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Review

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DNAzyme-Mediated Assays for Amplified Detection of Nucleic Acids and Proteins

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The discovery of RNA enzymes (ribozymes) has inspired the exploration of using DNA molecules for biological catalysis. Although natural DNA enzymes (DNAzymes) have not been found, synthetic DNAzymes have been identified using an *in vitro* selection technology.[1] DNAzymes with desirable catalytic functions can be obtained from a random-sequence DNA library after repeated rounds of selective enrichment. Since the first DNAzyme was reported by Breaker and Joyce in 1994,[2] hundreds of DNAzyme sequences have been identified and these DNAzymes have various catalytic activities.[3−5]

DNAzymes have several practical advantages when compared to protein enzymes and ribozymes and thus have been extensively used for developing analytical techniques.[6−18] For example, DNAzymes are more chemically and thermally stable than protein enzymes and ribozymes, which makes DNAzymes suitable for point-of-care testing and on-site analysis. DNAzymes are amenable to various DNA amplification techniques, which is important for improving the sensitivity of assays. Advances in chemical synthesis of DNA drastically reduce the cost of the production of DNAzymes as compared to the generation of proteins. DNAzymes can be readily conjugated to various fluorescent tags, functional molecules, and solid surfaces, allowing the adaption of DNAzymes into diverse detection formats. In addition to biosensing, DNAzymes have been used for other applications, such as the *in vivo* degradation of mRNA[19,20] and the construction of logic gates[11−14] and DNA nanomachines.[11,14,21]

The catalytic activity of RNA-cleaving DNAzymes generally requires metal ions as cofactors,[6] such as Pb^{2+}, Zn^{2+}, Mg^{2+}, Mn^{2+}, Ca^{2+}, Cu^{2+}, UO^{2+}, and Na^{+}. Therefore, many DNAzyme-based sensors have been developed for the detection of these metal ions and have been summarized in several comprehensive reviews.[7,10,15,22,23] More recently, increasing efforts have been devoted to developing DNAzyme-based assays for the amplified detection of nucleic acids and proteins. This review highlights the principles and various strategies for the development of these assays. These assays commonly use two classes of DNAzymes, peroxidase-mimicking DNAzymes and RNA-cleaving DNAzymes, because of their appealing catalytic activities and well-characterized structures.

STRUCTURES AND FUNCTIONS OF PEROXIDASE-MIMICKING DNAZYMES AND RNA-CLEAVING DNAZYMES

Peroxidase-Mimicking DNAzymes. Peroxidase-mimicking DNAzymes are a group of guanine-rich DNA sequences that form G-quadruplexes and exhibit peroxidase-like activity after binding to hemin (Figure 1).[24−26] A G-quadruplex can be

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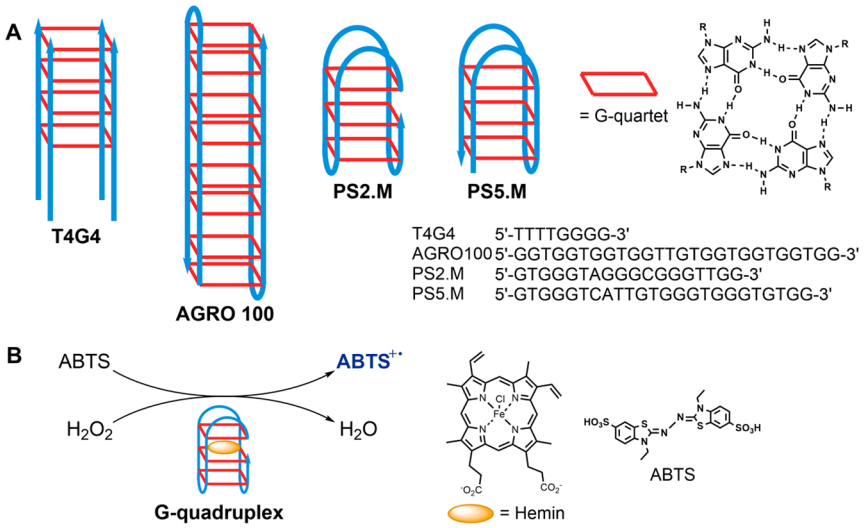


