Trends in Biotechnology

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Review

RNA-Cleaving NAzymes: The Next Big Thing in Biosensing?

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Nucleic acid enzymes (NAzymes) are nucleic acid molecules with catalytic activity. A subset, the RNA-cleaving NAzyme, is characterized by its substrate of choice: an RNA unit. These enzymes have been used for diverse applications, including biosensor development, akin to their protein counterparts. Owing to their function as both biorecognition elements and signal generators, robust bioassays based entirely on NAzyme molecules have been developed. Additionally, unique mechanisms for integration with other biorecognition elements and signal generation methods have been explored to realize ultrasensitive, specific, and user-friendly biosensors. Furthermore, NAzyme-based bioassays have already broken into the *in vitro* diagnostics market, with more promise in the pipeline.

Highlights

RNA-cleaving nucleic acid enzymes (NAzymes) are increasingly used in biosensor development, edging closer to their protein counterparts.

NAzymes are used for direct biorecognition of targets (e.g., metal ions and nucleic acids) and direct signal generation (e.g., using fluorophore-labeled substrates).

NAzyme-based biosensors can also employ auxiliary molecules, like aptamers and antibodies, for target recognition.

The nucleic acid substrates of NAzymes have also been combined with other methods for signal generation, such as electrochemical and nucleic acid amplification reactions.

The market readiness of NAzyme-based biosensors is still in its infancy, although a handful of NAzyme tests are already available to purchase.

Nucleic Acid (NA) Enzymes

Enzymes are pivotal in catalyzing reactions that underpin cellular structure and function, as well as for developing industrial bioprocesses and biosensors, amongst other applications. Although protein enzymes have been predominately utilized in this context since they were reported in 1962 [1], NA-based enzymes (NAzymes) joined this field in the 1980s. NA-based catalysis has advanced since seminal work on ribozymes [2] and DNA-based enzymes (DNAzymes) [3]. Unprecedented diversity in the DNAzyme sequences and structures, generated via ***in vitro* selection** (see Glossary), made them stand out among their naturally evolved ribozyme- and protein counterparts for catalyzing a diverse group of reactions, including catalysis of NA substrates [4]. Although the latter can be both DNA [5–7] and RNA in nature, NAzymes cleaving RNA substrates (i.e., RNA-cleaving NAzymes; Box 1) have been the most extensively studied and utilized catalysts within the field of NA-driven NA catalysis [8,9] and will be the focus of this review. For generation, structure, and (biosensing) applications of different NAzymes, the reader is referred to other excellent reviews [8–12]. More specifically, we will evaluate the use of RNA-cleaving NAzymes in developing biosensors, focusing on the different designs proposed for recognition and binding of a myriad of target molecules, and the various signal generation approaches used for integration with these NAzymes. Furthermore, unlike previously published work, this review will assess the current readiness level of these RNA-cleaving NAzyme-based biosensors with respect to their commercial availability and potential for in-field use (Figure 1, Key Figure).

RNA-Cleaving NAzymes in Biosensing

The first building block of a typical biosensor is the biorecognition element, used for specific target recognition and capture. In biosensors with RNA-cleaving NAzymes, this has been accomplished by either direct target interaction with the NAzyme (Figure 1A) or by employing other molecules (such as **aptamers** and antibodies) for target binding, which in turn activates the NAzyme (Figure 1B). Each has its merits and niche of applicability, as delineated further with a focus on matrix effects in target binding. The second key building block in a biosensor is the signal generation element, which in these biosensors relies on the NAzyme substrate. As such, signal is measured by directly analyzing the substrate, such as through cleavage of a **molecular beacon** style

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Box 1. RNA-Cleaving NAzymes: What and Where Are They From?

RNA-cleaving NAzymes were first reported by Santoro and Joyce [77]. These NAzymes, that are DNA in nature, cleave the linkage between two distinct bases in the cleavage site of the NA substrate, thus resulting in two fragments. After an initial reported cleavage of a full RNA sequence, the DNAzyme repertoire has been expanded to also include enzymes that cleave DNA sequences harboring RNA bases only in the cleavage site. Increasingly, there was a shift in choice of substrates towards the latter, especially for *in vitro* applications, perhaps owing to the higher stability of DNA versus RNA sequences. In any case, this catalytic cleavage of the substrate is responsible for signal generation.

RNA-cleaving NAzymes, unlike their RNA counterparts, are generated *in vitro* via a multiround selection process [78] that begins with a library of random DNA sequences ligated with a potentially cleavable RNA substrate sequence. After multiple incubation, separation, and amplification rounds in reaction environments of interest, the final catalytic DNA sequence is amplified, cloned, and sequenced before being utilized for different applications. As such, NAzymes with unique sequences, target specificities, and even sample compatibilities have been generated with this method. These catalytic sequences have also undergone further engineering, such as adaptations for activity at different temperatures [56,58], inclusion of aptamer domain [79,80], and dividing DNAzyme into **split DNAzymes** [81] or multicomponent NAzymes (MNAzymes), the latter incorporating target binding arms [34].

The RNA-cleaving DNAzymes consist of two segments: the core segment and the flanking substrate-binding segment that recognizes and binds the substrate via Watson-Crick base pairing. Although the first RNA-cleaving DNAzyme reported was GR-5, the 8–17 core and 10–23 core NAzymes are the most extensively studied and used classes of RNA-cleaving DNAzymes (Figure IA) [82]. GR-5, 8-17 core, and 10-23 core DNAzyme have, respectively, 15, 13, and 15 nucleotides in their core sequences and require divalent metal ions for their catalytic action. While GR-5 and 10–23 core DNAzyme are selective for either Pb^{2+} or Mg^{2+}, respectively, the 8–17 core functions in the presence of either of the two. Contrary to unimolecular DNAzymes, MNAzymes comprise of two partzymes, each containing half of the catalytic core, one substrate binding arm, and one facilitator binding arm. In the presence of a facilitator, the partzymes assemble in a functional form, thus leading to their catalytic action (Figure IB). Each NAzyme has preference for a particular dinucleotide combination in the cleavage site of the substrate NA, which allows for further tailoring of the substrate sequence to realize multiplex reactions. Although promising in activity, RNA-cleaving NAzymes are slower than their RNA and protein counterparts, leaving room for further improvement [83].

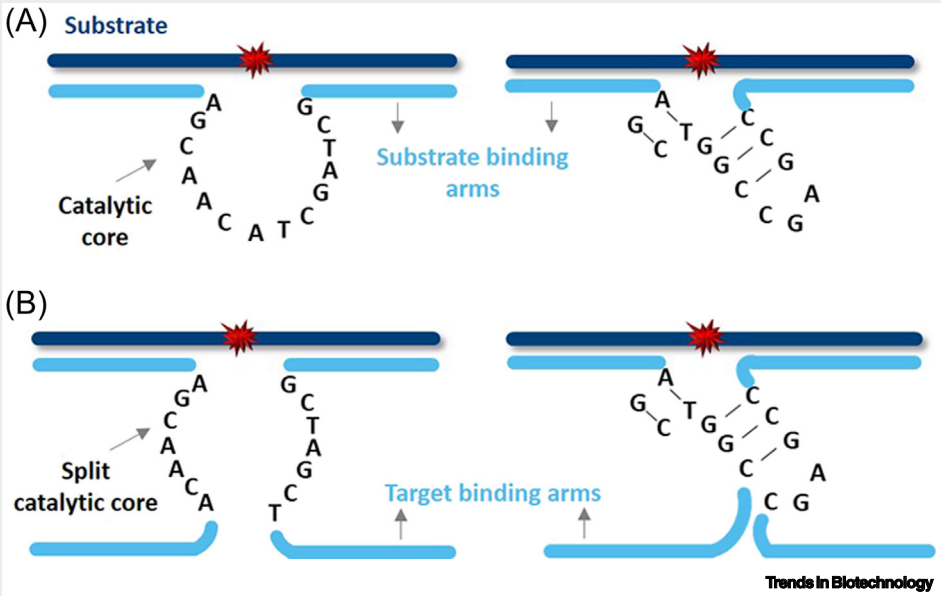


Figure I. The 10–23 and 8–17 Core Nucleic Acid Enzymes (NAzymes). (A) The 10–23 (left) and 8–17 (right) core DNAzymes are depicted, with core sequence (black letters), substrate binding arms (light blue), substrate sequence (dark blue), and cleavage site (red) indicated. (B) Multicomponent NAzymes derived from the 10–23 (left) and 8–17 (right) core DNAzymes are depicted, with split core sequences (black letters) and target binding arms (light blue) indicated (sequences based on references [34,78].

