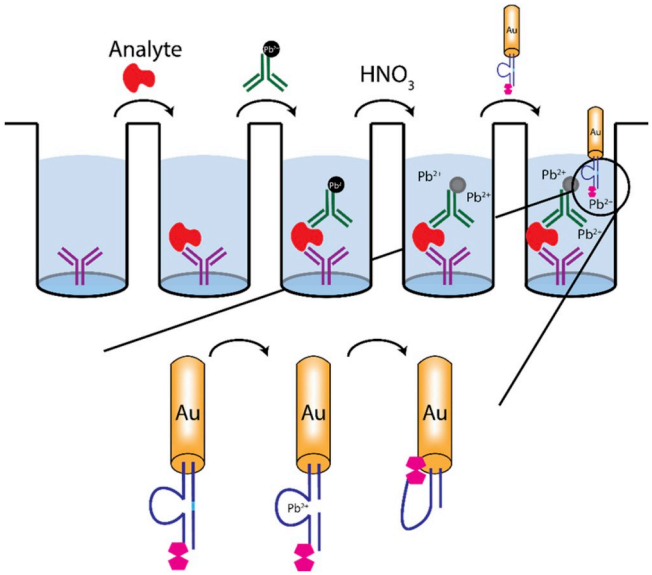

Fig. 11. The utilization of the 8–17 DNAzyme as a lead-activated readout following a traditional sandwich ELISA. The detection antibody is conjugated to a PbS nanoparticle. Free lead ions are generated by acid stripping, and the supernatant is added to a gold electrode with the 8–17 DNAzyme on its surface (top row). Cleavage of the DNAzyme allows a ferrocene molecule to come in contact with the gold electrode, generating an electrical current (bottom row) [90]. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

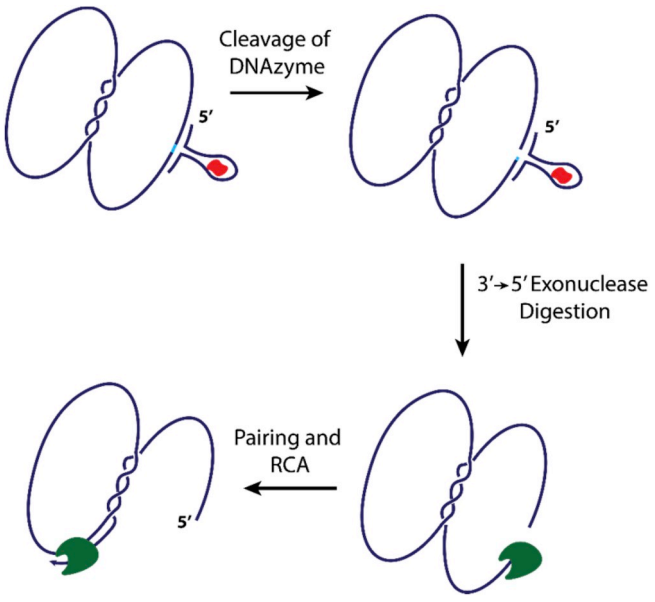

Fig. 12. This assay demonstrates the use of a DNAzyme to generate a primer for Rolling Circle Amplification (RCA). The cleavage of the EC1 DNAzyme leaves a cyclic phosphate on the 3′ end. Upon end processing with PNK, the phi29 DNA polymerase (green) can excise back to a region that can pair with the other circle, and then it can extend the primer using the second concatenated circle as a template [94]. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

substrates, it is one of the fastest cleaving DNAzymes available, and it is catalytically active in the presence of the Mg^{2+} that is available in the cell. With a simple alteration of the binding arm sequence, it can be made to hybridize to a specific target location, such as the bcr-abl fusion mRNA [100], the HIV-1 Integrase mRNA [101], and the latent membrane protein (LMP1) mRNA of the Epstein-Barr Virus [102]. The