Figure 1. Representative peroxidase-mimicking DNAzymes. (A) The G-quadruplex structures and sequences of T4G4, AGRO100, PS2.M, and PS5.M.[26,31] Reproduced from Travascio, P.; Witting, P. K.; Mauk, A. G.; Sen, D. *J. Am. Chem. Soc.* **2001**, *123*, 1337−1348 (ref 26). Copyright 2011 American Chemical Society. Reproduced from G-quadruplex aptamers with peroxidase-like DNAzyme functions: Which is the best and how does it work? Li, T.; Dong, S. J.; Wang, E. K. *Chem.-Asia J.*, Vol. *4*, Issue 6 (ref 31). Copyright 2009 Wiley. (B) Oxidation of ABTS by H_2O_2 to $ABTS^{+\bullet}$, a reaction catalyzed by G-quadruplex DNAzyme.

formed by one, two, or four DNA strands, although unimolecular G-quadruplexes are most frequently used. The presence of K^+ is vital for stabilizing the structure of G-quadruplexes;[24,26] however, NH_4^+ can replace K^+ without deteriorating enzymatic activity.[27] G-quadruplexes can have parallel, antiparallel, or mixed structures, which differ in hemin-binding affinities and peroxidase-like activities.[27−30] Parallel and mixed structures have stronger binding affinity to hemin and higher enzymatic activity than antiparallel structures.[27] The observed rate constants (k_{obs}) of DNAzymes containing mixed and parallel G-quadruplex structures, PS2.M and EAD ($C(TG_3)_4A$), were 1.11 ± 0.011 s^{-1} and 3.62 ± 0.28 s^{-1}, respectively, whereas the k_{obs} was only 0.045 ± 0.009 s^{-1} for a DNAzyme containing an antiparallel G-quadruplex structure, a thrombin aptamer.[28]

Like horseradish peroxidase (HRP), peroxidase-mimicking DNAzymes can catalyze the oxidation of ABTS [2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid] by H_2O_2 to $ABTS^{\bullet+}$. The resulting color change is commonly used for colorimetric detection. The peroxidase activity of peroxidase-mimicking DNAzymes depends on the concentration of H_2O_2, but unlike HRP, it does not depend on the concentration of ABTS.[31] This implies that the transfer of oxygen from H_2O_2 to Fe(III) in hemin is a rate-limiting step of the DNAzyme-catalyzed ABTS oxidation. Luminol and tetramethylbenzidine (TMB) are also commonly used as substrates of HRP and peroxidase-mimicking DNAzymes for generating chemiluminescence (Luminol) and colorimetric (TMB) signals.

RNA-Cleaving DNAzymes. RNA-cleaving DNAzymes can catalyze the cleavage of RNA substrates or ribonucleotides embedded in a chimeric DNA substrate.[2,32,33] Three RNA-cleaving DNAzymes, the 8-17, 10-23, and Mg^{2+}-dependent DNAzymes, are widely used for biosensing because of their small catalytic cores and robust activities.[32,33] The numerical names 8-17 and 10-23 do not mean the size of the DNAzymes but instead reflect the experimental procedures from which these DNAzymes were selected.[32] Specifically, 8-17 represents the 17th clone in the eighth round of DNAzyme selection experiments, and 10-23 represents the 23rd clone in the 10th round.[32]

The structures of the 8-17, 10-23, and Mg^{2+}-dependent DNAzymes contain a three-way junction comprising of a catalytic core flanked by two binding arms (Figure 2). The catalytic core is responsible for the cleavage of RNA substrates, whereas the binding arms are used to hybridize to substrates through Watson−Crick base pairing. The sequences of the binding arms in these DNAzymes have little effect on the catalytic activity.[32,33] The cleavage site of the substrate is a dinucleotide junction located between the two binding arm domains within the substrate. The 10-23 DNAzyme can cleave all purine-pyrimidine junctions (denoted as R-Y in Figure 2A), whereas the 8-17 DNAzyme is more restrictive and can only cleave an N-G junction.[32,34] The cleavage mechanism involves attacking of the adjacent phosphodiester linkage by the 2′-hydroxyl group of the upstream nucleotide within the cleavage junction (Figure 2E). Two cleavage products are formed, one containing a 2′,3′-cyclic phosphate and the other containing a 5′-hydroxyl group.[35]

