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DNAzymes as key components of biosensing systems for the detection of biological targets

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ABSTRACT

DNAzymes are synthetic functional nucleic acids that have found widespread use in biosensing applications both for molecular recognition and signal generation. Two classes of DNAzymes have proved particularly effective for use in proof-of-concept biosensing systems, namely RNA-cleaving DNAzymes (RCDs) and peroxidase mimicking DNAzymes (PMDs). RCDs catalyze the site-specific cleavage reaction of an RNA dinucleotide junction, generating two cleavage fragments. PMDs are capable of catalyzing peroxidation reactions of chromophores, thereby generating a measurable signal. Herein, we review the use of these DNAzymes in biomedical assays and diagnostics, and show that this emerging field should have great promise for biosensor development over the next few decades.

1. Introduction

Deoxyribozymes, often denoted as DNAzymes, are a specific class of functional nucleic acids, which are single-stranded DNA molecules capable of catalyzing a specific chemical reaction. Some DNAzymes are also activated upon binding a specific target, and therefore can be used as a molecular recognition element (MRE) to detect this target. These properties, in addition to their prolonged intrinsic stability and relatively low synthetic cost, make DNAzymes an excellent choice for inclusion in biosensing platforms (Ma and Liu, 2020; Silverman, 2009).

Researchers in North America have done pioneering work in the field of DNAzymes. Inspired by the discovery that RNA has catalytic properties, which earned Sidney Altman and Thomas Cech the Nobel Prize in chemistry in 1989, several groups in North America used a simple and powerful *in vitro selection* technique (Ellington and Szostak, 1990; Tuerk and Gold, 1990) to make ground-breaking discoveries of catalytically active DNA species in the early 1990s (Li and Breaker, 1999). The first DNAzyme ever reported was GR-5, an RNA-cleaving DNAzyme (RCD) discovered by the Joyce group in 1994 (Breaker and Joyce, 1994). RCDs like GR-5 are engineered to cleave a specific dinucleotide junction of RNA (Fig. 1A). When the catalysis of an RCD requires activation by another molecule, this RCD can be used to design a detection system for this molecule. For example, GR-5 requires Pb^{2+} as a cofactor to support its catalytic activity, and therefore, it can be used to design a biosensing assay or device to detect Pb^{2+} (Fig. 1B). A large number of target-responsive RCDs have been discovered over the past 25 years, and many of them, such as 17E (Figs. 1C) and 39E (Fig. 1D), have been examined as MREs. In fact, RCDs constitute the most dominant class of DNAzymes in biosensing studies (M. Liu et al., 2017a; Safdar et al., 2020).

In addition to molecular recognition, biosensing systems need to have a signal producing module. Many DNAzyme-based biosensors take advantage of another DNAzyme to achieve this goal: peroxidase mimicking DNAzymes (PMDs). A classic example is PS2.M, which was discovered as the porphyrin-binding DNA aptamer (Li et al., 1996). PMDs like PS2.M consist of a guanine-rich (G-rich) sequence capable of forming a G-quadruplex, which, in the presence of hemin, can catalyze an oxidation reaction between hydrogen peroxide and a chromophore, such as ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)) and TMB (3,3′,5,5′-tetramethylbenzidine), producing a color change (Fig. 2) (Travascio et al., 1998). The same DNAzyme can also act as a fluorescent reporter, as it can bind specific fluorogenic molecules, such as protoporphyrin IX (PPIX), to produce a significantly enhanced fluorescence signal (Li et al., 2010).

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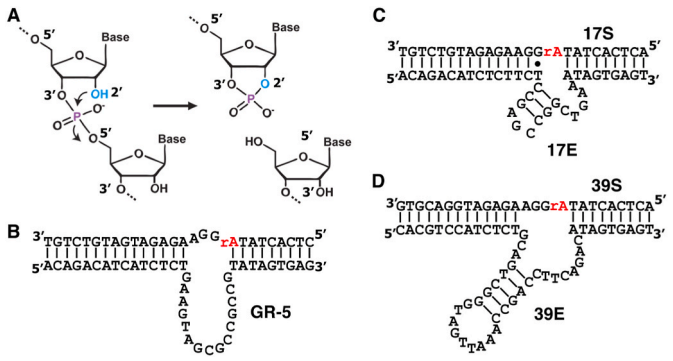

Fig. 1. RNA-cleavage DNAzymes (RCDs). Transesterification reaction (A) catalyzed by most RCDs reported to date, including GR-5 (B), 17E (C) and 39E (D). rA in B-D stands for adenosine ribonucleotide; other letters represent deoxyribonucleotides.

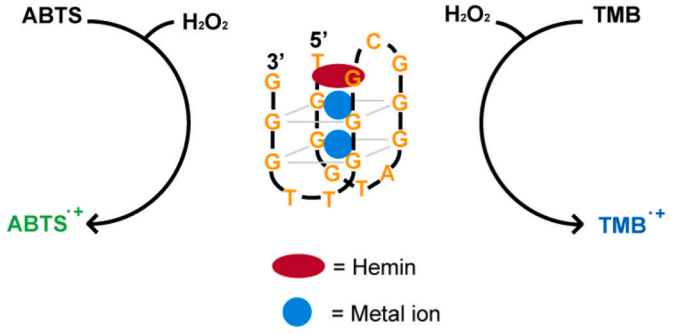

Fig. 2. Peroxidase mimicking DNAzyme (PMD). The specific PMD shown here is PS2.M, which can catalyze the oxidation of ABTS (left) or TMB (right) to produce a color change. Reprinted with permission from Chang D, Zakaria S, Deng M, Allen N, Tram K, Li Y. Integrating Deoxyribozymes into Colorimetric Sensing Platforms. Sensors. 2016, 16, 2061 (Chang et al., 2016). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Extensive work has been done in North America both in initiating the field of DNAzymes and in developing DNAzyme-based biosensing methods. The two senior authors of this review have a long-standing interest in developing DNAzymes as key components of bioassays and biosensors to detect biological targets. Therefore, we will focus this review on some of our own work published in this area, and at the same time, we will strive to capture the remarkable accomplishments achieved by some of the other North American researchers in this auspicious field. Of note, this work does not constitute a comprehensive review of all DNAzyme-based biosensing studies. Rather, this focused review will place emphasis on North American researchers' work to advance DNAzyme-based biosensing as it pertains to the detection of targets relevant to biomedical applications. Specifically, we will discuss relevant advancements made by us and others in North America towards using DNAzymes to achieve molecular recognition of biological targets, signal transduction, and signal amplification. Our key motivations are to showcase the breadth of potential clinical applications for DNAzyme-based biosensors and to encourage further research and development of DNAzymes towards establishing clinically useful diagnostic tests.

2. RCDs and PMDs: the two dominant DNAzyme classes for biosensing applications

2.1. RCDs

As previously noted, GR-5 was the first identified RCD (Breaker and Joyce, 1994). Briefly, this DNAzyme was identified from a synthetic single-stranded DNA library that was subjected to multiple rounds of incubation with a buffer containing Pb^{2+}, partitioning of active from inactive sequences, and amplification of active ones by polymerase chain reaction (PCR), to create an enriched library for the next round of selection. By this process, a DNAzyme was selected that underwent specific intramolecular cleavage at an embedded ribonucleotide site, in the presence of Pb^{2+} (Breaker and Joyce, 1994). Interestingly, RCDs were originally conceived for therapeutic applications. However, as we will discuss in several examples below, the potential of RCDs for biosensing applications, especially in environmental monitoring, was soon realized; GR-5, as well as many other RCDs, would go on to become the most commonly used recognition elements in DNAzyme-based biosensing (Lake et al., 2019). Furthermore, the advantages of biosensing RCDs, which specifically cleave a single ribonucleotide embedded in an otherwise all-DNA sequence, have now been appreciated, propelling the use of RCDs forward into biosensing of diverse targets, including metal ions and bacteria.

The identification of the 8–17 and 10–23 DNAzymes by the Joyce group was another notable advancement in the field (Santoro and Joyce, 1997). Interestingly, 8–17 has been identified in several independent DNAzyme selection experiments, including one reported by the Lu group who identified a Zn^{2+} and Pb^{2+} dependent variant of 8–17, which they called 17E (Fig. 1C) (Li et al., 2000; Zhou et al., 2017a). This reoccurrence was hypothesized to occur due to the "tyranny of the short motif" where there is a high probability of the relatively short active domain randomly existing within a combinatorial library (Hollenstein, 2015; Li and Lu, 2009; Silverman, 2009; Zhou et al., 2017b). This challenge has been addressed by researchers by the utilization of longer selection templates (Li and Lu, 2009).

In 2007, the Lu group also described a DNAzyme with unprecedented 1-million fold selectivity for UO_2^{2+} against 19 other metal cations with various charges (Liu et al., 2007). The DNAzyme, called 39E (Fig. 1D), could detect as low as 43 pM of UO_2^{2+}. The success of this DNAzyme has made uranyl a common ion cofactor investigated with DNAzyme-based sensing platforms. Additionally, its success led to a pivotal innovation, with its incorporation into a sensor platform utilizing a personal glucose meter as an electrochemical reader for point of care biosensing (Lake et al., 2019).

Through efforts of several groups, including the ones led by Yi Lu and Juewen Liu, many metal-ion dependent RCDs have been discovered and utilized for the development of a variety of excellent sensors for metal ions, which have been reviewed elsewhere (Lake et al., 2019; Zhou et al., 2017b). Researchers were also interested in the selection of DNAzymes which were catalytically active in the presence of non-metal cofactors and/or targets. Early progress saw the report of an RNA-cleaving DNAzyme activated by the amino acid histidine, selected by the Breaker group in 1998 (Roth and Breaker, 1998). Over a decade later, the Li group reported the selection of a fluorogenic RNA-cleaving DNAzyme, called RFD-EC1, that was catalytically active in the presence of *E. coli*. As described below, these bacteria-targeting DNAzymes are more appropriately termed aptazymes, as they contain both an aptamer domain for target recognition and an allosterically controlled DNAzyme domain for catalysis.