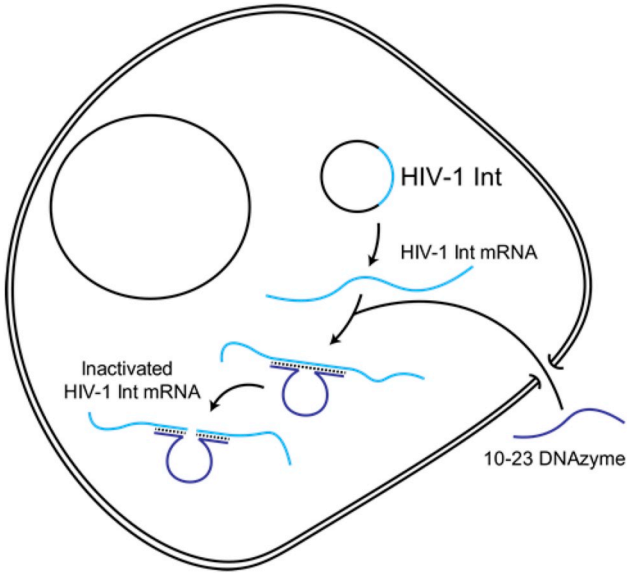

Fig. 13. The 10–23 DNAzyme injected into cells can inactivate HIV-1 Int mRNA transcribed from a plasmid, in HeLa, U-87, and Jurkat cells [101].

HIV-1 Integrase mRNA cleavage by 10–23 is shown in Fig. 13. In all three cases, the DNAzyme is capable of cleaving the target both in vitro and in cell culture, inhibiting the protein's expression and, in the case of LMP1, making a nasopharyngeal carcinoma more responsive to radiation therapy and decreasing tumor vascular permeability [103]. DNAzymes, which intrinsically have a longer half-life in serum when compared against RNA and protein [104], can also include modifications that make them more stable. The LMP1-cleaving DNAzyme was designed with phosphorothioates on the first and last two bases of the sequence to enhance its survivability in serum [103]. Similarly, a muscle acetylcholine receptor (AChR) DNAzyme included locked nucleic acids within the ends of the binding arms, which not only increased stability of the DNAzyme in serum, but also increased cleavage efficiency [105], corroborating an existing body of work regarding the stability of synthetic DNA in cells [106].

While all these studies [100–105] used transfection of the DNAzymes into the target cells, several have pointed out that transfection of the DNAzyme is one of the major hurdles to overcome, as transfection rates vary across cell types and agents [107]. (Delivery is, of course, a serious hurdle for all nucleic-acid based therapies.) Bacterial cells have also been targeted by insertion of a DNAzyme [108]. In most cases, these proof-of-concept publications utilize electroporation to introduce the DNAzyme into the cell. Two examples of this method successfully reduced β-lactamase production in cells. The first is a modified 10–23 DNAzyme that was used to cleave *blaR1* mRNA and consequently reduce methicillin resistance in MRSA through the disruption of the *blaR1-blaZ* signaling pathway [109]. The di-DNAzyme illustrated in the allosteric section (Fig. 9) was designed to inhibit ampicillin resistance in an *E. coli* strain. It is a construct of two 10–23 DNAzymes built into one sequence, cleaving two sites in a β-lactamase mRNA simultaneously for enhanced inactivation [110].

A study using a DNAzyme that targets the isocitrate lyase (icl) mRNA in tuberculosis utilized isoniazid, an antibiotic against tuberculosis, to make the cell wall more permeable to the DNAzyme. This increased inhibition of ICL expression in bacteria within macrophages, as well as a subsequent decrease in the survivability of tuberculosis [111]. Regardless of the mechanism of DNAzyme introduction, the reliance on exogenous delivery of the DNAzymes is a barrier to therapeutic efficiency. C-Dz, a variant of the 10–23 DNAzyme cloned into an M13 vector, is capable of replicating in the cell [112]. The circular DNAzyme allows for similar if not higher inhibition of β-lactamases as compared

to the linear DNAzyme, with the additional advantage of intracellular generation of the DNAzyme.