Glossary

Alkaline phosphatase (ALP): an enzyme catalyzing dephosphorylation that is commonly found in various organisms. It is frequently used to mediate electrochemical reactions and also serves as a biomarker for certain human diseases.
Aptamer: an oligonucleotide or peptide that binds a specific target molecule and is thus routinely used in biosensors.
Competitive bioassay: a type of bioassay design that relies on competition between target and its mimic for binding a restricted number of bioreceptors.
Electrochemical reaction: a reaction that involves transfer of electrons, typically between electrode and the molecules in surrounding electrolyte.
Electrophoretic separation: separation of molecules based on their size and charge.
Gel electrophoresis: a technique in which porous gel matrix, perfused with ion-containing buffers, is used to separate molecules based on their size and charge.
Hybridization chain reaction: protein enzyme-free fluorescent signal amplification method, via sequential hybridization of nucleic acid probes.
***In vitro* selection:** process of generating NA sequences that bind a specific ligand, via several cycles of positive and negative selection and subsequent enrichment.
Metal organic frameworks (MOF): multidimensional structures comprising of metal ions coordinated with organic ligands.
Methylene blue (MB): a redox indicator, which is blue in color in oxidized condition and colorless when reduced.
Molecular beacon: a hairpin shaped nucleic acid labeled with a fluorophore (F) and a quencher (Q). An increase in distance between the F and Q results in a measurable increase in fluorescence.
Partzyme: one-half of the MNAzyme, comprising one-half sequence for each of the three components (i.e., assembly facilitator binding, substrate binding, and the catalytic core).
RNA-induced silencing complex (RISC): a multiprotein complex with a short RNA sequence (e.g., siRNA) incorporated in it. Messenger RNA that has complementarity with this siRNA sequence is bound and cleaved by the complex.

substrate, resulting in an increase in fluorescence or through cleavage of substrates labelled with gold nanoparticles (AuNPs) in mass-based sensors (Figure 1C). Alternatively, the substrate is combined with various downstream reactions for signal generation and amplification, either **electrochemical reactions** or NA amplification (Figure 1D). Both strategies are discussed further in this review, with a focus on sensitivities achieved by different signal generation approaches.

Biorecognition in NAzyme-Based Biosensors

NAzymes as Biorecognition Elements for Direct Target Binding

RNA-cleaving NAzymes have been widely utilized as biorecognition elements for binding of metal ions, RNA, and DNA targets. Metal ions are shown to be essential for the activity of RNA-cleaving NAzymes as they induce changes in the 3D folding and conformation of the NAzymes to trigger catalysis [13,14]. Thus, relying on this characteristic, the 8–17 core and GR-5 DNAzymes, were extensively used for detecting the highly poisonous lead ions (Pb^{2+}), which have long-lasting effects on human health and environment [15–18], primarily from water bodies [19,20] (Figure 2A). Although similar to the 8–17 core, the GR-5 DNAzyme demonstrated a unique feature: an unprecedented selectivity for Pb^{2+}, even in the presence of other confounding metal ions [18]. Similarly, NAzymes having specific binding activity for ions such as uranium, copper (Cu^{2+}), and zinc (Zn^{2+}) have been described. Uranium ion detection was demonstrated, interestingly, even from soil samples at ambient temperature [21], whereas NAzymes with specificity for Cu^{2+}, magnesium (Mg^{2+}), and Zn^{2+} have been used for *in vivo* sensing and imaging applications [22–25]. These reports validated the robustness of NAzymes as biorecognition elements for target recognition across different matrices.

NAzymes can also simultaneously use more than one cofactor metal ion for activity. For instance, the 8–17 core NAzyme that primarily binds Pb^{2+} showed binding with both calcium (Ca^{2+}) and Mg^{2+} in the absence of Pb^{2+} [26]. This selectivity furthermore resulted in 8–17 core variants that had: (i) varying specificity for Ca^{2+} and Mg^{2+}, (ii) enzymatic activity at different rates, and (iii) functionality in serum. As such, these unique NAzymes were used to measure the relative concentration of Ca^{2+} and Mg^{2+} in human blood serum, where presence of Pb^{2+} is rare [26].

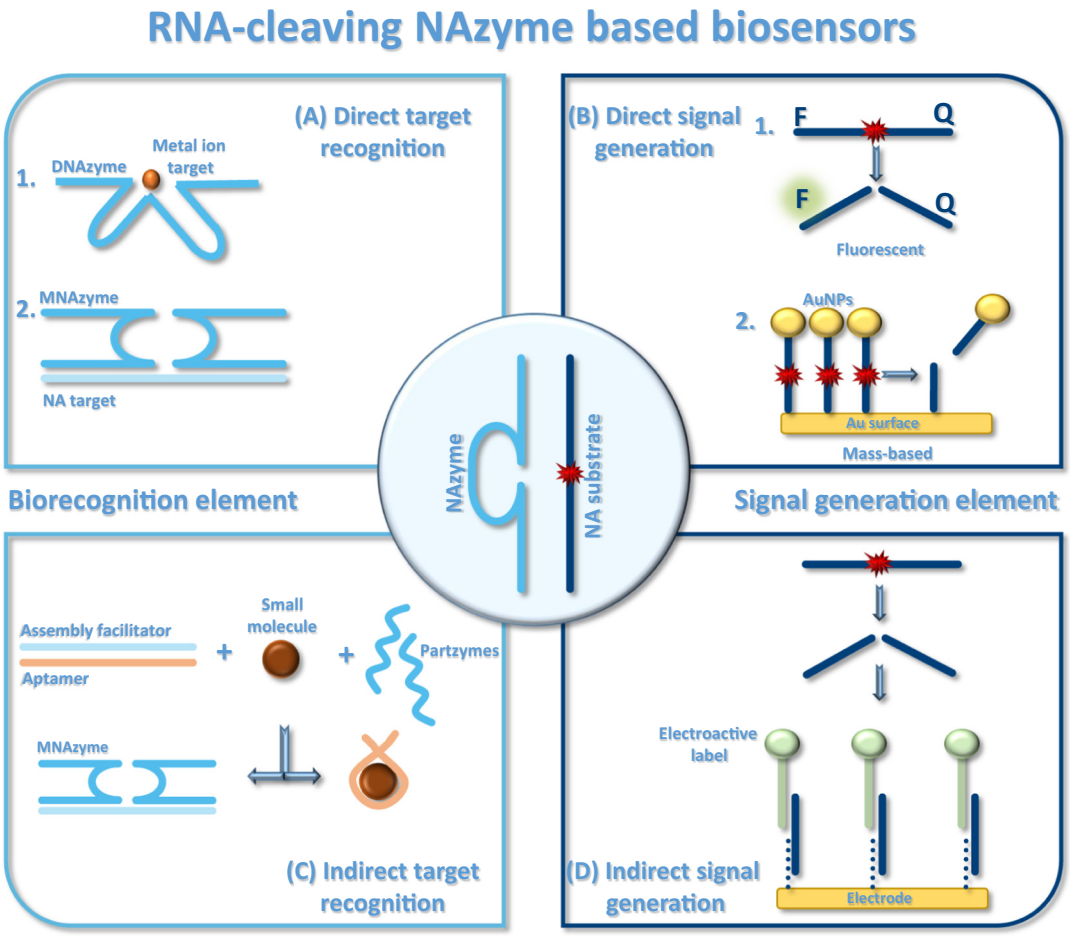
Although NAzymes are mostly used for divalent metal ion recognition, they have shown promise for monovalent ion binding as well. Torabi and coworkers demonstrated via *in vivo* imaging studies generation of the first Na^{+}-specific NAzyme with high catalytic rates and specificity [27]. A more recent unique approach was described by Xu and colleagues, where multicomponent NAzymes (MNAzymes) were used as a selective biorecognition elements for silver (Ag^{+}) detection [28] (Figure 2B). Essentially, MNAzyme sequences were designed to acquire their active confirmation only when the cytosine-Ag-cytosine bridge was formed, thereby resulting in an inactive enzyme in the absence of Ag^{+} [28]. The progress in this sector is slow because of the lower clinical and environmental relevance of monovalent metal ions: di- and, less often, tri-valent ions are more important for monitoring and understandably receive more focus.

Besides acting as biorecognition elements for metal ions, DNAzymes and MNAzymes can also bind NAs. This mode of target binding is supported by their NA nature, which enables hybridization between the target and these catalytic molecules through the standard Watson-Crick base pairing. As such, DNAzymes have demonstrated specificity for viral RNA binding [29] as well as impressive selectivity between wild type RNA molecules and those carrying **single nucleotide polymorphisms** [30]. In another instance, since the 10–23 core DNAzyme exhibits superior specificity for uridine compared with the analog pseudouridine, it served as a fitting biorecognition element for detecting pseudouridine in an RNA target. Essentially, the presence of pseudouridine

Rolling circle amplification (RCA): an isothermal nucleic acid amplification method that uses a circular template and RNA/DNA polymerases to amplify short RNA/DNA primer.
Single nucleotide polymorphism: a variation in nucleic acid sequence, which occurs at single base position. Commonly referred to as SNP.
Split DNAzyme: a DNAzyme that is split in half, such that each fragment contains one-half of the core and one-half of the substrate binding arm.
Subzyme: a DNAzyme-based amplification system, where presence of active NAzymes triggers downstream DNAzymes for amplified signal generation.
Surface plasmon resonance: an optical biosensing technique used for measuring biological interactions. Typically, the change in refractive index of a thin metal film, due to binding of analyte, is recorded as a measure of analyte concentration.

Key Figure

RNA-Cleaving Nucleic Acid Enzyme (NAzyme)-Based Biosensors



Biorecognition elements	Signal generation elements
Ligase [39]	ALP [64-68]
PCR primer [40, 41]	Methylene Blue [47,69]
Aptamer [43,46]	Glucose oxidase [33,46]
Nucleic acid [43,44]	MOFs [72]
Antibodies [47]	ECL dyes [16, 74]
	Nanomaterials [17, 71, 74]
	Digital PCR [75]

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in target NA precluded its cleavage and thereby blocked the downstream **rolling circle amplification (RCA)** [31].

Contrary to DNAzymes, used so far only for direct RNA detection, MNAzymes have been used for both RNA [32,33] and DNA target binding [34–37] and have thus expanded the repertoire of bioassays relying on NAzymes as biorecognition elements (Figure 2C). Interestingly, MNAzymes could capture with high specificity an miRNA target spiked in serum samples and from cellular extracts, despite the presence of cellular RNA [32,33]. For DNA target binding, MNAzymes were utilized in conjunction with PCR, also enabling multiplexed detection. PCR assisted in amplification of the target sequences, resulting in an increased number of target sites for functional MNAzyme assembly and, thus, superior sensitivity and specificity [35]. More recently, MNAzyme-based multiplexed target binding was demonstrated, at ambient temperature, from a nasopharyngeal swab matrix [37].