Generally, RCDs can be further divided into multiple subclasses depending on the sequence (cis, trans or split), if their substrate region is fixed or variable, whether the sequence is modified (unmodified RCDs, catalytic molecular beacons, and fluorogenic RCDs), and if the activity of the DNAzyme is regulated by an aptamer (aptazyme). Cis or trans refers to the interconnectedness of the substrate and catalytic domain. In the cis form, the RCD is a single molecule where the substrate region and catalytical domain are joined into one sequence. In contrast, RCDs in the trans form consist of a substrate strand and a catalyst strand. An important distinction between cis and trans is that though in the cis form, RCDs are catalytically active, only RCDs in the trans form are actually enzymes since they can catalyze multiple turnovers. For

biosensing, the catalytic region of trans DNAzymes are sometimes split, so that catalysis requires the assembly of three or more individual sequences, rather than the assembly of a substrate strand and a catalyst strand.

The next important distinction worthy of discussion is whether the substrate binding region is fixed. Some RCDs, such as the 8–17 and 10–23 DNAzymes, have some flexibility in the sequence of the substrate and the binding arms within the catalyst strand. As such, the sequences of the binding arms can be altered to recognize a specific substrate sequence. RCDs are often modified with either a fluorophore or a fluorophore/quencher pair to create RNA-cleaving fluorogenic DNAzymes (RFDs). Such modifications can also be done to the library to allow for the selection of RCDs that inherently carry signaling properties. Though both catalytic molecular beacons and fluorogenic RFDs incorporate a fluorophore/quencher pair, catalytic molecular beacons tend to have terminal modifications whereas fluorogenic RFDs are internally modified, flanking the cleavage site.

Finally, the intramolecular cleavage that defines the catalytic activity of an RCD is often highly dependent on metal-DNA interactions. Therefore, most RCDs tend to be metal-ion dependent. However, some RCDs have been derived to be activated by non-metal targets. In these cases, specific metal ions may still be required (as a co-factor for catalysis). As noted above, these RCDs are referred to as aptazymes, where the cleavage activity is dependent on the interaction of the recognition sequence with the non-metal target, rather than the metal ion alone. RCDs activated by bacterial targets are an example of this class.

2.2. PMDs

In parallel to many seminal discoveries on RCDs, researchers in North America have also made significant contributions investigating the catalytic properties of G-rich DNA sequences. A series of papers published by the Sen group in the late 1990s led to the development of PMDs (Travascio et al., 1998) which have been abundantly applied as reporter probes in biosensing systems (Peng et al., 2018). A particular advantage of the PMD class is that they are single-stranded molecules capable of forming G-quadruplex structures (Liu et al., 2020). Further, their properties are compatible with multiple readouts such as colorimetric, chemiluminescent, and electrochemical methods. This has allowed multiple methods to be developed to control formation of PMDs and their signaling modalities to develop diverse and highly sensitive assays. For example, common strategies have included incorporating the PMD directly in the sensor as a signal transducer, combining the PMD with nanomaterials to enhance the signal transducing effect, incorporating the PMD into a molecular beacon, the cleavage of which leads to the exposure of a PMD domain for controlled signal transduction, exploitation of a split DNAzyme assembly for controlled signal transduction, or the use of isothermal amplification strategies like rolling circle amplification (RCA) to create multiple copies of the PMD in one functional DNA superstructure, thereby amplifying signal transduction (Peng et al., 2018). Given the programmability of DNA, it is also possible to develop biosensing platforms integrating both RCDs and PMDs as core components, which will be highlighted in the sections to follow.

3. RCD and PMD based biosensors

3.1. General use of RCDs and PMDs in biosensor design

Many published studies have utilized various DNAzymes for the design of biosensors to detect a broad range of targets. In general, DNAzymes can be used to fulfill functions of molecular recognition and signal production of a biosensor. As we have pointed out earlier, DNAzymes can occupy the role of MRE so long as their activities are dependent on the presence of a target of interest, as demonstrated by GR-5 for Pb^{2+} detection and 39E for UO_2^{2+} sensing. In the next section, we will discuss some specific examples of target-responsive RCDs as the MRE to detect biologically important targets.

PMDs have also been widely used as an important component of many biosensors as they offer a simple way to produce a signal if their activity can be coupled to target binding. There are general ways to link the formation of a functional PMD to target binding. One common strategy involves target-activated assembly or disassembly of a functional PMD. Most biosensors using this strategy contain a split PMD, a recent example of which was provided by Xiao, Yu and colleagues (Luo et al., 2019). Another strategy relies on target-induced conformational changes to impact the activity of PMD. In this approach, the molecular recognition element (MRE) is often strategically linked to the sequence of a PMD such that the activity of the PMD unit is regulated by the binding state of the MRE. For example, in a recent study, Lou et al. used structure-switching aptamers to regulate the activity of a PMD (Gao et al., 2019). In their design, the PMD is functional in the absence of target; target binding to the aptamer, however, disrupts the structure formation of the PMD unit, resulting in the reduction of peroxidase activity. A third approach is the production of PMD via DNA synthesis that is linked to target binding. For example, RCA regulated by aptamer-target binding has been used by our groups to produce repetitive copies of a PMD as part of the DNA amplicon for signal production (Liu et al., 2015).

DNAzymes can also be incorporated into a biosensor to take on the roles of both molecular recognition and signal generation. As demonstrated in a recent study by Cao et al., a cis-acting RCD for L-Histidine (L-His) was employed as the MRE (meaning that the RCD cleaves itself in the present of L-His), releasing a small DNA fragment. The released fragment can then act as a trigger to initiate a hybridization chain reaction (HCR), generating functional PMD units for signal production (He et al., 2019).

Our groups have also described a dual DNAzyme-enabled all-DNA sensing system designed to detect *E. coli*. The sensor utilized a 4-way junction (4WJ) structure with the input controlled by an RCD and the output linked to the generation of a PMD (Fig. 3A) (Zhou et al., 2020). In response to binding of its target (*E. coli*), the RFD-EC1 DNAzyme cleaves its substrate named DNA3, releasing two DNA fragments (P1 and P2). In the absence of the target, DNA3 remains intact and is capable of interacting with DNA molecules 1, 2, and 4/5 to form a 4WJ. In doing so, DNA5 is displaced from the DNA4/5 complex, allowing it to act as a trigger for catalytic hairpin assembly (CHA) for signal amplification. The recycling of DNA5 results in many copies of PMD units, which bind PPIX to produce a fluorescent signal. The core principle of this biosensor is the coupling of the assembly-mediated strand release (ASR) reaction whereby DNA5 is released to the linked CHA-split G quadruplex reassembly (CHA-SGR) process. Given that the presence of the target halts the formation of the 4WJ and eliminates the CHA trigger, the signal output of this biosensor is inversely proportional to the amount of target in solution, and thus it is a turn-off sensor.

The system can also be configured into a turn-on sensor, whose signal output is proportional to the amount of target in solution (Fig. 3B). In this design, the trigger for CHA is DNA6, which becomes unavailable, due to the 4WJ formation, if there is no target to assist the RCD to cleave DNA3. However, in the presence of the target, DNA3 is cleaved and the 4WJ is unable to form, allowing DNA6 to trigger the CHA-SGR process.

The examples highlighted in this section serve to provide an introduction to common uses of RCDs and PMDs as key components of DNAzyme based biosensors. In the next few sections, we will discuss selected studies where RCDs and/or PMDs have been utilized to design specific sensors for biological targets relevant to human health. Given the availability of many excellent reviews that have highlighted the advances of DNAzyme-based metal ion sensing (Lake et al., 2019; Ma and Liu, 2020; McGhee et al., 2017; Zhou et al., 2017b), this area of research will not be discussed in this review.

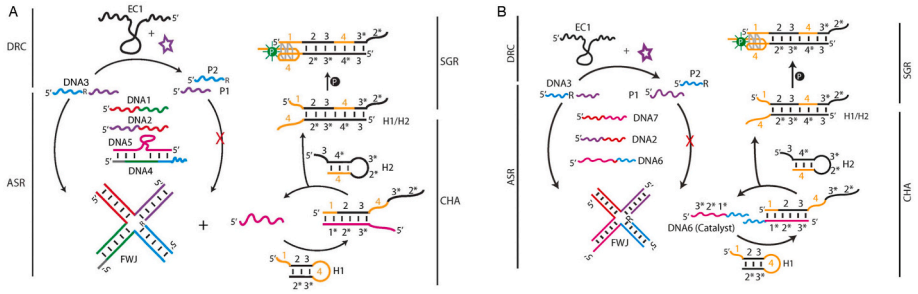

Fig. 3. Two all-DNA biosensing platforms that use an RCD for *E. coli* recognition, PMD for signal generation and 4WJ formation as a regulator. (A) Turn-off design. (B) Turn-on design (see text for detailed descriptions). Reprinted (adapted) with permission from Zhou Z, Brennan JD, Li Y. A Multi-component All-DNA Biosensing System Controlled by a DNAzyme. *Angew Chem. Int. Ed.* 2020, 59, 10401–10405. Copyright 2020 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim.

3.2. Representative PMD biosensors rationally designed for the detection of biological molecules

Important advances have been made in using DNAzymes as key components for the detection of specific biologically important molecules, which include small molecules, proteins and RNA. For example, Lee et al. recently described a PMD-utilizing assay for the detection of microRNA-205 (miRNA-205), a biomarker associated with lung cancer (Kim et al., 2019). The key design here is the use of two DNA probes that are complementary to miRNA-205 (Fig. 4A), one of which is placed on a paramagnetic bead and functions as the MRE, and the second that brings in the PMD. The target miRNA-205 acts as a linker between the MRE and the PMD. This allows the DNAzyme to be attached to the paramagnetic bead in the presence of the miRNA. Upon separation and addition of hemin to the reaction mixture, the PMD catalyzes the reaction between Amplex Red and H_2O_2, generating resorufin that can be detected by chemiluminescence.