4. Conclusion and outlook

Here we have provided an exhaustive list of the nucleic-acid cleaving DNAzymes reported to date, along with their probable cleavage mechanisms and optimal operating conditions. Through this detailed assembly of DNAzyme sequences, we have clarified conserved regions among DNAzymes, which can offer insights and guidance to researchers looking to adopt DNAzymes for sensing and therapeutics applications. As summarized in Table A1, nucleic acid-cleaving DNAzymes provide functional capabilities that are available under an wide range of operational conditions, allowing for the utilization of thermostable, simple, and cheap components in a variety of reactions. Additionally, the long list of discovered sequences (catalogued in Table A2) allows for the rational design of future DNAzymes, as well as the development of novel DNAzyme sequences for biological targets and cations of high importance. More specific or faster variants of the existing DNAzyme options are constantly being developed, and the field is expanding as completely novel DNAzymes are discovered through SELEX experiments.

Of particular importance to the field is the need to develop DNAzyme sequences with tighter and more selective binding to metal ions or other cofactors. Cofactor promiscuity or high cofactor concentrations needed to initiate cleavage are currently detrimental to the application of many sequences, as they impact the specificity and sensitivity of the assays. Optimization efforts such as the development of the GR5 DNAzyme for exclusive use of lead as the cleavage cofactor have begun, but this optimization needs to be completed for a wider range of metal ions. With increased speed, specificity and stability, DNAzymes will be poised to replace complex and expensive molecules, such as antibodies and enzymes in biosensing applications; at the same time, the amplification power of functional nucleic acids can reduce the need for complex instrumentation, radically simplifying medical diagnostics and therapeutics in the decades to come.

Funding

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Appendix

Table A1
Compendium of DNAzyme properties

DNAzyme, reference	Substrate	Cleavage Site Preference	Required Cation	Small Molecule Promoter	Small Molecule Inhibitor
8-17 [2]	RNA = Chim	All but rCˆrU	Pb^{2+} > Zn^{2+} > Mg^{2+}		
10-23 [37]	RNA = Chim	All but rAˆ(rC/T/rU)	Mg^{2+}		
GC DNAzyme [43]	Chim > RNA	–	Mg^{2+} and Mn^{2+}		
S4 [49]	Chim > RNA	NrUˆC	Mn^{2+}	K^+ = Na^+	
S9 [49]	Chim > RNA	NrCˆC	Mn^{2+}		
S21 [49]	Chim > RNA	NrUˆT	Mn^{2+}		
EtNa [48]	Chimera	TrAˆGG	Na^+	Ethanol	
G3 [61]	Chimera	NrAˆN	Na^+ = NH_4^+		
NaA43 [62]	Chimera	TrAˆGGAA*	Na^+	Ce^{3+}	
Ce13d [46]	Chimera	TrAˆGGAA*	Ce^{3+}	Na^+	
E6 [47]	Chimera	NrAˆN	Mg^{2+}	Na^+	
CT10.3.29 M [50]	Chimera*	NrXˆN X: U = C » A = G	Mg^{2+} and Mn^{2+}		
S15 [49]	Chimera	NrCˆT	Mn^{2+}		
DZ1 [60]	Chimera	NrUˆN	Mn^{2+} > Co^{2+} > Ni^{2+} > Zn^{2+} > Cd^{2+}		
DZ7 [60]	Chimera	NrXˆN X: G = A = U > C	Mn^{2+} > Co^{2+} > Pb^{2+}		
Ag10C [51]	Chimera	TrAˆGGAA*	Ag^+ » Hg^{2+}		
E_{Hg}0T [53]	Chimera	TrAˆGGAA*	Hg^{2+}		
39 [54]	Chimera	TrAˆGGAA*	UO_2^{2+}		
GR5 [55]	Chimera	TrAˆGGAA*	Pb^{2+}		
PSCu10 [56]	Chimera	TrA† ˆGG*	Cu^{2+}		
DEC22-18 [57]	Chimera	FrAˆQ	Co^{2+}		
MgZ [58]	Chimera*	FrAˆQ	Mg^{2+}		
5J-A28 [59]	Chimera	FrAˆQ	Mg^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}		
DZ15 [63]	Chimera*	NrAˆN*	Na Citrate, pH 4–4.5	K^+	Cd^{2+}
DZ27 [63]	Chimera*	NrAˆN*	Na Citrate, pH 4–4.5		Cd^{2+}
pH3DZ1 [64]	Chimera*	NrAˆN*		pH 3	
pH4DZ1 [64]	Chimera*	NrAˆN*	Mn^{2+} = Cd^{2+} > Ni^{2+}	pH 4	
pH5DZ1 [64]	Chimera*	NrAˆN*	–	pH 5, Mn^{2+}	
pH6DZ1	Chimera*	NrAˆN*	Mn^{2+} and Ni^{2+}	pH 6.0	
Pistol-Like	DNA	–	Cu^{2+}	Hydrogen Peroxide	
PLDz [32]	DNA	–	Cu^{2+} > Co^{2+} > Mn^{2+}	Ascorbic Acid	Fe^{2+}
F8-X [31]	DNA	CTˆCG	Cu^{2+}	Hydrogen Peroxide, Mn^{2+}, DTT	
10MD5 [72]	DNA	ATGˆT	Zn^{2+}	Mn^{2+} > Cd^{2+} > Ca^{2+}	
Class I [71]	DNA	GTTGAˆAG	Zn^{2+}		
Class II [71]	DNA	NˆAGNATCTT	Zn^{2+}		