The 8-17 and 10-23 DNAzymes exhibit higher catalytic activities compared to other RNA-cleaving DNAzymes and the hairpin and hammerhead ribozymes.[32] DNAzymes with higher catalytic activities in DNAzyme-based assays are desirable as they can achieve highly sensitive detections in a reasonably short time. Several 8-17 variants have been reported, which show different catalytic activities and metal ion dependencies.[34,36−40] Lu and co-workers[41,42] selected a Pb^{2+}-dependent 8-17 variant, named 17E. They reported an observed rate constant (k_{obs}) of 5.75 min^{-1} in the buffer (pH = 6.0) containing 100 μM Pb^{2+}. Li and co-workers compared the activity of different 8-17 variants and found the largest k_{obs} (∼10 min^{-1}) from an 8-17NG variant that cleaved the G−G junction.[43] The 10-23 DNAzyme exhibited the highest activity (k_{obs} = ∼10 min^{-1}) in the presence of Mn^{2+}, although this DNAzyme was initially selected by using Mg^{2+} as the cofactor.[35] The activity of Mg^{2+}-dependent DNAzyme (E6) is much lower than those of the 8-17 and 10-23 DNAzymes. The k_{obs} of E6 is

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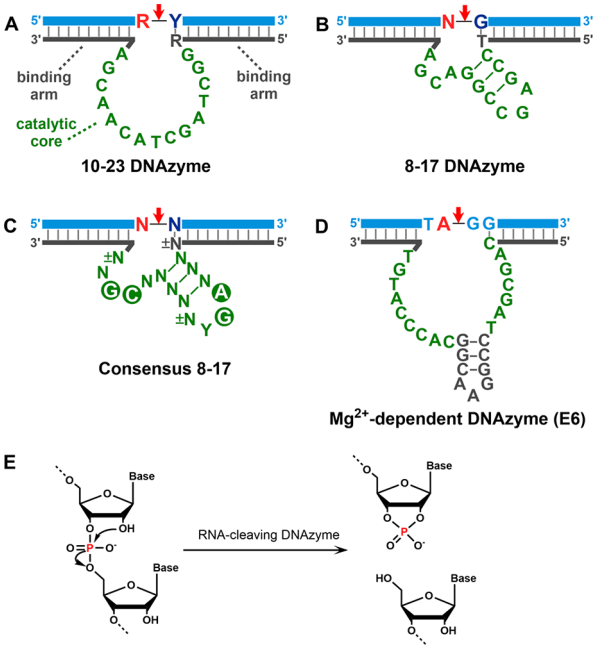


Figure 2. Representative RNA-cleaving DNAzymes, including the 10-23 DNAzyme (A),[32] the classic 8-17 DNAzyme (B),[32] the consensus 8-17 DNAzyme (C),[6] and the Mg^{2+}-dependent DNAzyme E6 (D).[33] The red arrows indicate the cleavage site in the substrate strand. Reproduced with permission from Santoro, S. W.; Joyce, G. F. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 4262−4266 (ref 32). Reprinted from *Chem. Biol.*, Vol. 2, Breaker, R. R.; Joyce, G. F.; A DNA enzyme with $Mg^{(2+)}$-dependent RNA phosphoesterase activity, pp 655−660 (ref 33). Copyright 1995, with permission from Elsevier. (E) RNA cleavage reaction catalyzed by a RNA-cleaving DNAzyme.[6,35] Reproduced from Liu, M.; Chang, D.; Li, Y. *Acc. Chem. Res.* **2017**, *50*, 2273−2283 (ref 6). Copyright 2017 American Chemical Society. Reproduced from Santoro, S. W.; Joyce, G. F. *Biochemistry* **1998**, *37*, 13330−13342 (ref 35). Copyright 1998 American Chemical Society.