Our groups have shown PMDs can been used as reporter molecules for miRNA detection using RCA on paper (Liu et al., 2016a). In this assay, a paper device is made by printing a biological ink comprising all the reagents for two different reactions: a specific DNA probe that can hybridize with one end of the miRNA target, a circular DNA template that binds the other end of the miRNA, phi29 DNA polymerase and dNTPs needed for RCA. The miRNA in a test sample will first be captured by a DNA probe and binds the circular DNA template to initiate RCA. The circular DNA template is designed to contain the antisense sequence of a specific PMD so that the RCA reaction will produce many copies of PMDs for signal generation. Therefore, the paper device represents an all-in-one, equipment-free biosensor enabling colorimetric detection. It should be noted that the bioink also contains pullulan, a natural polysaccharide that, upon drying, forms a thin, oxygen impermeable film to stabilize proteins (Jahanshahi-Anbuhi et al., 2014) and RNA (Hsieh et al., 2017). The film readily dissolves upon contacting aqueous solution to allow an intended biochemical reaction to occur. This approach has been used in many other paper sensors reported by our groups for the detection of other targets, which will be discussed in the later sections of this article.

PMDs can also be combined with a DNA aptamer to achieve the detection of the target that binds the DNA aptamer. For example, Xiao, Yu and colleagues developed a biosensing system based on cooperative binding of split DNA probes for the detection of small molecules including cocaine and methylenedioxypyrovalerone (MDPV) (Luo et al., 2019). The MRE is composed of a cocaine-binding split aptamer (CBSA), with each half of the aptamer containing a segment of the PMD (Fig. 4B). Through cooperative binding of the target, the two aptamer segments combine and are able to bring the two PMD segments together, facilitating the assembly of the whole PMD for signal production. They termed the system "CBSAzyme" and demonstrated the versatility of the

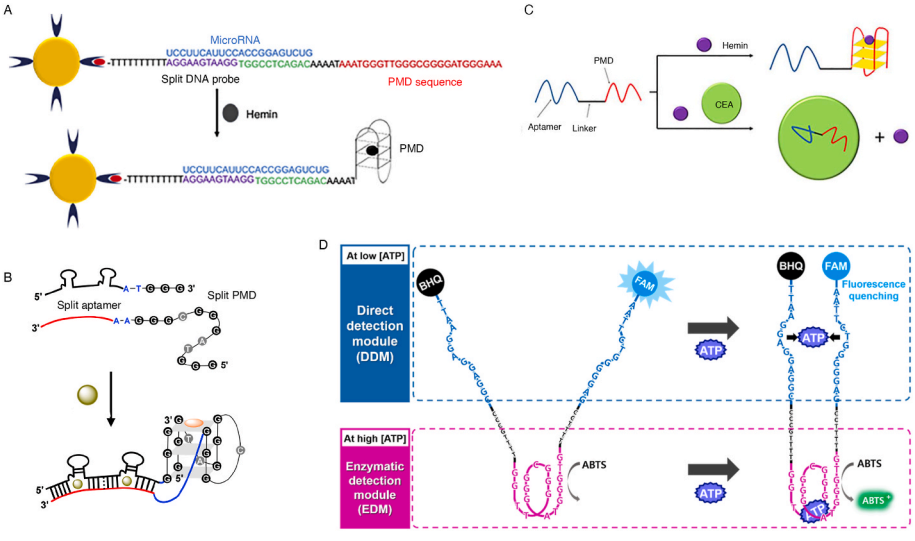

Fig. 4. Biomarker detection using biosensors containing a PMD unit. (A) MiRNA detection using a split DNA probe. Reprinted from Kim K, Park P, Lee JH. Cost-effective monitoring of microRNA-205 applied as a biomarker using G-quadruplex DNAzyme and 1,1'-oxalyldiimidazole chemiluminescence. *J Pharm Biomed Anal.* 2019, 175:112780. Copyright 2019, with permission from Elsevier. (B) Small molecule detection using a split aptamer and a split PMD. Reprinted (adapted) with permission from Luo Y, Yu H, Alkhamis O, et al. Label-Free, Visual Detection of Small Molecules Using Highly Target-Responsive Multimodule Split Aptamer Constructs. *Anal Chem.* 2019, 91, 7199–7207. Copyright 2019 American Chemical Society. (C) Protein detection using a single sequence containing a DNA aptamer and a PMD. Reprinted from Khang H, Cho K, Chong S, Lee JH. All-in-one dual-aptasensor capable of rapidly quantifying carcinoembryonic antigen. *Biosens Bioelectron.* 2017, 90, 46–52. Copyright 2017, with permission from Elsevier. (D) ATP detection using a combined ATP-binding DNA aptamer and a PMD. Reprinted (adapted) with permission from Kang B, Park SV, Soh HT, Oh SS. A Dual-Sensing DNA Nanostructure with an Ultrabroad Detection Range. *ACS Sensors.* 2019, 4, 2802–2808. Copyright 2019 American Chemical Society.

sensing platform by applying it to detect cocaine, MDPV and 10 other synthetic cathinones (psychoactive stimulants).

Aptamers and PMDs can also be combined into a single sequence to function as a bifunctional sensor. For example, Lee et al. took this approach for carcinoembryonic antigen (CEA) detection by joining a CEA-binding DNA aptamer with a PMD with a short linker composed of 5 adenines (Fig. 4C) (Khang et al., 2017). CEA is a biomarker directly linked to colorectal cancer, and used faithfully in clinical diagnosis, disease progression, and treatment response. In the absence of CEA, the PMD domain is capable of binding hemin to function as a peroxidase. However, the binding of CEA to the aptamer domain impedes the hemin binding ability of the PMD, reducing its peroxidase activity. Consequently, the aptamer-CEA binding acts as a PMD off-switch, resulting in an inversely proportional relationship between target and biosensor signal.

Soh, Oh and colleagues recently described a biosensor for the detection of ATP by combining an ATP-binding DNA aptamer and a PMD (Fig. 4D) (Kang et al., 2019). The sensing system offers two different types of readouts that together can achieve a dynamic detection range over 5 orders of magnitude. It has a direct detection module (DDM) made of a split ATP-binding aptamer (top) and an enzymatic detection module (EDM) comprised of an ATP-enhanced PMD (bottom) (Kong et al., 2010). ATP binding to the top DDM module induces aptamer assembly and places the fluorophore and quencher pair at the end of the aptamer next to each other. This event results in a fluorescence decrease that correlates with ATP levels at relatively low (micromolar) concentrations. Because ATP is also known to enhance the peroxidase activity of PMDs, at higher concentration of ATP, the bottom EDM module exhibits enhanced oxidation of ABTS (Kang et al., 2019).

3.3. *Representative RCD biosensors rationally designed for the detection of biological molecules*

When combined with aptamers as MREs, RCDs can be rationally designed to produce aptazymes, which can be integrated into biosensors for the detection of biological targets. Sensors in this type are often called “aptazyme sensors”. In this section, we will discuss a few key studies in this area.

An early example of a rationally designed aptazyme was reported by the Li group, who designed an aptazyme for the detection of ATP (Fig. 5A) (Tram et al., 2015). The sensor was constructed with an ATP-binding DNA aptamer and an RCD capable of cleaving L-RNA, the enantiomer of D-RNA (natural RNA). Therefore, this sensor functions effectively in complex biological samples due to the fact that ubiquitous RNases cannot cleave L-RNA substrates (Tram et al., 2015). The design uses an aptamer-containing substrate strand (S1-Apt1) with a sequence design in which part of the aptamer engages part of the substrate into a hairpin structure that prevents the access of the substrate by the DNAzyme. However, in the presence of ATP, the hairpin structure gives way to the formation of a ligand-aptamer complex, making the substrate fully accessible to the DNAzyme (Fig. 5A).

Juewen Liu’s group also reported an RCD-based aptazyme for ATP detection using the same ATP binding DNA aptamer but a different RCD named EtNa (Fig. 5B), which remains functional in 50% ethanol (Yu et al., 2018). They applied this sensor for the detection of AMP. Therefore, this sensor is unique in the sense that it can still function in high concentrations of an organic solvent.

Ma and colleagues have described an aptazyme for prostate specific antigen (PSA) detection using an RCD, a PSA-binding DNA aptamer and graphene oxide (GO) (Yan et al., 2020). The RCD and the aptamer are combined into a hairpin structure so that the DNAzyme is not available for cleaving its fluorophore-tagged substrate in the absence of PSA (Fig. 5C). Under this condition, the substrate strand is absorbed by GO and the fluorescence is quenched. However, in the presence of PSA, PSA-aptamer binding frees up the DNAzyme, which cleaves the substrate and generates shorter cleavage products that are not adsorbed efficiently by GO, resulting in a higher level of fluorescence.