F = Fluorophore (Fluorescein-dT), Q = Quencher (DABCYL-dT).
* = only one variant tried, so other sequences may be possible.
ˆ = cleavage site.
† = Phosphorothioate on the 3′ side of the cleavage site.

Table A2
List of DNAzyme Sequences (all 5′–3′)

DNAzyme, reference	Enzyme (Catalytic) Strand or self-cleaving sequence	Substrate (Cleaved) Strand, if the reaction is bimolecular
8-17 [2]	NNNNTCCGAGCCGGACGANNNN	NNNNNrGˆNNNN
10-23 [37]	NNNNAGGCTAGCTACAACGANNNN	NNNNUrAˆNNNN
GC DNAzyme [43]	NNNNCGGCCCGCGGCGCGANNNN	NNNNTrGˆGGNNNN
S4 [49]	NNNNCTAGGGTGGGGTTAGAGTGGANNNN	NNNNTrUˆCGTGNNNN
S9 [49]	NNNNCTACTGCTTTACTGGCGGCCANNNN	NNNNTrCˆCGTGNNNN
S21 [49]	NNNNAGTATATCAAGTGAATGGCANNNN	NNNNTrCˆTGTNNNN
EtNa [48]	NNNTTCTCACAGCGTACTCGCTAAGGTTGTNNNN	NNNNTrAˆTTNNNN
G3 [61]	NNNNGGGACGAATTCTAATACGACTCACTATrAˆGGAAGAGATGGCGACAACTCTTTACCCAAGAAGGGGTGCGTACTATGCTACCTTATTAACGTGA CGGTAAGCTTGGCACCNNNN	
NaA43 [62]	NNNNCAGGTCAAAGGTGGGTGAGGGGACGCCAAGAGTCCCCG CGGTNNNN	NNNNTrAˆGGAANNNN
Ce13d [46]	NNNNAGGTGAAAGGTGGGGTGCGAGTTTTACTCGTTNNNN	NNNNrAˆGGAAGNNNN
E6 [47]	NNNNCAGCGATCCGGAACGGCACCCATGTNNNN	NNNNTrAˆGGNNNN
CT10.3.29 M [50]	CAAATGATCGGTGGGTAGCAACTGAAAGGCGGTTGCAATGCG GATGGATTGTACGGTC	GTCCGTGCTrUˆTGGTTCAATTTG
S15 [49]	NNNNAGTATATCAAGTGAATGGCANNNN	NNNNTrCˆTGTNNNN
DZ1 [60]	NNNNCTGGTACCGACTTATGGTCCTCCATAACATCGGGCTATG CGTANNNN	NNNNYGCYrUˆTGGNNNN
DZ7 [60]	NNNNGGCACGGCGGGGTCCTATGTGGAGACACCTTTAGGTAA TGTGTGTANNNN	NNNNTGCYrGˆYGGTTCNNNN
Ag10C [51]	NNNNTAGGTGATTTCCACGATTATGCGGAAACAGGGCAGCGT NNNN	NNNNrAˆGGNNN
E_{Hg}0T [53]	NNNNTGTCGGGAAACCGACCTTCGACANNNN	NNNNTrAˆGGAANNNN
39 [54]	NNNNTGCAGTCGGGTAGTTAAACCGACCTTCAGACANNNN	NNNNArAˆGGAANNNN
GR5 [55]	NNNNTGAAGTAGCGCCGCCGTANNNN	NNNNArAˆGGAANNNN
PSCu10 [56]	NNNNACCAGGCCNNNN	NNNNrA†ˆGNNNN