only 0.039 min^{-1} in HEPES buffer (pH = 7.0) containing 10 mM Mg^{2+} ion.[33]

The sequences of the binding arms in the 8-17, 10-23, and Mg^{2+}-dependent DNAzymes do not affect the catalytic activity of these DNAzymes. Therefore, these RNA-cleaving DNAzymes can be used to cleave various substrates by designing the sequences of the binding arms to complement with those of the substrates of interest. This programmable feature allows these DNAzymes to be readily adapted into the various design configurations without affecting the catalytic activity.

AMPLIFIED DETECTION OF NUCLEIC ACIDS AND PROTEINS USING PEROXIDASE-MIMICKING DNAZYMES

In the past decade, there has been an explosion in the use of peroxidase-mimicking DNAzyme to detect various analytes, including nucleic acids, proteins, metals, and small metabolites.[9,44−48] In this section, we focus on various strategies using peroxidase-mimicking DNAzymes as the signal transducer for the detection of nucleic acids and proteins. We organize these strategies by addressing the intrinsic properties of DNAzymes as both enzymes (catalytic) and DNA (amplifiable).

Direct Use of Peroxidase-Mimicking DNAzymes as Signal Transducer. Peroxidase-mimicking DNAzymes can be conjugated to affinity ligands (e.g., antibodies) and used directly as signal transducers.[49−52] For nucleic acid analysis, the DNA probe that recognizes the target nucleic acid sequence can be simply extended to include the peroxidase-mimicking DNAzyme sequence. Peroxidase-mimicking DNAzymes have been used to generate colorimetric,[53−55] chemiluminescence,[56] fluorescence,[57] chemiluminescence resonance energy transfer (CRET),[58−60] and electrochemical signals.[61,62]

A sandwich binding format has been combined with the use of peroxidase-mimicking DNAzymes for the detection of nucleic acids and proteins (Figure 3A).[62−66] The peroxidase-mimicking DNAzyme sequence was added to the 5′ or 3′ end of either a DNA probe for nucleic acid detection or an aptamer for protein detection. Target molecules were first captured by an immobilized probe, and the DNAzyme-containing DNA probes or aptamers were then used to bind to the captured target, forming a sandwich complex. After the unbound DNA probes or aptamers were removed through washing, the substrates were added to generate detection signals. For example, Pavlov et al.[63] used a peroxidase-mimicking DNAzyme to develop a chemiluminescence assay for DNA detection. A capture DNA probe immobilized on an Au-coated glass plate was used to hybridize to one part of the target DNA. The reporter DNA probe labeled with a DNAzyme hybridized to another part of the target DNA. The excess, unbound reporter DNA was removed by washing. Luminol and H_2O_2 were then added. The oxidation of luminol was catalyzed by the peroxidase-mimicking DNAzyme to generate the chemiluminescent signals for detection. The sensitivity of this peroxidase-mimicking DNAzyme-based assay was ∼2 orders of magnitude lower than that of the analogous analysis using HRP.

Use of Peroxidase-Mimicking DNAzyme-Conjugated Nanomaterials as Signal Transducer. Conjugating multiple DNAzyme strands onto nanomaterials for signal transduction further improved detection sensitivity. Nanomaterials, such as gold nanoparticles (AuNPs)[56,61,67,68] and graphene oxide (GO),[67,69] have been commonly used because DNAzymes can be easily conjugated onto the surface of these nanomaterials that have large surface-to-volume ratios.