It is also possible to initiate the catalytic activity of an RCD using an antibody as an MRE. This was demonstrated in a recent report by Kraatz, Chen, Huang and colleagues (Li et al., 2020). In this work, an electrochemical biosensing device for CEA and α-fetoprotein (AFP) detection was designed in which an RCD acts as a DNA walker on hollow carbon nanospheres (HCS). CEA and AFP are involved in the diagnosis of hepatocellular carcinoma. The CEA/AFP antibody was conjugated to two different DNA molecules: one used as an anchor DNA placed on HCS and the other as the RCD. Through antibody and CEA/AFP binding, the DNAzyme becomes attached to HCS and then functions as the DNA walker to cleave substrate strands placed on HCS. With time, one DNAzyme walker cleaves multiple substrate strands and generates many copies of intermediate DNA (iDNA) to achieve signal amplification. The iDNA then creates Y-shaped DNA structures with two capture DNAs on an electrode that carry the redox probe methylene blue (MB) and

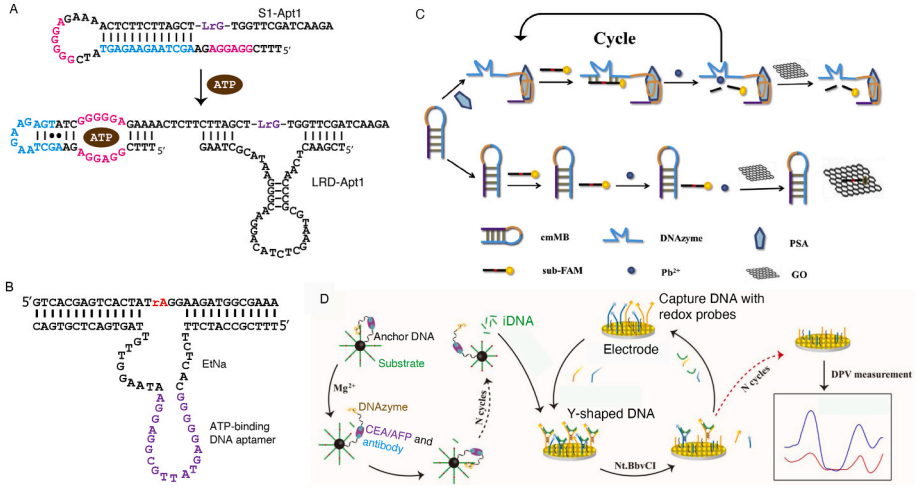

Fig. 5. Detection of biomolecules using RCD based biosensors. (A) ATP detection using an RCD that cleaves L-RNA. Reprinted from Tram K, Xia J, Gysbers R, Li Y. An Efficient Catalytic DNA that Cleaves L-RNA. *PLoS One*. 2015, 10, e0126402. Copyright 2015 Tram et al. (B) AMP detection using EtNa, an RCD that is functional in 50% ethanol. Reprinted (adapted) with permission from Yu T, Zhou W, Liu J. Ultrasensitive DNAzyme-Based Ca^{2+} Detection Boosted by Ethanol and a Solvent-Compatible Scaffold for Aptazyme Design. *ChemBioChem*. 2018, 19, 31–36. Copyright 2018 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim. (C) Protein detection using an RCD, PSA binding DNA aptamer and graphene oxide (GO). Reprinted from Yan Y, Ma C, Tang Z, Chen M, Zhao H. A novel fluorescent assay based on DNAzyme-assisted detection of prostate specific antigen for signal amplification. *Anal Chim Acta*. 2020, 1104, 172–179, Copyright 2020, with permission from Elsevier. (D) Detection of CEA and AFP using a DNAzyme walker and electrochemistry. Reprinted from Li X, Qiu Q, Ni X, et al. Detection of carcinoembryonic antigen and α-fetoprotein exploiting a 3D DNA walker strategy. *Sensors Actuators, B Chem*. 2020, 319, 128327. Copyright 2020, with permission from Elsevier.

ferrocene (Fc), respectively. The Y-shaped DNA structures are cleaved by the nicking enzyme Nt.BbvCI for another round of signal amplification. The end result is the decrease of the redox signal, which can be measured by differential pulse voltammetry (DPV) (Fig. 5D).

3.4. Detection of metal ions inside cells using RCDs

Another recent area of DNAzyme research has been centered around the use of metal ion dependent RCDs to detect intracellular metal ions (metal ion imaging). The core principle behind metal ion imaging relies on the activation of a DNAzyme by a specific metal ion cofactor. As such, by quantifying an RCD's catalytic activity, researchers can harness the signal generated to demonstrate the presence of the metal ion in question and correlate the signal intensity to the concentration of a metal ion. For such applications, it is important to overcome the issues of non-specific cleavage of RNA-containing substrates and degradation of DNAzymes by intracellular nucleases. To overcome these barriers the Lu group investigated a photoactivation strategy in 2014. This strategy involved the design of a photoactivateable DNAzyme where the substitution of a 2′-O-nitrobenzyl adenosine from the scissile adenosine ribonucleotide of the 8–17 DNAzyme allowed it to be delivered into cells intact, and only activated in the presence of its target, Zn^{2+}, upon irradiation with 365 nm light (Hwang et al., 2014). In 2016, Zhang, Tan and colleagues examined the use of L-DNAzymes, the enantiomeric form of the standard D-DNAzymes, as L-nucleic acids are completely resistant to endogenous nucleases (Cui et al., 2016). They have shown that an L-RCD can be activated by the same metal ion for the related D-RCD.

The groups of Lu and Zhu came up a strategy that also enabled the use of 8–17 for the detection of Zn^{2+} ion inside cells (Fig. 6A), avoiding premature DNAzyme activation and degradation upon entering a cell, which constitutes further hurdles for *in vivo* DNAzyme-based biosensing (Wang et al., 2017). The design features a three-stranded DNAzyme precursor (TSDP) consisting of an enzyme strand with a black hole quencher (BHQ2) at the 3′-end, a substrate strand with Cy5 at the 5′-end, and a Linker DNA strand with an SH group at the 5′-end. Upon hybridization, the DNAzyme within TSDP is inactive. Via conjugation to a gold nanoshell (AuNS), the system is protected from inappropriate cleavage and is placed under more regimented control. In response to near-infrared illumination (NIR), the DNAzyme is released from the precursor by virtue of the rise in local temperature, allowing it to adopt its active conformation and undergo substrate cleavage in the presence of its metal-ion cofactor Zn^{2+}. In doing so, it generates a fluorescent signal, effectively demonstrating the presence of the metal ion cofactor in question.

A significant challenge for detecting metal ions inside cells is their low concentration. The Lu and Jiang groups tackled this challenge through the use of CHA (Fig. 6B) (Wu et al., 2017). The RCD employed in this study was Na43, a Na^{+}-specific DNAzyme. The 2′-hydroxyl of the ribonucleotide at the cleavage site was protected with nitrobenzyl, a photocaged group (PG), which was used to protect the substrate from non-specific cleavage. Irradiation at 365 nm removed nitrobenzyl, allowing the DNAzyme to cleave the substrate in the presence of Na^{+}. Doing so releases a cleavage fragment, which proceeds to act as an initiator for CHA.

The photo-deprotection via irradiation at 365 nm limits the applicability of DNAzyme sensors to cellular and *in vivo* sensing, as the 365 nm light has poor penetration into tissue and can potentially cause phototoxicity to living cells or animals. To overcome these issues, the Lu group used lanthanide-doped upconversion nanoparticles (UCNP), which allowed more effective and safer near-infrared illumination. Photocaging with nitrobenzyl was once again used to protect the 2′-hydroxyl at the cleavage site, but the DNAzyme was attached to the UCNP. Using this improved strategy, the Lu group achieved Zn^{2+} sensing within cells and zebrafish embryos with the use of 8–17 (Yang et al., 2018).

Another interesting example is the Lu and Zheng groups' design of a DNAzyme-based fluorogenic biosensor for Mg^{2+} detection in living cells (Fig. 6C) (Lin et al., 2019). Particularly important is the fact that this

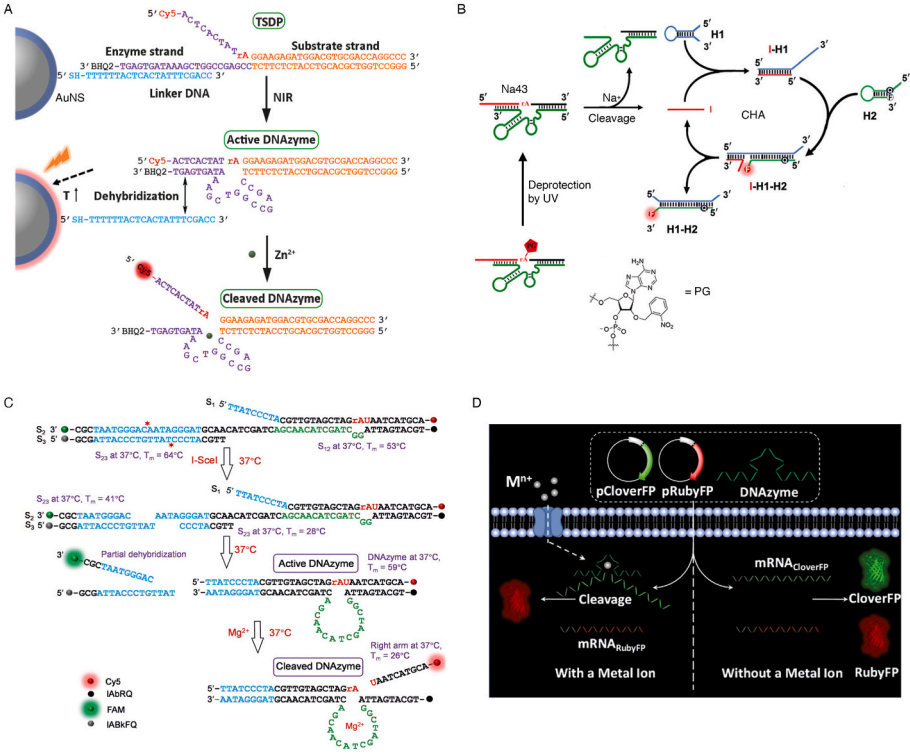

Fig. 6. Detection of metal ions inside cells using RCD based biosensors. (A) Zn^{2+} detection with an RCD coupled to AuNS. Reprinted (adapted) with permission from Wang W, Satyavolu NSR, Wu Z, et al. Near-Infrared Photothermally Activated DNAzyme–Gold Nanoshells for Imaging Metal Ions in Living Cells. *Angew Chem. Int. Ed.* 2017, 56, 6798–6802. Copyright 2017 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim. (B) Sodium ion detection using Na43, photocaged substrate and CHA. Reprinted (adapted) with permission from Wu Z, Fan H, Satyavolu NSR, et al. Imaging Endogenous Metal Ions in Living Cells Using a DNAzyme–Catalytic Hairpin Assembly Probe. *Angew Chem. Int Ed.* 2017, 56, 8721–8725. Copyright 2017 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim. (C) Mg^{2+} detection using 10–23 and a homing endonuclease I-SceI. Reprinted (adapted) with permission from Lin Y, Yang Z, Lake RJ et al. Enzyme-Mediated Endogenous and Bioorthogonal Control of a DNAzyme Fluorescent Sensor for Imaging Metal Ions in Living Cells. *Angew Chem. Int. Ed.* 2019, 58, 17061–17067. Copyright 2019 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim. (D) Detection of metal ions through genetically encoded fluorescent sensors controlled by a metal-ion dependent RCD. Reprinted (adapted) with permission from Xiong M, Yang Z, Lake RJ, et al. DNAzyme-Mediated Genetically Encoded Sensors for Ratiometric Imaging of Metal Ions in Living Cells. *Angew Chem. Int. Ed.* 2020, 59, 1891-1896. Copyright 2020 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