DEC22-18 [57]	NNNNCGTTGTCATTGGCACACGGAGGTTTACTGAGTGGTAAC CACGTAGCATGNNNN	NNNNCGTGCFrAˆQGGTTCGNNNN
MgZ [58]	NNNNCAGGTCGGGGCCGAAATATAGGATATTTTGGGAGGCTA TGNNNN	NNNNCFrAˆQGNNNN
5J-A28 [59]	NNNNTGATCGAGGAACCAAATATTGTAATATTGATGCCTGGC GGCAGTCGGTACCGAGGTCGGTACGCATGGCACCCGCATCG	NNNNGATGTGTCCGTGCFrAˆQGGTCGATTCNNNN
DZ15WS [63]	NNNNTrAˆGGAAGAGATGGCGACATCTCTTACAAACCCCAAACCTTCTCTT	
DZ27WS [63]	NNNNTrAˆGGAAGAGATGGCGACATCTCCTCACCTCAAGCGACTTCTCTCG	
pH3DZ1 [64]	GTAGTACGAGGAAATAGGGGGAGAGTGGTGTAGGCTTGAAGG TGCCACGTC	NNNNTGCFrAˆQGGTTCGANNN
pH4DZ1 [64]	GAGAAACATCTTTGAGGGATAAGCCGCCGATAGAGCGGAAGC GACTTGGTTGTAGCTG	NNNNTGCFrAˆQGGTTCGANNN
pH5DZ1 [64]	TGAATAGGGTCTCGGGCATAAATTACGGAAACGGTTTTAATTT TCTAGTGGAAAGGTCCGATAACGAG	NNNNTGCFrAˆQGGTTCGANNN
pH6DZ1 [64]	ATCGGACAAGGGAGGCACTGGAGGTTGAGGTAGTGAGCGTTG GTTAACGCCGGACAAAGGGAAGCATGGT	NNNNTGCFrAˆQGGTTCGANNN
Pistol-Like	NNNNATACGACTCACˆTATAGGAAGAGATGGCGACATAGTTAAGAGCTCGGGGTAGGCGGGAACAACGTTCACGTTGTGTNNNN	
PLDz [32]	GAATTCTAATACGAˆCTCAGAATGAGCTGGGCCTCTTTTTAAGAAC	
F8-X [31]	NNNNCˆTGCNNNNNNNNNGTGTGTGGATGCCGGGTCCGNNN	
10MD5 [72]	NNNNAACATAACTCCCTGTCGATATCGTTTGCGTGGGTGAATA GATGCNNNN	NNNNTATATGˆTNNNN
Class I [71]	NNNNACGTAGTTGAGCTGTCNNNNNNNGACGTTGAˆAGCGTNNNN	
Class II [71]	NNNNˆAGCATCTTTGGCNNNNNGCTAGGGGAATAAATCTTTGGGCANNNN	
RFA-EC1 [92]	CACGGATCCTGACAAGGATGTGTGCGTTGTCGAGACCTGCGACCGGAACACTACACTGTGTGGGATGGATTTCTTTACAGTTGTGCAGCTCCGTCCG ACTCTTCCAGCFrNˆQGGTTCGATCAAGA	
AAI2-5 [113]	GGAACGGTTAGATCTGATACCTTAGCGAAGGTGTGGTTGGC	ACTCTTCCTAGCFrAˆQGGTTCGATCAAGA

F = Fluorophore (Fluorescein-dT), Q = Quencher (DABCYL-dT).
ˆ = cleavage site.
† = Phosphorothioate at the 3′ side of the cleavage site.

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