Functionalization of AuNPs with DNA has been well-studied and widely used for biosensing. Likewise, AuNPs can serve as an effective scaffold for conjugating DNAzymes. Niazov et al. conjugated multiple reporter DNA strands, each containing a peroxidase-mimicking DNAzyme sequence, onto the surface of a 13 nm AuNP (Figure 3B).[56] These functionalized AuNPs were then used for the amplified detection of target DNA and telomerase activity. The target DNA was sandwiched by a capture DNA probe conjugated onto an Au-coated glass plate and a reporter DNA on the AuNP. Upon the addition of luminol and H_2O_2, multiple DNAzyme strands on the AuNP catalyzed the oxidation of luminol by H_2O_2, generating an amplified chemiluminescent signal. As a result of the amplification, greater than 10-fold improvement in sensitivity was achieved as compared to the use of a single DNAzyme. The sensitivity improvement depended on the number of the DNAzyme strands conjugated onto individual AuNPs, but too high a density of DNAzyme strands might not be favorable for the formation of the G-quadruplex structure.

To test the activity of telomerase, Niazov et al. conjugated a primer sequence of the telomerase onto a plate. The telomerase in a HeLa cell extract extended the primer to produce repeating units of telomeres. The telomeres were hybridized to the reporter DNA conjugated on the AuNP for signal generation. This method was able to detect the activity of telomerase extracted from as few as 1000 cells.

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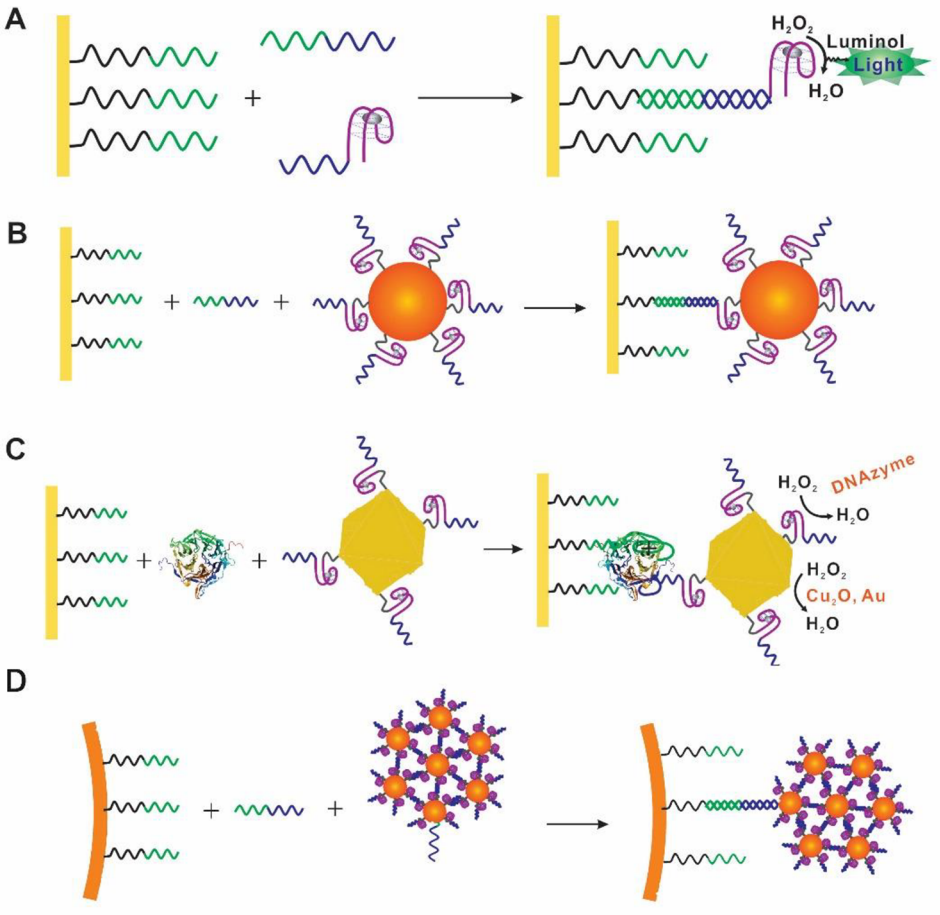