biosensor is controlled in an endogenous and bioorthogonal manner, by harnessing the properties of a homing endonuclease, namely I-SceI. The principle upon which bioorthogonal control can be achieved is the infrequency of I-SceI substrates. In fact, the substrate recognition sequence has been reported to occur by chance only once in 7×10^{10} base pairs within genetic material, making it very unlikely to interact with the intracellular genome and have detrimental effects (Lin et al., 2019). The 10–23 DNAzyme was used for this study and was modified to contain the 18-base pair substrate recognition sequence of I-SceI. The attachment of the recognition sequence rendered the DNAzyme nonfunctional by prohibiting it from adopting its active conformation. However, upon cleavage by the homing endonuclease, the cis-acting DNAzyme is released, and is free to adopt its catalytically active conformation. In the presence of Mg^{2+}, the DNAzyme proceeds to self-cleave, generating a detectable fluorescent signal, thus enabling intracellular Mg^{2+} imaging.

An additional elegant study is Lu and Zhang's work on ratiometric imaging of metal ions through genetically encoded sensors, all of which is mediated through a DNAzyme-target interaction (Fig. 6D) (Xiong et al., 2020). The ratiometric signal in this biosensing platform is provided by DNAzyme-controlled expression of two genetically encoded fluorescent proteins (FPs), Clover2 and Ruby2. To initialize the biosensing cascade, plasmids containing encoded sequences for Clover2 and Ruby2 as well as a metal-ion dependent RCD are transfected into a cell. The DNAzyme is chosen such that it is a trans acting DNAzyme, which adopts its catalytically active form in the presence of its specific metal-ion cofactor (the metal ion of interest). Following plasmid transfection, the FP sequences are transcribed into mRNA, which act as the substrates for the RCD. As such, FP expression is subject to mRNA cleavage and degradation by the DNAzyme. Additionally, the DNAzyme is designed to only hybridize with the Clover2 mRNA, meaning Ruby2 mRNA would not be subject to DNAzyme cleavage. Thus, in the absence of target, both Clover2 and Ruby2 are expressed in relatively equal amounts, leading to a relative expression ratio of one. In the presence of the metal ion of interest, the DNAzyme adopts its catalytically active conformation, leading to the cleavage of Clover2 mRNA, limiting its expression. Subsequently, the Clover2 to Ruby2 expression ratio is proportionately lower. The success of this biosensing platform was demonstrated with both the Mg^{2+}-activated 10–23 DNAzyme and the Zn^{2+}-specific 8–17 DNAzyme.

3.5. Detection of nucleic acid targets inside cells using RCDs

Although intracellular sensing is dominated by metal ion detection, efforts have also been made in the realm of RNA sensing in living cells. The Le group has described an RCD-AuNP based molecular motor for miRNA detection in cancer cells (Fig. 7A) (Peng et al., 2017). The AuNPs were decorated with many substrate strands (DNA tracks) and a few 8–17 molecules locked by a complementary DNA sequence. Intracellular binding of a target miRNA frees the DNAzyme to cleave its substrates on the AuNP one after another, generating a progressively enhanced fluorescence signal.

Katz, Kolpashchikov, Minko and colleagues also developed an interesting approach for detecting specific RNA molecules inside cells using a split DNAzyme approach (Fig. 7B) (Bakshi et al., 2017). The two DNAzyme halves, named DZa and DZb, were designed to hybridize to a specific mRNA sequence. Doing so permitted them to come together in close enough proximity to form the trans-acting catalytically active core, resulting in enzymatic activity and fluorescence emission. A fascinating feature of this biosensor is the magnetic field dependence, which enables temporal control over the biosensing system. This is achieved by employing two distinct species of magnetic beads (MBs), and conjugating them to two components of the biosensor, specifically the DNAzyme and its substrate (named F-sub in Fig. 7B). Cleavage of the substrate by the DNAzyme can only occur when the MB-conjugated substrate strand is present within the cell *and* upon application of a magnetic field designed to bring the two bead species together. Thus, this design is able to confer temporal control over the biosensing system by allowing components to diffuse within the cell and controlling the timing of DNAzyme and substrate strand interaction, in effect controlling the timing of signal generation.

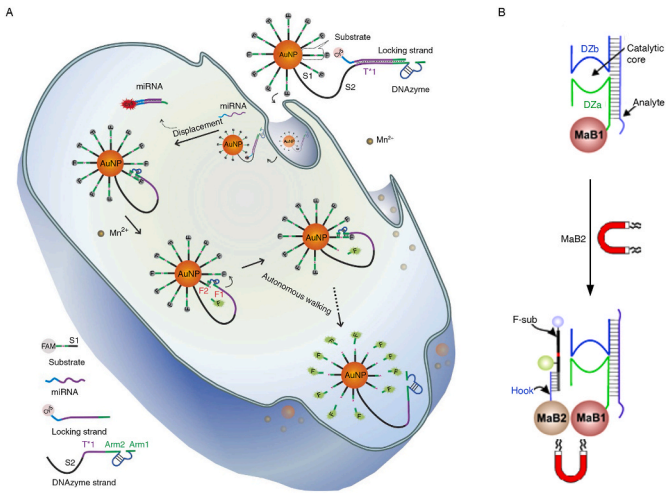

Fig. 7. Detection of nucleic acid targets inside cells using RCDs. (A) Intracellular miRNA detection using an RCD walker on AuNPs. Reprinted (adapted) with permission from Peng H, Li XF, Zhang H, Le XC. A microRNA-initiated DNAzyme motor operating in living cells. *Nat Commun.* 2017, 8, 14378. Copyright 2017 Peng et al. (B) A split DNAzyme approach. Reprinted (adapted) with permission from Bakshi SF, Guz N, Zakharchenko A, et al. Magnetic Field-Activated Sensing of mRNA in Living Cells. *J Am Chem Soc.* 2017, 139, 12117–12120. Copyright 2017 American Chemical Society.

Though DNAzymes have demonstrated significant potential for intracellular biosensing, a particular challenge faced is the active delivery of DNAzymes to specific locations and/or sub-cellular structures in living systems. More innovations are required to further broaden applications of DNAzymes in this area.

3.6. Deriving RCDs activated by bacterial targets

Infections by pathogenic bacteria are a major threat to public health as they cause many costly outbreaks around the world each and every year, making rapid and facile detection of pathogenic bacteria highly important. Several research groups in North America, including ours, have been interested in examining RCDs as a platform to achieve the detection of specific bacteria. To date, RCDs have been developed for *E. coli* (Ali et al., 2011), *Clostridium difficile* (Shen et al., 2016), *Helicobacter pylori* (Ali et al., 2019), *Klebsiella pneumoniae* (Ali et al., 2019), *Vibrio anguillarum* (Gu et al., 2019), and *Legionella pneumophila* (Rothenbroker et al., 2020). In this section we will discuss the selection and properties of these RCDs.

Bacteria-responsive RCDs have been selected using a common *in vitro* selection strategy that involves the use of a complex mixture derived from a specific bacterium as the positive selection target and a similar mixture from control bacteria as the counter selection target. The Li group pioneered this technology in a study published in 2011 in which they derived an RCD named RFD-EC1. The RNA-cleaving Fluorogenic DNAzyme (RFD) library used for selection utilized sequences with a ribonucleotide (R in Fig. 8A), flanked by two nucleotides modified with a fluorophore and a quencher (F and Q in Fig. 8A and B), that can be specifically activated by a target present in *E. coli* (Ali et al., 2011). The cleavage reaction separates the fluorophore and quencher, and thus generates a fluorescence signal that can be used for biosensor development. RFD-EC1 was selected using a subtractive selection method, wherein negative selection was first done using the crude extracellular mixture (CEM) of a non-target bacterium, in this case *Bacillus subtilis*

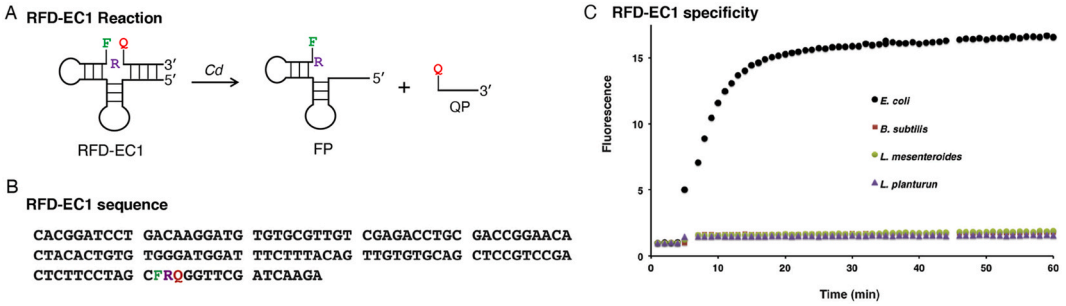

Fig. 8. RFD-EC1, an RCD that can be specifically activated by *E. coli*. (A) Schematic reaction of RFD-EC1. (B) The sequence of RFD-EC1. (C) RFD-EC1 specificity measured by the fluorescence enhancement upon incubation with *E. coli* and the named control Bacteria. Reprinted from Zhang W, Feng Q, Chang D, et al. In vitro selection of RNA-cleaving DNAzymes for bacterial detection. *Methods*. 2016, 106, 66–75, Copyright 2016, with permission from Elsevier.