These reports further reinforced the robustness, specificity, and flexibility offered by NAzymes for target binding, thus opening avenues for further innovation. An unlikely addition to the field was direct detection of bacteria using a DNAzyme, where the DNAzyme was activated upon interaction with bacterial lysate, on a paper matrix [38]. This application has significant potential, but it relies on strength of *in vitro* selection to yield specific NAzymes.

Employing Auxiliary Molecules for Target Recognition in NAzyme-Based Biosensors

NAzyme-based bioassays have also integrated other biorecognition elements (e.g., aptamers, antibodies) to detect diverse targets (Figure 1B). In this mode, the biorecognition element binds to the target, which initiates a cascade that results in NAzyme generation, unblocking, or its change to functional conformation, each being discussed further in detail. Firstly, there are several known examples for NAzyme generation: ligation of multiple NA strands into an active DNAzyme via ATP-driven ligase, which serves as a biorecognition element for ATP [39] (Figure 3A); through PCR primers harboring complement of DNAzyme, where the target triggers the PCR, thereby generating amplicons with embedded active DNAzyme [40,41]; and **RNA-induced silencing complex (RISC)** for detection of argonaute proteins (Ago2). In this example, in the presence of Ago2, the functional RISC complex cleaves the substrate, thus releasing the RCA primer and subsequently triggering RCA-dependent generation of DNAzyme [42].

Secondly, the NAzymes can be present in the bioassay mix in their complete, albeit blocked, configuration and utilized in a **competitive bioassay** format for target detection. Bone and coworkers utilized an ATP-specific aptamer, blocked with complementary oligonucleotide, as a biorecognition element. Upon ATP binding with aptamer, the complementary oligonucleotide was freed up and initiated a DNA-only cascade that activated the NAzyme [43] (Figure 3B). In

Figure 1.

For a Figure360 author presentation of Figure 1, see the figure legend at https://doi.org/10.1016/j.tibtech.2020.04.012.

Biosensors comprise of two key building blocks: biorecognition and signal generation elements. (A) In RNA-cleaving NAzyme-based biosensors, the NAzyme can serve as the biorecognition element for direct target recognition, for instance, metal ion binding by DNAzyme and nucleic acid (NA) binding by multicomponent NAzyme (MNAzyme). (B) NAzymes can rely on the help of other biorecognition elements for the target recognition, such as aptamers for small molecule binding [other examples depicted in (E)]. (C) The NA substrate of NAzymes has been used for direct signal generation by labeling of the substrate with reporter molecules like a fluorophore (F) and quencher (Q) for fluorescence readout (1) or with gold nanoparticles (AuNPs) for mass-based readout (2), amongst other strategies. (D) Indirect signal generation is achieved via other signal generation elements, such as employing electroactive labels to bind with cleaved NA substrate that is captured on an electrode, resulting in electrochemical reactions [other examples are depicted in (E)]. (E) Overview of different biorecognition and signal generation elements employed in (A) and (D), respectively. Abbreviations: ALP, alkaline phosphatase; ECL, electrochemiluminescence; MOFs, metal organic frameworks. See [16,17,33,39–41,43,44,46,47,64–69,71,72,74,75].

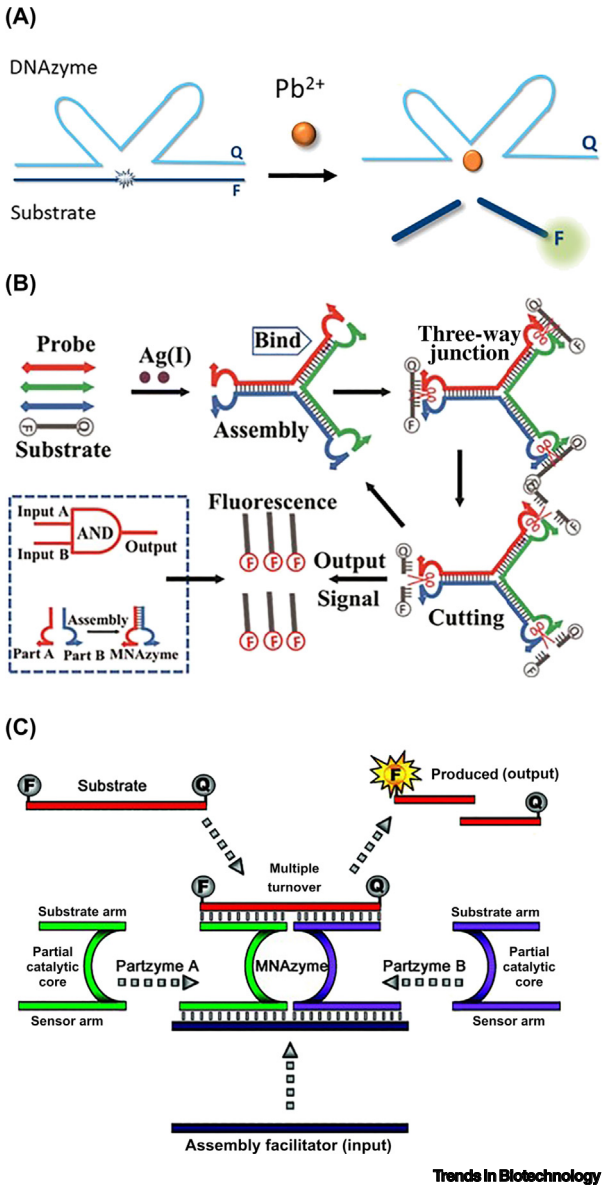

Figure 2. Nucleic Acid Enzymes (NAzymes) as Biorecognition Elements for Direct Target Binding. (A) The 8–17 core DNAzyme was used as a biorecognition element for detection of lead ions (Pb^{2+}). The 8–17 DNAzyme, with quencher (Q) on one end, hybridized with its fluorescently labeled substrate sequence [fluorophore (F)] in such a way that F–Q pair was adjacent upon hybridization. In the presence of target, the DNAzyme became catalytically active and cleaved the substrate, resulting in fluorescence increase [20]. (B) Multicomponent NAzyme (MNAzyme) used as a biorecognition element for silver ion (Ag^{+}) detection, based on the cytosine-Ag-cytosine bridge formation principle. MNAzyme components were designed so that they harbor a cytosine-cytosine mismatch, which was only stabilized in the presence of Ag^{+} target. The latter enables formation of a three-way, catalytically active MNAzyme junction that cleaved F–Q-labeled substrate, resulting in fluorescence generation [28]. (C) Nucleic acid (NA) detection in MNAzyme-based biosensors. In the presence of target, acting as assembly facilitator, the partzymes assembled into functional MNAzymes and catalyzed cleavage of the F–Q-labeled substrate, resulting in increased fluorescence due to an increase distance between F–Q pairs [34]. A adapted, with permission, from [20,28,34].

another report, real-time monitoring of an NA target was demonstrated, by utilizing an NA oligonucleotide as a biorecognition element, hybridized with the DNAzyme. In the presence of target, the NA dissociated from the DNAzyme and preferentially bound the NA target [44].

Thirdly, formation of functional NAzyme conformation strategy is used for protein target detection in NAzyme-based biosensors. This involves aptamers and antibodies as biorecognition elements for activation of downstream NAzyme cascades [45–47]. For instance, a platelet-derived growth factor BB (PDGF-BB) target was sandwiched between two aptamers, one coupled to the sensor surface and the other to ZnS nanoparticles (NPs) [46]. Once this sandwich conformation took place, the addition of silver ions (Ag^{+}) triggered a cation exchange reaction that released Zn^{2+} for actuation of the downstream DNAzyme. An MNAzyme in a tripartite configuration was

exploited to detect carcinoembryonic antigen (CEA) by use of three antibodies, each labeled with either a **partzyme** or assembly facilitator, for target capture [47] (Figure 3C).

These reports highlight that NAzyme-based biosensors, albeit simplistic at first glance, demonstrate diverse target binding mechanisms. The modular nature of MNAzymes has undoubtedly contributed to this aspect, even more than the original DNAzymes, which warrants further focus.

Signal Generation in NAzyme-Based Biosensors

Direct Signal Generation with NAzyme

To directly measure catalytic activity of RNA-cleaving NAzymes, four methods have primarily been used, namely **electrophoretic separation**, fluorescence generation, mass change, and **colorimetric** analysis, each based on analyzing the cleaved substrate oligonucleotides (Table 1). The seminal method involved electrophoretic separation of the cleaved fragments, where an increase in concentration of shorter fragments compared with longer ones indicated an increased activity of the NAzyme [3]. Alternatively, for a plethora of biosensors, fluorescence is the method of choice to directly generate a signal. In this context, molecular beacon-style NAzyme substrates, having a fluorophore (F) and a quencher (Q) on each end, have been extensively utilized since cleaving the substrate results in loss of quenching due to increased distance between F–Q pairs. This approach has been combined to date with PCR systems, spectrophotometers, and microscopy and will be discussed further in detail.