(CEM-BS) to remove non-catalytic sequences (no cleavage), followed by positive selection using CEM from *E. coli* (CEM-EC) to identify catalytic sequences that cleaved the ribonucleotide linkage. After several repeated rounds of selection, a highly selective RFD was obtained that showed cleavage when exposed to *E. coli*, but no cleavage with control bacteria such as *Bacillus subtilis*, *Leuconostoc mesenteroides*, and *Lactobacillus planturum* (Fig. 8C) (Zhang et al., 2016).

The Li group reported another bacteria-responsive RCD, denoted RFD-CD1, which was not only species-specific (as it does not recognize other species of bacteria) but also strain-specific (Shen et al., 2016). RFD-CD1 was selected after many rounds of counter-selection against the combined CEM from *E. coli*, *B. subtilis*, CD630 (a non-BI/027 strain of *C. difficile*), as well as positive-selection with the CEM from the antibiotic-resistant BI/027-H strain of *C. difficile*. Not surprisingly, this RCD was only active with the BI/027 strain of *C. difficile*.

The Brennan and Li groups worked together to carry out an *in vitro* selection experiment to isolate RCDs that could be specifically activated by *H. pylori*, a key pathogen responsible for gastric carcinoma, gastric and duodenal ulcers (Ali et al., 2019). A highly specific RFD, denoted DHp3T4, was obtained after many rounds of counter-selection with the CEM from 5 different bacteria (*E. coli*, *C. difficile*, *Salmonella typhimurium*, *B. subtilis*, and *Listeria monocytogenes*) and the CEM from *H. pylori* as the positive-selection target. This DNAzyme has also been incorporated into a paper-based sensor for *H. pylori* detection, which will be discussed in the next section.

In a most recent study published by Li, McConnell and colleagues, a new RCD named LP1 was isolated for *L. pneumophila,* a deadly bacterial pathogen that has caused numerous Legionnaires' disease outbreaks (Rothenbroker et al., 2020). The ultimate goal of this work was to use the derived RCD to monitor *L. pneumophila* in cooling tower water. In this case it was highly desirable to have an RCD that could detect different strains of *L. pneumophila* so its application would not be restricted by geographical location. For this consideration, *L. pneumophila* serogroups 1, 2, and 3 were used for positive selection. Two other bacteria, *Pseudomonas aeruginosa* and *K. pneumoniae*, were used as the control bacteria for the counter-selection, as they often co-exist with *L. pneumophila*. Subsequently, LP1 was assessed against many different bacterial species and 5 different *L. pneumophila* strains. It was found that this DNAzyme could be activated by any of the *L. pneumophila* strains but not by other bacterial species. Additionally, this RCD exhibited a remarkable detection limit of 10 colony forming units (CFU) without signal amplification (Rothenbroker et al., 2020).

Beyond the work described above that has been done in the groups of Li and Brennan, two other studies on the selection of RCDs for bacterial activation have recently been published. In one study, Ali, Zhao and colleagues developed an RCD named KP6 to recognize *K. pneumoniae* (Ali et al., 2019). This DNAzyme is capable of differentiating *K. pneumoniae* from other species of bacteria, but it is not strain-selective. Specifically, KP6 is not able to distinguish between drug-resistant versus drug-susceptible strains of *K. pneumoniae*. In another study, Liu and colleagues derived an RCD, named VAE-2, for the recognition of *Vibrio anguillarum*, a pathogenic marine bacterium. VAE-2 differs from all the RCDs discussed above in the sense it is not an RFD, as it was selected from a DNA library containing a ribonucleotide that was not flanked by fluorophore and quencher labels (Gu et al., 2019).

Preliminary studies to identify the molecular targets that activate each of the 6 RCDs described above demonstrated that the target was a protein, though the identity of the majority of the protein targets has not been deciphered at this moment, except for RFD-CD1. In this case, the target was identified as a truncated version of TcdC (a transcription factor) that is unique to the BI/027 strain of *C. difficile* (Shen et al., 2016). It will be interesting to identify the targets that activate the other RCDs to determine if there are any common features to these RCD-activating species.

3.7. Biosensing assays and devices engineered with bacteria-targeting RCDs

Fluorogenic RCDs can be easily adapted to design fluorescence assays. The most straightforward approach is to simply monitor the change in fluorescence intensity upon target-activated cleavage, which was initially demonstrated by the Li group in a fluorescence assay for *E. coli* detection using RFD-EC1 (Fig. 9A) (Aguirre et al., 2013). The optimized assay could achieve a detection limit of 1000 CFU/mL without a culturing step, and could detect a single CFU following a 4-h bacterial culturing step.

A trans-acting version of RFD-EC1 was also employed by Hong and colleagues to achieve extremely sensitive *E. coli* detection following a short (1 h) culturing step (Zaouri et al., 2019). It was shown that this method was able to detect a single CFU of *E. coli*. The fluorescence assay was also tested with a large number of other species of water-borne bacteria that are known to produce a high rate of false positives in currently available commercial assays. The DNAzyme-culturing assay demonstrated an impressive ability to distinguish between *E. coli* and these interfering bacteria.

Fluorogenic RCDs can also be used to produce fluorescent paper-based sensors for bacterial detection. Our groups have demonstrated this through the creation of an all-in-one paper sensor that is printed with RFD-EC1 and lysozyme trapped within a film composed of pullulan and trehalose (Fig. 9B) (Ali et al., 2017), which confer long-term stability to RFD-EC1. By including lysozyme, the paper sensor does not require additional lysing and sample preparation steps, improving ease of use. In the presence of *E. coli*, the DNAzyme proceeds to self-cleave resulting in fluorescence emission that can be detected using a fluorescence scanner. The printed paper sensor generated a signal readout within 5 min of coming into contact with intact *E. coli* cells (Fig. 9B) and provided a low limit of detection (100 CFU/mL) without the need for sample enrichment or signal amplification (Ali et al., 2017). The KP6 DNAzyme has also been used to create a printed 96-well paper sensor to allow for simultaneous examination of multiple samples for

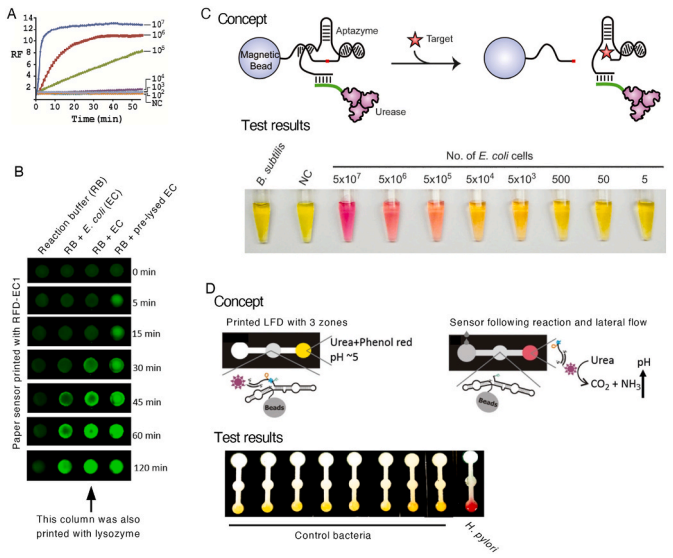

Fig. 9. RCD-based assays and devices for bacterial detection. (A) *E. coli* detection via a fluorescence assay with RFD-EC1. Reprinted (adapted) from Aguirre S, Ali M, Salena B, Li Y. A Sensitive DNA Enzyme-Based Fluorescent Assay for Bacterial Detection. *Biomolecules*. 2013, 3, 563–577, Copyright 2013 Aguirre et al. (B) A fluorescent paper sensor printed with RFD-EC1. Reprinted (adapted) from Ali MM, Brown CL, Jahanshahi-Anbuhi S et al. A Printed Multicomponent Paper Sensor for Bacterial Detection. *Sci Rep*. 2017, 7, 12335, Copyright 2017 Ali et al. (C) A solution-based colorimetric assay for *E. coli* using RFD-EC1/urease conjugates coupled to magnetic beads. Reprinted (adapted) with permission from Tram K, Kanda P, Salena BJ et al. Translating bacterial detection by DNAzymes into a litmus test. *Angew Chem Int Ed.* 2014, 53, 12799-802, Copyright 2014 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim. (D) A paper-based lateral flow device (LFD) printed with agarose beads containing DHp3T4 for *H. pylori* detection. Reprinted (adapted) with permission from Ali MM, Wolfe M, Tram K et al. A DNAzyme-Based Colorimetric Paper Sensor for *Helicobacter pylori*. *Angew Chem Int Ed*. 2019, 58, 9907–9911, Copyright 2019 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

K. pneumoniae (Ali et al., 2019). It is noteworthy that the pullulan film formation in these paper sensors could significantly extend their longevity, as shown by consistent target detection with the paper sensor after 6 months of storage at room temperature. The enhanced RNA preservation is due to the decreased spontaneous degradation of RNA and protection of RNA against digestion by RNases (Hsieh et al., 2017).