The NA nature of NAzymes enabled their unique integration with PCR-based readout methods, as PCR provides not only real-time fluorescence detection but also temperature control for NAzyme activity at elevated temperatures. Todd and colleagues first demonstrated target-dependent generation of PCR amplicons that included DNAzyme sequence. This DNAzyme could cleave an F–Q labeled substrate, which enabled fluorescence-based detection down to ten or fewer copies [40]. After development of MNAzymes, this bioassay was revamped through utilizing the amplicon as assembly facilitator and using universal F–Q labeled substrates for signal generation. The new design obviated the need to include an NAzyme sequence in PCR primers**,** thereby enabling a more generalized approach. This methodology was extensively validated and resulted in multiplexed assays with superior specificity [35,48,49], including detection from clinical samples [50], while retaining sensitivities of ≤15 copies. Interestingly, the recently introduced **subzymes** presented a novel NAzyme-based signal amplification strategy that increases the rate of NA detection [51] and demonstrated a remarkable 1 fM limit of detection (LOD) in under 30 minutes [52].

Next to PCR, spectrophotometers were also extensively reported as platforms for realizing NAzyme-based readout in various bioassays [28,36,37,53–56] that reached competitive LODs when detecting ions (uranyl, 13 pM [55] and 4 pM [54]; Ag, 5 pM [28]), NAs (20 f. [53]), and PDGF-BB protein (22 pM [57]). However, these bioassays generally required preparation and incubation steps at elevated temperatures, with a final end point fluorescence measurement at ambient temperature [28,53–55]. Remarkably, some novel strategies circumvented such elevated temperature requirements. For instance, Gao and colleagues used copolymers as NA chaperones for improved NAzyme activity [36,58], whereas Ven and colleagues redesigned NAzymes [56] for establishing (multiplexed) detection of NA at ambient temperature, while reaching sub-nanomolar detection limits [37].

Microscopy was also utilized for NAzyme-based signal measurement in bead-based and live cell analyses. For example, NAs were detected in multiplexed bioassay from nasopharyngeal swab matrix in femtoliter-sized microwells, realizing sub-picomolar detection limits [37] (Figure 4A). Additionally, microscopy has enabled NAzyme-based imaging in living cells for miRNAs [59],

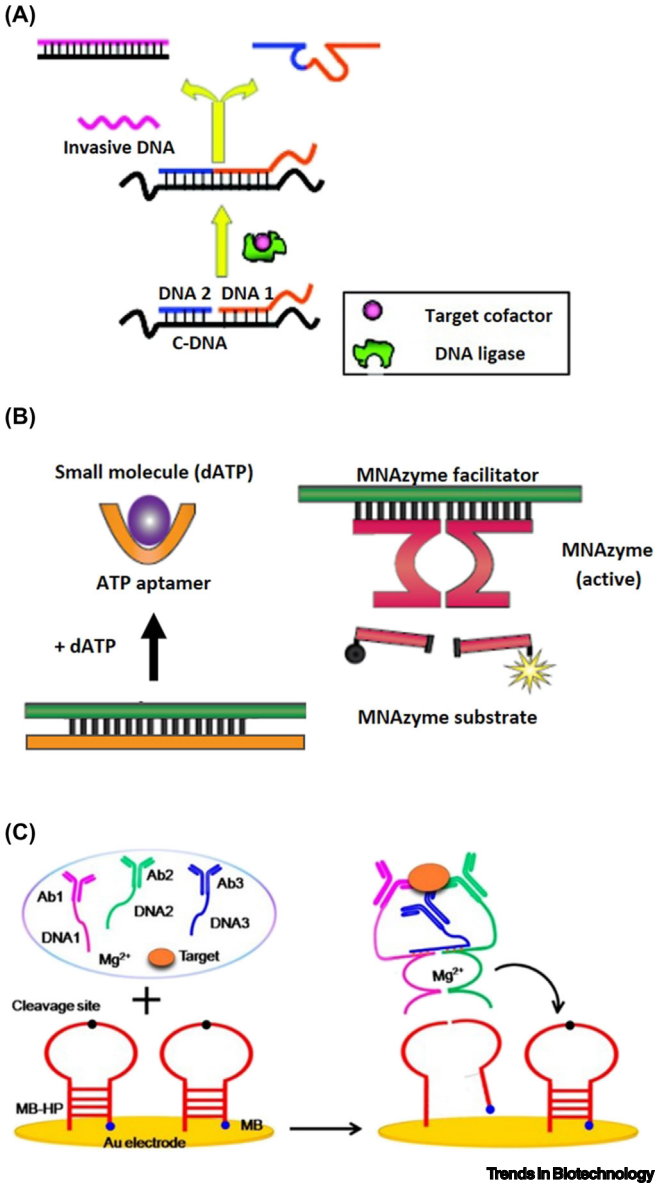

Figure 3. Employing Auxiliary Molecules for Target Recognition in Nucleic Acid Enzyme (NAzyme)-Based Biosensors. (A) An ATP ligase enzyme was used as a biorecognition element for ATP detection. The presence of ATP activated the enzyme that ligated DNA1 and DNA2 strands, which resulted in generation of the 8–17 core DNAzyme. The cleavage of fluorophore (F)–quencher (Q)-labeled substrates caused generation of fluorescence signal [39]. (B) An ATP aptamer was used as a biorecognition element for ATP detection. In the presence of target, the aptamer preferentially bound ATP and made the assembly facilitator available for generating the functional multicomponent NAzyme (MNAzyme). Cleavage of the nucleic acid (NA) substrate by MNAzyme can result in direct fluorescence increase (depicted in figure) or in unblocking of the additional DNAzyme that triggers the DNA-only cascade-mediated amplified signal generation [43]. (C) Antibodies were used as biorecognition elements for carcinoembryonic antigen (CEA) protein detection, in conjunction with MNAzymes. In the presence of target, the three antibodies (each bound with either one of the partzymes or the assembly facilitator) captured the CEA protein, which resulted in formation of active MNAzymes for signal generation [47]. Adapted, with permission, from [39,43,47].

Na^+ ions [27], or even Zn^{2+} and Cu^{2+} ions in a multiplex manner [23,24]. These bioassays not only demonstrated the power of NAzymes for complex matrix and *in vivo* biosensing, but have also presented new directions for development of innovative sensing approaches.

More recently, fluorescence was also combined with electrophoretic separation, to circumvent the use of toxic intercalating dyes, for the detection of uranyl ions using DNAzymes [21] and NA target in undiluted, human serum using MNAzymes [60].

Although promising, these studies still left room for better sensitivities coupled with user-friendly operation, owing to dependence on skilled personal for polyacrylamide **gel electrophoresis**. Moreover, NAzyme-based signal detection has been also explored with mass-sensitive systems. In this context, AuNPs have been removed from either fiber optic **surface plasmon resonance** (FO-SPR) surface or from the surface of quartz crystal microbalance (QCM) biosensor. In both cases, the target-dependent, DNAzyme-mediated cleavage of the substrates triggered, respectively, an SPR or frequency shift due to the removal of AuNPs, resulting in LODs of 1.4 nM [44] for NA target (Figure 4B) or 14 nM for Pb^{2+} detection (Figure 4C) [61].

As ease of readout is crucial for user-friendly biosensors, detection with the naked eye is of paramount interest. For instance, DNAzyme-tethered AuNPs were used for detection of dengue virus RNA down to 0.06 nM RNA, by deshielding the AuNPs, leading to a change in color from red to transparent [29]. Another MNAzyme-based bioassay presented a somewhat modified approach whereby the substrate strand comprised of a linker NA sequence required for AuNP aggregation. Consequently, in the presence of target NA, the linker was cleaved, leading to dispersion of AuNPs and change in color from blue to red [62]. This strategy reported a remarkable 50 pM LOD for NA target, which was impressive for colorimetric detection. In addition to NPs, microparticles have also enabled visual detection in NAzyme-based bioassays. For instance, a combination of magnetic microparticles and polystyrene microparticles were used in conjunction with GR-5 DNAzyme to detect Pb^{2+} ions with naked eye and enabled a 0.44 parts per billion (ppb, ~2.12 nM) LOD, which is 35 times below the 15 ppb permissible concentration [63] (Figure 4D).

NAzyme-based signal generation has thus shown compatibility with diverse applications, ranging from *in vitro* and live cell imaging, to solid surface-based detection, strengthening its outlook for biosensor development. In this context, fluorescence-based detection strategies have clearly received an overwhelming amount of attention in NAzyme-based biosensors, owing likely due to availability of reliable readout methods. These bioassays have not only demonstrated the power of NAzymes for target detection in complex matrices and living cells, but also presented new directions for development of innovative sensing approaches as seen through integration with mass-sensitive systems. Nevertheless, although frequently used, fluorescent readout methods still suffer from a major weakness: different fluorophore-labeled substrate sequences need to be designed and manufactured for different target molecules, often requiring stringent optimization before a bioassay can be implemented. In addition, fluorescence-based methods are less likely to be compatible with the point-of-care applications, contrary to the colorimetric approaches that are more interesting in this context. However, the latter still requires further advancement before their widespread deployment as they must incorporate an internal quantifying approach to remove operator bias.