While the examples provided above utilize a fluorescence output, it is often desirable to have a simple colorimetric output that can be read by eye. Two examples published by our groups have demonstrated that release of urease from an RCD can be used for this purpose. The first example utilized RFD-EC1 to detect *E. coli* in solution (Fig. 9C) (Tram et al., 2014), and the second used DHp3T4 for *H. pylori* detection in a paper-sensor format (Fig. 9D) (Ali et al., 2019). In both cases, cleavage of the DNAzyme by the relevant bacteria released a DNA strand carrying urease. After separation, using either magnetic beads (for *E. coli* detection; Fig. 9C), or flow along paper (for *H. pylori* detection, Fig. 9D), the colorimetric signal was generated through the hydrolysis of urea by urease, raising the pH of the reporting solution, and ultimately resulting in a color change in a phenol red indicator from yellow to red. The RCD in these examples was immobilized on either magnetic beads (for *E. coli* detection) or agarose beads (for *H. pylori* detection) and bound to a urease molecule at the 5′-end of the RCD (distal to the cleavage site). This enabled the separation of the urease-containing fragment from the RCD segment attached to the bead in the presence of the bacterium. The *E. coli* sensor was able to detect 5000 CFU/mL, and in the case of the HP sensor, it was possible to detect *H. pylori* directly in stool samples with no sample pretreatment, and with a high degree of sensitivity (10^4 CFU/mL).

The Li, Brennan and Liu groups have investigated micrometer-sized functional nucleic acid superstructures, denoted as 3D DNA, for making paper sensors (Fig. 10A) (Liu et al., 2018b). 3D DNA with a diameter of over 1 μm was produced by RCA in the presence of high salt concentrations, and contained repeating sequences of RFD-EC1. These very large DNA structures, when printed onto cellulose papers, are physically absorbed by the paper surface and become immobilized (Carrasquilla et al., 2015). These RCD-containing 3D DNA structures showed great resistance to degradation by nucleases, significantly reduced nonspecific protein adsorption, and a much higher surface loading density (in comparison to the monomeric DNAzyme). These properties make 3D DNA ideally suited for development of paper-based biosensors.

The groups of Li and Filipe have recently published another RCD-utilizing biosensing system involving cis-acting bacteria-responsive RCDs attached to agarose beads and a paper strip printed with a capture DNA probe designed to capture the fluorophore-carrying cleavage product. The idea is to use the paper strip, incubated with the bead-bound RCDs in the target-containing test sample, to capture the cleavage product and concentrate it onto a smaller predetermined surface area to increase the detection sensitivity (Samani et al., 2020). Using RFD-EC1, the system is capable of detecting *E. coli* down to 10 CFU/mL. A 2-plex assay has also been designed for the detection of both *E. coli* and *K. pneumoniae* with the use of EC1 and KP6; the cleavage product from each RCD is captured by the matching DNA probe printed onto a circular zone in a paper strip (Fig. 10B). In addition to fluorescence-based detection, a colorimetric detection assay using the urease-facilitated litmus test described above has also been implemented (Samani et al., 2020).

Lastly, Filipe, Didar and colleagues designed a food wrap biosensor, intended to be included in food packaging to offer real-time assessment of the presence of bacterial contamination in various packaged perishable foods (Yousefi et al., 2018). Once again, the MRE is the cis-acting RFD-EC1, combining the task of molecular recognition and signal generation. The DNAzyme is covalently linked to a cyclo-olefin polymer, which provides multiple advantages to the biosensor, including stability, transparency, and flexibility. Upon target binding, the RCD proceeds to self-cleave, releasing the quencher-containing segment, leading to fluorescence emission. The amount of fluorescence can be detected using a fluorescence scanner. Biosensors of this type can be readily implemented in food packaging for monitoring the presence of bacteria in food.

3.8. *Amplified biosensing assays with bacteria-targeting RCDs*

The ability to detect extremely low concentrations of bacterial cells is particularly important for early diagnosis of disease. To do so, it is often necessary to incorporate signal amplification strategies. Given RCDs are composed of DNA, DNA amplification can be used to achieve signal amplification. In Section 3a, we discussed two examples of using RFD-EC1 and CHA to achieve sensitive detection of *E. coli*. Here we will discuss two other strategies demonstrated by our groups where RCA is exploited to amplify the cleavage event of RFD-EC1 for *E. coli* detection. The first study used a DNA catenane made of two topologically linked single-stranded DNA rings connected through a strong linking duplex (Fig. 11A) (Liu et al., 2016b). This design ensures the inability of either component ring to trigger RCA. However, by designing one ring to contain the cleavage site of RFD-EC1, the DNAzyme can linearize the RNA-containing ring, enabling the other ring to act as the template for RCA. This sensing system is capable of detecting *E. coli* down to 10 CFU/mL (Fig. 11B).

Another example is DNAzyme feedback amplification (DFA), a mechanism of cross feedback between RCA and an RCD (M. Liu et al., 2017b). DFA relies on the tenet of producing RCDs via RCA and using these RCDs to produce more DNA primers for RCA. This amplification

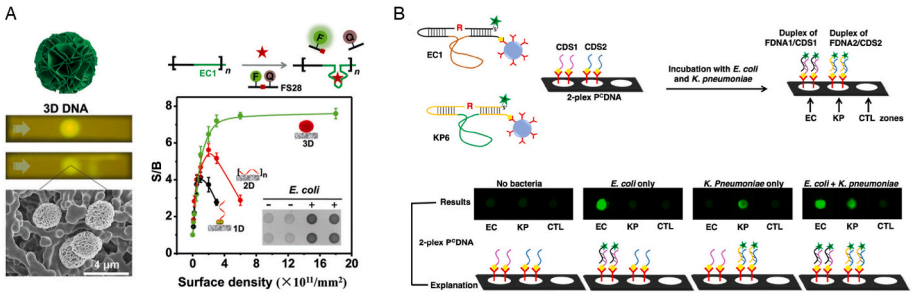

Fig. 10. Two additional RCD-utilizing paper sensors for bacterial detection. (A) *E. coli* detection with a paper sensor printed with 3D DNA containing repetitive units of RFD-EC1. Reprinted (adapted) with permission from Liu M, Zhang Q, Kannan B et al. Self-Assembled Functional DNA Superstructures as High-Density and Versatile Recognition Elements for Printed Paper Sensors. Angew Chem Int Ed Engl. 2018, 57, 12440–12443, Copyright 2018 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim. (B) A 2-plex surface-to-surface product enrichment strategy for the detection of *E. coli* and *K. pneumoniae* with the use of EC1 and KP6 immobilized on agarose beads. Reprinted with permission from Samani SE, Chang D, McConnell EM et al. Highly Sensitive RNA-Cleaving DNAzyme Sensors from Surface-to-Surface Product Enrichment. Chembiochem. 2020, 21, 632–637, Copyright 2020 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim.

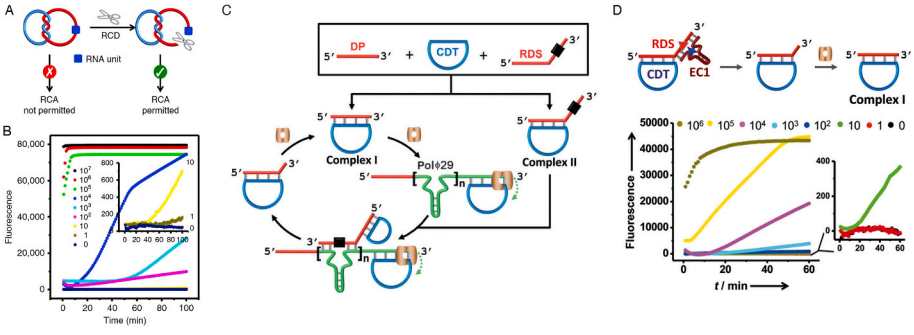

Fig. 11. Amplified bacterial detection using RFD-EC1 and RCA. (A) A 2-ring DNA catenane approach. The blue circle cannot act as the template until linearization of the red ring by the RCD. (B) Monitoring RCA reactions in response of increasing CFU of *E. coli*. Reprinted (adapted) from Liu M, Zhang Q, Li Z et al. Programming a topologically constrained DNA nanostructure into a sensor. *Nat. Commun.* 2016, 7, 12074, Copyright 2016 Liu et al. (C) DNAzyme feedback amplification (DFA). Exponential amplification is achieved here via the cross feedback mechanism between an RCD and RCA as shown. (D) Detection of *E. coli* via DFA. The bottom graph shows fluorescence increase in response of increasing CFU of *E. coli*. Reprinted (adapted) with permission from Liu M, Zhang Q, Chang D et al. A DNAzyme Feedback Amplification Strategy for Biosensing. *Angew Chem. Int. Ed.* 2017, 56, 6142–6146, Copyright 2017 Wiley-VCH, Verlag GmbH & Co. KGaA, Weinheim. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

strategy relies on the preassembly of two DNA complexes, complex I and complex II (Fig. 11C). The first complex consists of a DNA primer (DP) and a circular DNA template (CDT) that contains the RCD antisense sequence. The second complex contains the same circular DNA and the substrate for the RCD. The process begins with RCA over complex I, leading to the production of large numbers of RCDs, which proceed to cleave the substrate in complex II (Fig. 11C). By specifically designing the substrates such that their RCD-mediated cleavage releases more copies of the DNA primer, this method ensures exponential DNA amplification through DNAzyme feedback (Liu et al., 2018a). For *E. coli* detection, RFD-EC1 is used to process the substrate in complex II so that complex I is produced to enable RCA. DFA is able to detect as low as 10 CFU/mL E. *coli* without a culturing step (Fig. 11D).