Employing Auxiliary Molecules for Signal Generation in NAzyme-Based Biosensors

Many NAzyme-based bioassays have incorporated unique signal generation molecules to amplify the signal and thus attain sensitive target detection. Well-known signal generation approaches

Table 1. Signal Generation in NAzyme-Based Biosensors

Readout method	Signal generating strategy	Target molecule	Detection limit	Refs
Direct signal generation				
NA amplification	DNAzyme	NA	≤10 copies	[40]
	MNAzyme		≤15 copies	[35,48–50]
	Subzyme		10 pM	[51]
			1 fM	[52]
Spectrophotometry	DNAzyme	Uranyl ions	13 pM	[55]
			4 pM	[54]
		PDGF-BB	22 pM	[57]
		NA	20 fM	[53]
	MNAzyme	Silver ions	5 pM	[28]
		NA	2.1 pM	[58]
		Multiplex NAs	Low pM	[37]
Microscopy	DNAzyme	MicroRNA	49 pM	[59]
		Zinc and copper ions	High picomolar	[23,24]
		Sodium ions	3.1 ppm	[27]
	MNAzyme	Multiplex NAs	Sub-picomolar	[37]
Electrophoresis	DNAzyme	Uranyl ions	45 pM	[21]
	MNAzyme	NA	6 nM	[60]
Mass-based	DNAzyme	NA	1.4 nM	[44]
		Lead ions	14 nM	[61]
Visual detection	DNAzyme	Dengue viral RNA	0.06 nM	[29]
		Lead ions	~2.12 nM	[63]
	MNAzyme	NA	50 pM	[62]
Indirect signal generation				
Electrochemical reaction	Alkaline phosphatase	*H. pylori* DNA	50 fM	[64]
		DNA	79 pM	[65]
		MicroRNA	1.66 fM	[66]
		Lead ions	43 pM	[67]
		BCR-ABL gene	22 fM	[68]
	Methylene blue	CEA	Sub-picomolar	[47]
		ATP	Sub-picomolar	[69]
		Lead ions	120 pM	[70]
	Glucose oxidase	MicroRNA	65 pM	[33]
		PDGF-BB	0.11 fM	[46]
	Redox indicator	Lead ions	95 pM	[15]
	Quantum dots		6.1 pM	[17]
	Thionine-graphene nanocomposite		0.42 pM	[71]
	MOF		0.174 fM	[72]
	$Ru(bpy)_3^{2+}$ dye		8.4 pM	[16]
	Silver nanoparticles	Thrombin	37 aM	[73]
	Luminol with nanocomposite	Group B streptococci	68 aM	[74]

Table 1. (continued)

Readout method	Signal generating strategy	Target molecule	Detection limit	Refs
NA amplification	Digital PCR	Lead ions	500 pM	[75]
	RCA	Pseudouridine	5 nM	[31]
	CHA	MicroRNA	1 pM	[32]

include electrochemical reactions and NA amplification (both protein- and nonprotein-based approaches) (Table 1).

Among most prominent molecules that are employed in this context are those that result in electrochemical signal generation, such as **alkaline phosphatase (ALP)** and **methylene blue (MB)**. For instance, streptavidin-conjugated ALP was used for signal generation through binding with a biotinylated trigger, generated in the presence of target [64–68]. Detection of *Helicobacter pylori* DNA using DNAzyme coupled with ALP demonstrated an impressive LOD of 50 f. in buffer [64]. Incorporating additional amplification cascades upstream of ALP binding further improved the LOD of the bioassays to femtomolar range for detection of the BCR-ABL gene [68] and miRNA detection [66] (Figure 5A).

The electroactive compound MB was also frequently used for signal generation, where NA substrate functionalized with MB was decorated on the electrode. When the NAzyme acts, MB is removed from the proximity of the electrode, which causes a measurable electrochemical activity. Following this approach, ATP [69] and CEA [47] were detected, using a DNAzyme and MNAzyme, respectively, and achieving LOD values in the sub-picomolar range. As such, CEA detection is competitive with alternative commercially available systems. Pb^{2+} ion detection also benefitted from MB-incorporated DNAzyme-based assay, where signal was further amplified with inclusion of nanomaterials. This strategy achieved a respectable LOD of 120 pM in water bodies, making it promising for water quality assessment [70].

A glucose monitoring device, reliant on glucose oxidase, is likely the best-known example of an electrochemical biosensor and has been repurposed for signal generation in NAzyme-based assays. For instance, a DNAzyme-based sensor was designed to release invertase-conjugated NA strands in the presence of PDGF-BB protein target, achieving a remarkable 0.11 fM LOD using a glucometer for readout [46]. More recently, DNAzyme-based miRNA detection was shown using an NA hydrogel impregnated with amylase, that generated glucose in the presence of target [33].

Still other electroactive compounds have been made part of signal generation mechanisms in NAzyme-based bioassays, without the use of protein enzymes. In this context, several different strategies have been proposed for detection of Pb^{2+}. The most sensitive ones include thionine incorporated in a graphene-based nanocomposite (LOD of 0.42 pM) [71] and the use of **metal organic frameworks (MOF)**, such as streptavidin-conjugated Fe-MIL-101 (LOD 0.174 fM) [72]. Similar approaches were used for detecting other targets, such as thrombin. Here, electrostatic collection of Ag cations along the DNA backbone, mediated by aptazyme-based **hybridization chain reaction**, and their conversion into NPs via reduction resulted in ultrasensitive LOD of 37 aM [73].

Another key approach for signal generation in NAzyme-based bioassays utilized electrochemiluminescence generated by dyes [16,74]. For instance, $Ru(bpy)_3^{2+}$ dye was used with 8–17 DNAzyme for Pb^{2+} detection, achieving an LOD of 8.4 pM [16], whereas a combination of chemiluminescent luminol and CuMn-CeO_2 nanocomposites realized ultrasensitive and enzyme-free detection of group B streptococci with an LOD of 68 aM [74].

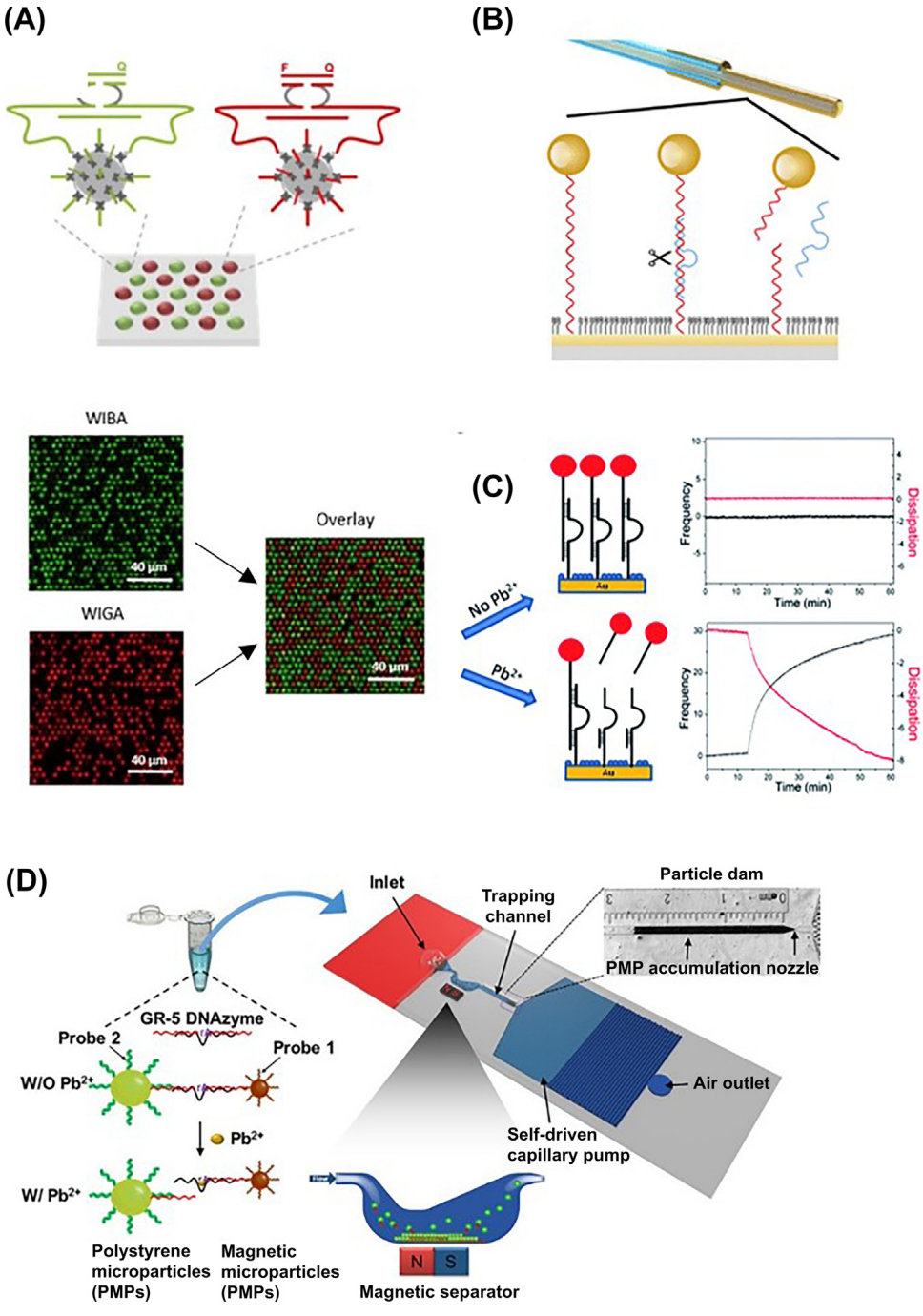

Figure 4. Direct Signal Generation with Nucleic Acid Enzymes (NAzymes). (A) Two multicomponent NAzymes (MNAzymes) immobilized on different populations of magnetic beads (MBs) were used for capturing two nucleic acid (NA) targets from nasopharyngeal swabs. These MBs, seeded in microwell arrays and incubated with a mixture of two substrates [labeled with FAM or HEX fluorophore (F) and quencher (Q) on opposite ends], were imaged via fluorescence microscopy (with WIBA and WIGA filters, respectively) for multiplex target detection [37]. (B) Fiber optic-surface plasmon

(Figure legend continued at the bottom of the next page.)

Because sensitive signal generation requires amplification, relying on NAs in this context seems to be a natural choice for NAzyme-based bioassays. GR-5 DNAzyme in combination with digital PCR (dPCR) was used for Pb^{2+} detection, where template DNA for dPCR was generated due to cleavage of substrate by GR-5 DNAzyme in the presence of target. This DNAzyme-dPCR bioassay achieved a competitive LOD of 500 pM for Pb^{2+} detection [75]. The 10–23 DNAzyme in combination with RCA successfully detected pseudouridine (Ψ), a biologically relevant analog of uridine (U), without reliance on radioactivity as in the traditional methods [31]. The DNAzyme cleaved the substrates containing U, and not Ψ, thus generating RCA primer. Analysis of the RCA product revealed the relative fraction of Ψ present in 5 nM total RNA sample, showing the power of this approach for sensitive and safe Ψ detection (Figure 5B). Another innovative signal generation method relies on protein enzyme-free NA amplification: catalytic hairpin assembly (CHA). Target mediated MNAzyme-based cleavage of substrate triggers CHA of oligonucleotide hairpins, one of which was modified with biotin, present in the mixture. The signal was measured based on SPR shift generated due to binding of streptavidin on the assembled CHA and resulted in LOD of 1 pM for miRNA detection, much lower than the protein enzyme-based counterparts [32].

These applications demonstrate the flexibility of integrating NAzymes with various signal generation processes and even for utilizing commercially available platforms. Electrochemical methods are predominantly favored, which is intuitive since the first biosensor ever developed was an electrochemical one. Since NAzymes render themselves for seamless integration with established electrochemical detection methods, this power is rightly exploited rather than reinventing the wheel. However, the use of protein enzymes in these strategies takes away from the NA-only potential of NAzyme-based biosensors, thus limiting their applications in more challenging conditions. The increased interest in use of nanomaterials, in lieu of protein enzymes, combines the best of both worlds, sensitive electrochemical signal without reliance on proteins, and seems to be where the future of signal generation lies in NAzyme-based biosensing.

Ready-to-Use Biosensors: Are We There Yet?

Biosensors relying on RNA-cleaving NAzymes have shown much promise, as described earlier. However, the road from the research laboratory to market shelves has its own twists and turns. Of all the technologies evaluated so far, DNAzyme-based heavy metal detectors and the MNAzyme-integrated PCR technologies have successfully made it to the market. The heavy metal detector, marketed by ANDalyze, Inc., provides water contamination testing solutions for Pb^{2+}, Hg^{2+}, Cu^{2+}, Zn^{2+}, and uranium ions (http://www.andalyze.com). The PCR technology, by SpeeDx, has enabled highly competitive *in vitro* diagnostics for infectious diseases and cancer. Their product line currently boasts six tests and at least three more in the pipeline (http://www.plexpcr.com). Although not all product lines are currently available in Europe and the US, partnerships such as with Biocartis, utilizing *Plex*Zyme and *Plex*Prime for their Idylla™ platform, are quickly bridging this gap.

resonance (FO-SPR) was used for NA detection using probes functionalized with gold nanoparticle (AuNP)-tethered substrates. In the presence of target, the active DNAzyme cleaved off the AuNP-tethered substrate from the FO probe, resulting in a measurable FO-SPR shift [44]. (C) A quartz crystal microbalance (QCM) surface functionalized with DNAzyme, hybridized with its substrate, was used for the detection of lead ions (Pb^{2+}). The substrate carried AuNP on the end farther from the QCM. Upon binding of Pb^{2+}, the active DNAzyme cleaved the AuNP-functionalized substrate, resulting in measurable QCM signal generation [61]. (D) Self-driven capillary pump based on a microfluidic platform was used for Pb^{2+} detection using DNAzyme substrate bound to magnetic microparticles (MMP) on one end and polystyrene microparticles (PMP) on the other. In the presence of target, DNAzyme cleaved the substrate, resulting in fragments bound with either MMP or PMP. The reaction flowed over a magnetic separator that only allowed cleaved PMP-bound fragment to flow and be collected in nozzle for readout [63]. Adapted, with permission, from [37,44,61,63].

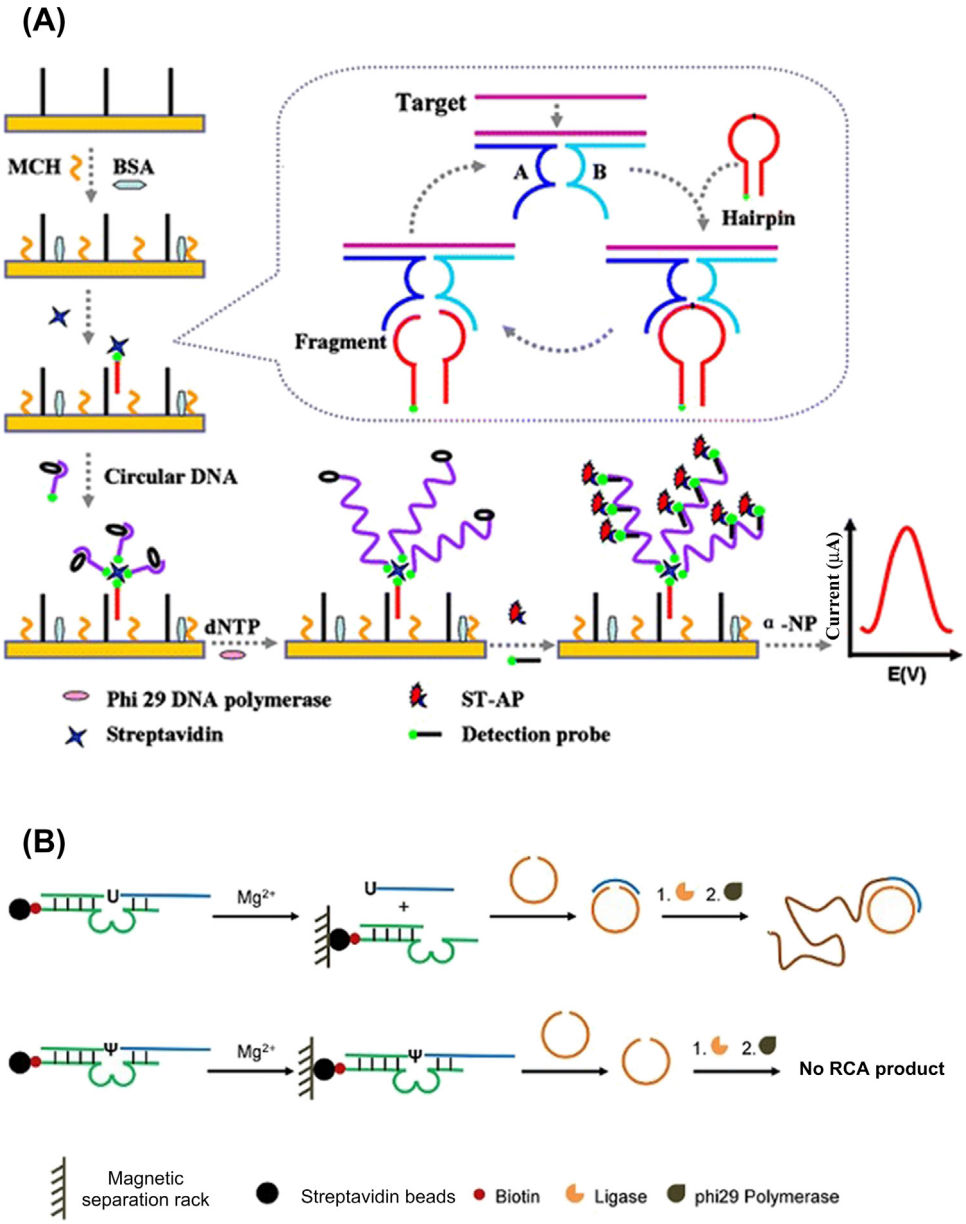

Figure 5. Employing Auxiliary Molecules for Signal Generation in Nucleic Acid Enzyme (NAzyme)-Based Biosensors. (A) Alkaline phosphatase (ALP) was used for microRNA detection via electrochemical signal generation. The biosensor relied on target-driven multicomponent NAzyme activity, that led to capture of cleaved substrate fragment on the electrode via hybridization with capture probes. These fragments bound with streptavidin through their biotin label and acted as sites for further collection of biotinylated fragments, including biotinylated circular DNA for rolling circle amplification (RCA) initiation in the presence of Phi 29 DNA polymerase. The RCA products were then hybridized with biotinylated detection probe, thus increasing the number of available biotin sites for binding with streptavidin-ALP (ST-ALP). The latter was responsible for amplified electrochemical signal generation via catalysis of α-naphthyl phosphate (α-NP) [66]. (B) RCA was used for signal generation in pseudouridine (Ψ) biosensor. Target in this case acted as DNAzyme substrate and, once cleaved, as RCA primer. In the presence of other RCA components, successful RCA product formation occurred in the presence of target, measured as a metric of target concentration [31]. Adapted, with permission, from [31,66].

Although no other NAzyme-based biosensors are currently found on the market, the use of a commercially available glucometer for readout foreshadows the ease of integrating NAzyme bioassays for commercial product development [33,46]. Moreover, the FO-SPR technology, applied for NAzyme-mediated NA detection [44], has also been commercialized for bioanalysis by FOx Biosystems (http://www.foxbiosystems.com). Additionally, hundreds of patents exist for DNAzyme/MNAzyme biosensors, revealing a repertoire of NAzyme-based biosensor technologies with the potential to embark on the journey from the lab to market.