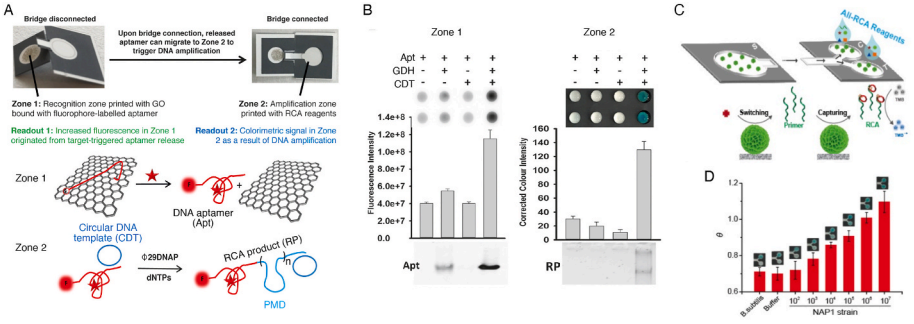

Fig. 12. Paper-based aptamer-PMD sensor for bacterial detection. (A) Detection of bacterial biomarkers using a 2-zone/2-piece paper sensor. (B) Use of the sensor for the detection of GDH (a biomarker of *C. difficile*). Reprinted (adapted) with permission from Hui CY, Liu M, Li Y, Brennan JD. A Paper Sensor Printed with Multifunctional Bio/Nano Materials. *Angew Chem. Int Ed.* 2018, 57, 4549–4553. Copyright 2018 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim. (C) Detection of bacterial biomarkers with a 3-zone, all-printed paper-based analytical device. (D) Detection of NAP1 strain of *C. difficile* using a DNA aptamer for toxin B. Reprinted (adapted) with permission from Liu M, Wang J, Chang Y et al. In Vitro Selection of a DNA Aptamer Targeting Degraded Protein Fragments for Biosensing. Angew Chem Int ED. 2020, 59, 7706–7710. Copyright 2020 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim.

3.9. Other strategies incorporating DNAzymes for bacterial detection

Bacterial detection can also be achieved using an aptamer as the MRE and a PMD as the output. Our groups have developed a 2-piece/2-zone paper sensor where aptamer binding is used to modulate PMD formation through RCA (Fig. 12A) (Hui et al., 2018). In Zone 1, a fluorophore-tagged aptamer is adsorbed by graphene oxide, which quenches the fluorescence. Target binding causes the release of the aptamer from the graphene surface, leading to fluorescence emission. Following a fluorescence read in Zone 1, the connecting bridge can be adjoined, which permits the aptamer to travel to Zone 2. Following 30 s of travel, the bridge is once again disconnected. Zone 2 corresponds to the signal amplification and generation step of this biosensing platform and is printed with pullulan and RCA-enabling components. Signal amplification is achieved through RCA. The primer is provided by the travelling aptamer, which triggers the amplification reaction. Following an hour of reaction time, a colorimetric signal can be detected upon addition of colorimetric assay reaction reagents. The signal is generated by way of mass production of PMD copies via RCA, which adopt their catalytically active conformation in the presence of hemin, H_2O_2 and TMB or ABTS (Fig. 12A). The sensor was validated with an RNA aptamer for ATP and a DNA aptamer for glutamate dehydrogenase (GDH, a biomarker specific for *C. difficile;* Fig. 12B).

Very recently, our groups, in collaboration with the Liu Lab, have developed an all-printed paper-based analytical device to detect pathogenic strains of *C. difficile* (M. Liu et al., 2020). The sensor is a 2-piece origami system (Fig. 12C). The first piece contains a sample zone (S zone) and the second piece is patterned with a bridge that connects with a test zone (T zone) and a control zone (C zone). The S zone is printed with a 3D DNA made of a DNA aptamer and a short DNA that binds the aptamer domain; the binding of the target to the aptamers in this zone will release the short DNA, which will migrate to the T zone to serve as the primer for RCA. The T zone is printed with a 3D DNA as the capture agent to bind the short DNA released from the S zone as well as the reagents for RCA. The C zone is printed with a different 3D DNA as well as all the reagents needed for RCA, including the DNA primer for RCA, which can be activated by Mg^{2+} migrated from the S zone. The RCA products at both T and C zones are designed to contain repetitive units of a PMD for producing colorimetric readouts. This sensor has been successfully applied for the detection of the NAP1 strain of *C. difficile* (Fig. 12D), with the use of a newly made DNA aptamer that recognizes toxin B, a biomarker expressed by all the infectious strains of *C. difficile*.

DNAzymes have also been used for bacterial typing via the detection of PCR products of specific bacterial genes. For example, Gerasimova, Rohde and colleagues were able to create a biosensing platform with a choice of fluorescent or colorimetric signal readout for the specific differentiation between tuberculous and nontuberculous mycobacteria (Wood et al., 2019). The core of the biosensor relies on a split trans-acting DNAzyme, which is able to assemble in response to a specific PCR product and catalyze the cleavage of a substrate strand. The substrate strand can be designed to contain the cleavage site between a fluorophore and quencher pair for fluorescence output or can contain a guanine-rich sequence which upon cleavage can adopt the PMD structure for colorimetric signal generation. The same team also created a similar fluorescence sensor to identify drug resistant strains of *Mycobacterium tuberculosis* (Bengtson et al., 2017). The core of the sensor is nearly identical to the example described above, employing a split RCD for simultaneous molecular recognition of strain-specific single nucleotide polymorphisms (SNPs) and signal transduction by cleaving a fluorogenic substrate.

3.10. Biosensing applications that incorporate DNAzymes and point-of-care (POC) meters

The ramifications of the success of DNAzymes in diagnostics are reflected by their incorporation in point-of-care (POC) applications, completing the bench-to-bedside journey. Lu and colleagues recently provided a comprehensive review of the transformation of laboratory-based diagnostic assays to POC diagnostics, placing large emphasis on widening the application of existing POC meters (Zhang et al., 2020). POC meters highlighted by Lu and colleagues include the glucose meter, pH meter, thermometer, and pressure meter (Zhang et al., 2020).

To date, repurposing the glucose meter has found the greatest success with DNAzymes, as evidenced by several published examples (Xiang and Lu, 2011; Su et al., 2012; Zhang et al., 2015; Ming et al., 2017; Zhang and Lu, 2018). The crucial principle in this reengineered device is overcoming the fidelity of the sensor signaling pathway which is designed to be triggered solely by the presence of glucose in the analyzed sample (Zhang et al., 2020). This feat was most commonly and successfully achieved by coupling a DNAzyme to a glucose-generating enzyme such as invertase or glucoamylase (Zhang et al., 2020). The DNAzyme is designed to take the role of a MRE specific for a new target, such as a protein biomarker or miRNA. By coupling the DNAzyme to the glucose generating enzyme, molecular recognition of the desired target induces the previously inactive enzyme to produce glucose from a readily available precursor like sucrose. The accumulation of glucose in the solution can be designed to correlate with the amount of principal target present, resulting in a user-friendly signal read-out.

Another similar POC platform which has seen successful incorporation of DNAzyme to broaden its analyte detection range are pregnancy test strips (PTS). Through the use of a one-step competitive displacement reaction mediated by DNAzymes or aptamers, Du and colleagues have demonstrated the ability to repurpose PTSs to detect non-human chorionic gonadotropin analytes like protein and nucleic acid biomarkers (Zhang et al., 2019).

Further research into testing real clinical samples and conducting clinical trials with suitable POC platforms is required to make these platforms useful in the hands of clinicians. This type of research is strongly encouraged.

4. Perspectives and future directions

Since their discovery, DNAzyme research has made major strides in clinical and non-clinical applications, specifically in the area of biosensing. Within this realm, it is evident that metal ion biosensing has been explored to greater lengths than biomolecule sensing for human health. As such, future research should focus on broadening the application of DNAzyme based biosensors to more clinical pathologies, beginning with adding to the growing number of DNAzymes for human pathology-derived targets. The relatively low number of DNAzymes selected for clinical pathologies also explains the propensity to employ PMDs in human health biosensors to exploit their signal generation capabilities. Given the degree of precision in molecular detection which can be achieved with iterative rounds of *in vitro* selection to obtain DNAzymes carrying high sensitivity and selectivity, adding to the growing library of RCDs capable of detecting biological molecules linked to human health will allow the broadening of their use as MREs and signal transducers for diagnostics and therapeutics. Compared to clinical applications from non-North American countries, North American contributions to clinical biomarker biosensing is comparatively limited. Given the versatility, sensitivity, elegance, and stability of DNAzyme-based biosensing platforms, researchers are urged to collaborate with clinicians to identify avenues to explore in terms of clinical applications, potential new targets for *in vitro* selection of DNAzymes, and patient sample acquisition for enhanced testing and sensor validation.

Further progression in DNAzyme based applications require more in-depth knowledge of the structures and catalytic mechanisms of DNAzymes so that we can make better DNAzymes or understand their limitations. Significant progress is being made in this area, exemplified by two studies where X-ray crystallography was used to determine DNAzyme structures. The groups of Höbartner and Pena reported the first crystal structure of a DNAzyme (the RNA-ligating 9DB1 DNAzyme) in

2016 (Ponce-Salvatierra et al., 2016) and their study not only confirms that DNA could form complex tertiary structures, but also suggests that the missing 2′-hydroxyl from the backbone may afford increased variability for possible tertiary conformations compared to RNA (Ponce--Salvatierra et al., 2016). The crystal structure of 8–17 has also been determined (H. Liu et al., 2017). In this work, three distinct structures allowed the research team to glean information about the pre-catalytic state and Pb^{2+}-accelerated catalysis, as well as important structural information about key nucleotides of the DNAzyme. Structural and mechanistic approaches for understanding DNAzyme catalysis have been described by Scott Silverman (2016) as well as by Cepeda-Plaza and Peracchi who have summarized the accomplishments and remaining challenges of structural investigation with the 8–17 DNAzyme as the most well-studied example (Cepeda-Plaza and Peracchi, 2020). Understanding the structure and mechanism of action of DNAzyme catalysis affords information that is important for the selection of novel DNAzymes as well as for biosensor design. The reaction conditions and kinetics of catalysis can more easily be optimized when the essential nucleotides and structure of a DNAzyme are known. Non-essential nucleotides can oftentimes be altered judiciously for biosensor design or application. Finally, signal to noise ratio can be decreased as a result of mechanistic optimization. Further attention towards probing the structure and mechanism of action of other DNAzymes is encouraged.

CRediT authorship contribution statement

Ioana Cozma: Conceptualization, Writing - original draft, Writing - review & editing. **Erin M. McConnell:** Conceptualization, Writing - original draft. **John D. Brennan:** Conceptualization, Writing - review & editing. **Yingfu Li:** Conceptualization, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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