In order to fully harness the potential of RNA-cleaving NAzymes for realizing field-deployable, commercially available biosensors, we posit that it is imperative to first fill a few gaps. Although protein enzymes are considered the gold standard for achieving competitive catalysis, a similar level of research has not yet been conducted on NAzymes, which are surely much more stable and insular to degradation compared with their protein counterparts. To this end, kinetics of NAzyme catalysis should be probed systematically and in greater detail, on a par with their protein counterparts (see Outstanding Questions). This understanding is crucial for not only choosing the optimal NAzyme, substrates, reaction buffers, and operating conditions for use in a biosensor, but also to generate new and improved NAzyme candidates that act as target receptors for diverse molecules (e.g., proteins and small molecules) at competitive rates. Moreover, recently a synthetic NA analog (XNA)-based RNA-cleaving NAzyme has shown superior activity compared with its native DNAzyme counterpart [76], demonstrating the untapped potential for generation of novel and highly competitive (X)NAzymes.

NAzyme-based biosensor development and commercialization requires substantial infrastructure and monetary fluidity, just like any other medical technology device, which is a challenge for new incumbents inching to break into the field. Additionally, a comfort bias that favors the protein-based receptors and signal generators over their NA counterparts is apparent in the biosensing field, which may surface in funding and investment decisions, thus making it difficult for these technologies to advance further. Research advancements delineated earlier can do their part in providing the scientific rationale to ward off this bias, thus leveling some of the bumps in the road faced by NAzyme-based biosensors to reach the market.

Outstanding Questions

Metal ion interaction with NAzymes: How do NAzymes coordinate and interact with the metal ions? Can these insights be used for intelligent selection of new NAzymes, or predict sequences that bind other metal ions?

NAzyme reaction kinetics: What factors govern the catalytic efficiency of the NAzymes? Can this understanding be used to accelerate their kinetics?

Target binding characteristics of NAzymes: Can NAzymes be generated to directly bind diverse target molecules, such as proteins and small molecules, akin to antibodies that bind DNA?

Utilizing the modular nature of MNAzymes: Can multisensing components be constructed based on this modular nature? Can such components potentially lead to construction of high-throughput multifunctional biosensors?

Concluding Remarks

RNA-cleaving NAzymes provide a flexible tool for innovative biosensor development, as showcased by their function as biorecognition elements and signal generators, in addition to integration with other molecules for robust target binding and sensitive signal generation. The promise of NAzymes for commercial biosensor development cannot be ignored and has injected a breath of innovation in a field otherwise dependent on protein enzymes. Still, efforts are needed to understand these NAzymes at a more fundamental level in order to break into the market with a new generation of ultrasensitive biosensors (see Outstanding Questions).

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References

1. Clark, L.C. and Lyons, C. (1962) Electrode systems for continuous monitoring in cardiovascular surgery. *Ann. N. Y. Acad. Sci.* 102, 29–45
2. Kruger, K. *et al.* (1982) Self-splicing RNA: autoexcision and autocyclization of the ribosomal RNA intervening sequence of tetrahymena. *Cell* 31, 147–157
3. Breaker, R.R. and Joyce, G.F. (1994) A DNA enzyme that cleaves RNA. *Chem. Biol.* 1, 223–229
4. Morrison, D. *et al.* (2018) DNAzymes: selected for applications. *Small Methods* 2, 1700319
5. Carmi, N. *et al.* (1998) Cleaving DNA with DNA. *Proc. Natl. Acad. Sci. U. S. A.* 95, 2233–2237
6. Dokukin, V. and Silverman, S.K. (2012) Lanthanide ions as required cofactors for DNA catalysts. *Chem. Sci.* 3, 1707–1714
7. Yue, G. *et al.* (2019) Highly selective fluorescence sensor for hydrogen sulfide based on the Cu(II)-dependent DNAzyme. *J. Lumin.* 207, 369–373
8. Zhang, J. (2018) RNA-cleaving DNAzymes: old catalysts with new tricks for intracellular and in vivo applications. *Catalysts* 8, 550

9. Liu, M. *et al.* (2017) Discovery and biosensing applications of diverse RNA-cleaving DNAzymes. *Acc. Chem. Res.* 50, 2273–2283
10. Gong, L. *et al.* (2015) DNAzyme-based biosensors and nanodevices. *ChemComm* 51, 979–995
11. Kwok, C.K. and Merrick, C.J. (2017) G-quadruplexes: prediction, characterization, and biological application. *Trends Biotechnol.* 35, 997–1013
12. Ma, L. and Liu, J. (2020) Catalytic nucleic acids: biochemistry, chemical biology, biosensors, and nanotechnology. *iScience* 23, 1
13. Hwang, K. *et al.* (2016) Biochemical and biophysical understanding of metal ion selectivity of DNAzymes. *Inorg. Chim. Acta* 452, 12–24
14. Liu, H. *et al.* (2017) Crystal structure of an RNA-cleaving DNAzyme. *Nat. Commun.* 8, 2006
15. Song, X. *et al.* (2019) A triply amplified electrochemical lead(II) sensor by using a DNAzyme and via formation of a DNA-gold nanoparticle network induced by a catalytic hairpin assembly. *Microchim. Acta* 186, 559
16. Wu, Y.F. *et al.* (2014) A novel signal-on DNAzyme-based electrochemiluminescence, sensor for Pb^{2+}. *Sens. Actuators B Chem.* 191, 60–66
17. Tang, S. *et al.* (2014) A novel electrochemical sensor for lead ion based on cascade DNA and quantum dots amplification. *Electrochim. Acta* 134, 1–7
18. Lan, T. *et al.* (2010) A highly selective lead sensor based on a classic lead DNAzyme. *Chem. Commun.* 46, 3896–3898
19. McGhee, C.E. *et al.* (2017) DNAzyme sensors for detection of metal ions in the environment and imaging them in living cells. *Curr. Opin. Biotechnol.* 45, 6
20. Han, S. *et al.* (2016) Practical, highly sensitive, and regenerable evanescent-wave biosensor for detection of Hg^{2+} and Pb^{2+} in water. *Biosens. Bioelectron.* 80, 265–272
21. Liu, J. *et al.* (2007) A catalytic beacon sensor for uranium with parts-per-trillion sensitivity and millionfold selectivity. *Proc. Natl. Acad. Sci. U. S. A.* 104, 2056–2061
22. He, Y. *et al.* (2014) DNAzyme self-assembled gold nanorods-based FRET or polarization assay for ultrasensitive and selective detection of copper(II) ion. *Biosens. Bioelectron.* 55, 285–288
23. Li, L. *et al.* (2015) Simultaneous imaging of Zn^{2+} and Cu^{2+} in living cells based on DNAzyme modified gold nanoparticle. *Anal. Chem.* 87, 4829–4835
24. Si, H. *et al.* (2018) Highly Sensitive fluorescence Imaging of Zn^{2+} and Cu^{2+} in living cells with signal amplification based on functional DNA self-assembly. *Anal. Chem.* 90, 8785–8792
25. Lin, Y. *et al.* (2019) Enzyme-mediated endogenous and bioorthogonal control of a DNAzyme fluorescent sensor for imaging metal ions in living cells. *Angew. Chem. Int. Ed.* 58, 17061–17067
26. Zhou, W. *et al.* (2016) In vitro selection in serum: RNA-cleaving DNAzymes for measuring Ca^{2+} and Mg^{2+}. *ACS Sensors.* 1, 600–606
27. Torabi, S.F. *et al.* (2015) In vitro selection of a sodium-specific DNAzyme and its application in intracellular sensing. *Proc. Natl. Acad. Sci. U. S. A.* 112, 5903–5908
28. Xu, Y. *et al.* (2019) Fluorometric detection of silver(I) using cytosine-Ag(I)-cytosine pair formation, DNA assembly and the AND logic operation of a multiple-component DNAzyme. *Microchim. Acta* 186, 522
29. Carter, J.R. *et al.* (2013) A novel dengue virus detection method that couples DNAzyme and gold nanoparticle approaches. *Virol. J.* 10, 201
30. Hong, C. *et al.* (2015) Fluorescence-based detection of single-nucleotide changes in RNA using graphene oxide and DNAzyme. *ChemComm.* 51, 5641–5644
31. Li, W. *et al.* (2019) A sensitive and radiolabeling-free method for pseudouridine detection. *Anal. Biochem.* 581, 9
32. Li, X. *et al.* (2016) A novel surface plasmon resonance biosensor for enzyme-free and highly sensitive detection of microRNA based on multi component nucleic acid enzyme (MNAzyme)-mediated catalyzed hairpin assembly. *Biosens. Bioelectron.* 80, 6
33. Si, Y. *et al.* (2019) Oligonucleotide cross-linked hydrogel for recognition and quantitation of microRNAs based on a portable glucometer readout. *ACS Appl. Mater. Interfaces* 11, 7792–7799
34. Mokany, E. *et al.* (2010) MNAzymes, a versatile new class of nucleic acid enzymes that can function as biosensors and molecular switches. *J. Am. Chem. Soc.* 132, 1051–1059
35. Mokany, E. *et al.* (2013) MNAzyme qPCR with superior multiplexing capacity. *Clin. Chem.* 59, 419–426
36. Gao, J. *et al.* (2015) MNAzyme-catalyzed nucleic acid detection enhanced by a cationic copolymer. *Biomater. Sci.* 3, 716–720
37. Safdar, S. *et al.* (2020) DNA-only, microwell-based bioassay for multiplex nucleic acid detection with single base-pair resolution using MNAzymes. *Biosens. Bioelectron.* 152, 3
38. Ali, M.M. *et al.* (2017) A printed multicomponent paper sensor for bacterial detection. *Sci. Rep.* 7, 12335
39. Lu, L.M. *et al.* (2011) A ligation-triggered DNAzyme cascade for amplified fluorescence detection of biological small molecules with zero-background signal. *J. Am. Chem. Soc.* 133, 11686–11691
40. Todd, A.V. *et al.* (2000) DzyNA-PCR: use of DNAzymes to detect and quantify nucleic acid sequences in a real-time fluorescent format. *Clin. Chem.* 46, 625–630
41. Peeters, B. *et al.* (2019) PCR amplified DNAzyme-amplicons for generic solid-phase antimicrobial resistence screening. In *20th International Conference on Solid-State Sensors, Actuators and Microsystems and Eurosensors XXXIII, Transducers 2019 and Eurosensors XXXIII*, IEEE
42. Zhang, D. *et al.* (2018) A dual signal amplification-assisted DNAzyme biosensor for ultrasensitive detection of argonaute 2 activity. *Chem. Commun.* 54, 13678–13681
43. Bone, S.M. *et al.* (2014) DNA-only cascade: a universal tool for signal amplification, enhancing the detection of target analytes. *Anal. Chem.* 86, 9106–9113
44. Peeters, B. *et al.* (2019) Real-time FO-SPR monitoring of solid-phase DNAzyme cleavage activity for cutting-edge biosensing. *ACS Appl. Mater. Interfaces* 11, 6759–6768
45. Xie, Y. *et al.* (2020) Proximity binding-triggered assembly of two MNAzymes for catalyzed release of G-quadruplex DNAzymes and an ultrasensitive homogeneous bioassay of platelet-derived growth factor. *Anal. Chem.* 92, 593–598
46. Hong, L. *et al.* (2017) Portable aptamer biosensor of platelet-derived growth factor-BB using a personal glucose meter with triply amplified. *Biosens. Bioelectron.* 95, 152–159
47. Ren, K. *et al.* (2015) Target-driven triple-binder assembly of MNAzyme for amplified electrochemical immunosensing of protein biomarker. *Anal. Chem.* 87, 1694–1700
48. Tabrizi, S.N. *et al.* (2016) Multiplex assay for simultaneous detection of mycoplasma genitalium and macrolide resistance using plexzyme and plexprime technology. *PLoS One* 11, e0156740
49. Bordin, A. *et al.* (2019) Evaluation of the SpeeDx Carba (beta) multiplex real-time PCR assay for detection of NDM, KPC, OXA-48-like, IMP-4-like and VIM carbapenemase genes. *BMC Infect. Dis.* 19, 571
50. Payne, M.S. *et al.* (2017) One-step simultaneous detection of *Ureaplasma parvum* and genotypes SV1, SV3 and SV6 from clinical samples using PlexPCR technology. *Lett. Appl. Microbiol.* 65, 153–158
51. Hasick, N.J. *et al.* (2019) Subzymes: regulating DNAzymes for point of care nucleic acid sensing. *Sensors Actuators B Chem.* 297, 10
52. Hasick, N. *et al.* (2020) Sensitive detection of nucleic acids using subzyme feedback cascades. *Molecules* 25, 1755
53. Liu, S. *et al.* (2015) Highly sensitive fluorescence detection of target DNA by coupling exonuclease-assisted cascade target recycling and DNAzyme amplification. *Biosens. Bioelectron.* 63, 99–104
54. Yun, W. *et al.* (2019) Entropy-driven catalytic reaction-induced hairpin structure switching for fluorometric detection of uranyl ions. *Microchim. Acta* 186, 653
55. Yun, W. *et al.* (2019) A dynamic, ultra-sensitive and "turn-on" strategy for fluorescent detection of uranyl based on DNAzyme and entropy-driven amplification initiated circular cleavage amplification. *Anal. Chim. Acta* 1068, 104–110
56. Ven, K. *et al.* (2019) Re-engineering 10–23 core DNA- and MNAzymes for applications at standard room temperature. *Anal. Bioanal. Chem.* 411, 205–215
57. Liu, Y. *et al.* (2020) Affinity binding-mediated fluorometric protein assay based on the use of target-triggered DNA assembling probes and aptamers labelled with upconversion nanoparticles:

application to the determination of platelet derived growth factor-BB. *Microchim. Acta* 187, 9
58. Gao, J. *et al.* (2015) Enhancement of deoxyribozyme activity by cationic copolymers. *Biomater. Sci.* 3, 308–316
59. Xue, C. *et al.* (2019) Y-shaped backbone-rigidified triangular DNA scaffold-directed stepwise movement of a DNAzyme walker for sensitive microRNA imaging within living cells. *Anal. Chem.* 91, 15678–15685
60. Zhou, W. *et al.* (2015) DNAzyme hybridization, cleavage, degradation, and sensing in undiluted human blood serum. *Anal. Chem.* 87, 4001–4007
61. Teh, H.B. *et al.* (2014) Highly sensitive and selective detection of Pb^{2+} ions using a novel and simple DNAzyme-based quartz crystal microbalance with dissipation biosensor. *Analyst* 139, 5170–5175
62. Zagorovsky, K. and Chan, W.C.W. (2013) A plasmonic DNAzyme strategy for point-of-care genetic detection of infectious pathogens. *Angew. Chem. Int. Ed.* 52, 3168–3171
63. Wang, G. *et al.* (2019) Microfluidic particle dam for visual and quantitative detection of lead ions. *ACS Sensors.* 5, 19–23
64. Sun, C. *et al.* (2010) Electrochemical DNA biosensor based on proximity-dependent DNA ligation assays with DNAzyme amplification of hairpin substrate signal. *Biosens. Bioelectron.* 25, 2483–2489
65. Diao, W. *et al.* (2016) Electrochemical DNA biosensor based on MNAzyme-mediated signal amplification. *Microchim. Acta* 183, 2563–2569
66. Yang, J. *et al.* (2016) Electrochemical strategy for ultrasensitive detection of microRNA based on MNAzyme-mediated rolling circle amplification on a gold electrode. *Microchim. Acta* 183, 3061–3067
67. Liu, S. *et al.* (2016) Ultrasensitive electrochemical DNAzyme sensor for lead ion based on cleavage-induced template-independent polymerization and alkaline phosphatase amplification. *Biosens. Bioelectron.* 83, 33–38
68. Duan, Y. *et al.* (2018) Detection of BCR/ABL fusion gene based on MNAzyme-mediated target-cycling and ssDNA-assisted cascade hybridization reaction. *Electroanalysis* 30, 2427–2433
69. Lu, L. *et al.* (2015) Highly selective and sensitive electrochemical biosensor for ATP based on the dual strategy integrating the cofactor-dependent enzymatic ligation reaction with self-cleaving DNAzyme-amplified electrochemical detection. *Biosens. Bioelectron.* 63, 14–20
70. Zeng, G. *et al.* (2016) Electrochemical DNA sensing strategy based on strengthening electronic conduction and a signal amplifier carrier of nanoAu/MCN composited nanomaterials for sensitive lead detection. *Environ. Sci. Nano.* 3, 1504–1509
71. Ma, Y. *et al.* (2019) DNAzyme assisted recycling amplification method for ultrasensitive amperometric determination of lead(II) based on the use of a hairpin assembly on a composite prepared from nitrogen doped graphene, perylenetetracarboxylic anhydride, thionine and gold nanoparticles. *Microchim. Acta* 186, 677
72. Yu, Z. *et al.* (2019) Highly efficient electrochemical detection of lead ion using metal-organic framework and graphene as platform based on DNAzyme. *Synth. Met.* 254, 164–171
73. Qian, Y. *et al.* (2015) A novel label-free and enzyme-free electrochemical aptasensor based on DNA in situ metallization. *Biosens. Bioelectron.* 74, 483–490
74. Ling, J. *et al.* (2019) An enzyme-free electrochemiluminescence biosensor for ultrasensitive assay of group B streptococci based on self-enhanced luminol complex functionalized CuMn-CeO_2 nanospheres. *Biosens. Bioelectron.* 127, 167–173
75. Zhang, T. *et al.* (2019) Ultrasensitive detection of Pb^{2+} based on a DNAzyme and digital PCR. *J. Anal. Methods Chem.* 2019, 3528345
76. Wang, Y. *et al.* (2020) Evaluating the catalytic potential of a general RNA-cleaving FANA enzyme. *ChemBioChem* 21, 1001–1006
77. Santoro, S.W. and Joyce, G.F. (1997) A general purpose RNA-cleaving DNA enzyme. *Proc. Natl. Acad. Sci. U. S. A.* 94, 4262–4266
78. Silverman, S.K. (2004) Deoxyribozymes: DNA catalysts for bioorganic chemistry. *Org. Biomol. Chem.* 2, 2701–2706
79. Achenbach, J.C. *et al.* (2005) Structure-switching allosteric deoxyribozymes. *Anal. Chim. Acta* 534, 41–51
80. Shen, Y. *et al.* (2006) Catalysis and rational engineering of trans-acting pH6DZ1, an RNA-cleaving and fluorescence-signaling deoxyribozyme with a four-way junction structure. *ChemBioChem* 7, 1343–1348
81. Ruble, B.K. *et al.* (2012) Mismatch discrimination and efficient photomodulation with split 10-23 DNAzymes. *Inorg. Chim. Acta* 380, 386–391
82. Peng, H. *et al.* (2018) DNAzyme-mediated assays for amplified detection of nucleic acids and proteins. *Anal. Chem.* 90, 190–207
83. Zhou, W. *et al.* (2017) Theranostic DNAzymes. *Theranostics* 7, 1010–1025