Figure 3. Assays using peroxidase-mimicking DNAzyme and nanomaterials conjugated with multiple peroxidase-mimicking DNAzymes. (A) A typical chemiluminescence assay for DNA detection directly using the peroxidase-mimicking DNAzyme for signal generation.[63] Reproduced from Pavlov, V.; Xiao, Y.; Gill, R.; Dishon, A.; Kotler, M.; Willner, I. *Anal. Chem.* **2004**, *76*, 2152−2156 (ref 63). Copyright 2011 American Chemical Society. (B) Conjugation of multiple peroxidase-mimicking DNAzyme strands onto an AuNP for amplified detection of target DNA.[56] Reproduced from Niazov, T.; Pavlov, V.; Xiao, Y.; Gill, R.; Willner, I. *Nano Lett.* **2004**, *4*, 1683−1687 (ref 56). Copyright 2011 American Chemical Society. (C) Binding of thrombin to aptamers to form the sandwich complex, followed by cocatalysis of H_2O_2 reduction by Au, Cu_2O, and peroxidase-mimicking DNAzyme.[70] Reprinted from *Biosens. Bioelectron.*, Vol. *99*, Chen, S., Liu, P., Su, K., Li, X., Qin, Z., Xu, W., Chen, J., Li, C., Qiu, J. Electrochemical aptasensor for thrombin using cocatalysis of hemin/G-quadruplex DNAzyme and octahedral Cu_2O−Au nanocomposites for signal amplification, pp 338−345 (ref 70). Copyright 2012, with permission from Elsevier. (D) Formation of nanonetwork through the assembly of the peroxidase-mimicking DNAzyme functionalized nanoparticles.[68] Reproduced from Xu, M. D.; Zhuang, J. Y.; Chen, X.; Chen, G. A.; Tang, D. P. *Chem. Commun.* **2013**, *49*, 7304−7306 (ref 68), with permission of The Royal Society of Chemistry.

Taking advantage of the large surface area and excellent electrical conductivity of graphene oxide (GO), Yi et al. achieved amplified electrochemical detection of thrombin.[69] They immobilized a thrombin-binding aptamer on a gold electrode and conjugated multiple thrombin-binding aptamers to the GO surface. Binding of thrombin to the aptamer on GO and on the gold electrode resulted in the formation of a sandwich complex. In the presence of hemin, the multiple aptamer sequences on GO formed G-quadruplex DNAzymes, which in turn generated electrochemical detection signals.

Au−Cu_2O nanocomposite has also been used to conjugate multiple peroxidase-mimicking DNAzyme strands. Chen et al.[70] synthesized an Au−Cu_2O nanocomposite by growing AuNPs on the surface of Cu_2O nanocrystals (Figure 3C). The thrombin-binding aptamer and electron transfer medium (toluidine blue) were conjugated to the surface of each nanocomposite. In the presence of target thrombin and hemin, the aptamer conformed into a hemin/G-quadruplex peroxidase-mimicking DNAzyme. As a result, the Au, Cu_2O and peroxidase-mimicking DNAzyme cocatalyzed the reduction of H_2O_2, promoting electron transfer of toluidine blue and generating electrochemical signals for thrombin detection.

The assembly of DNAzyme-functionalized nanoparticles into a nanonetwork further improved the sensitivity of DNAzyme assays.[68,71] Xu et al.[68] conjugated a capture probe onto a gold electrode surface and prepared two types of AuNPs conjugated with two reporter DNA probes (DNA1 and DNA2) (Figure 3D). The DNA1 probe was composed of a target-binding domain S1 and a DNAzyme sequence, whereas the DNA2 contained a complementary sequence of S1, S1*, and a DNAzyme sequence. Sandwich of the target DNA by the capture probe and DNA1 placed the DNA1-functionalized AuNPs onto the electrode. Upon the addition of DNA2-functionalized AuNPs, hybridization between S1 and S1* induced the formation of a nanonetwork. Therefore, a single binding event induced assembly of multiple DNAzyme-conjugated nanoparticles, resulting in amplified detection signals.

Use of Peroxidase-Mimicking DNAzymes to Construct Catalytic Beacons. The formation of the G-quadruplex is

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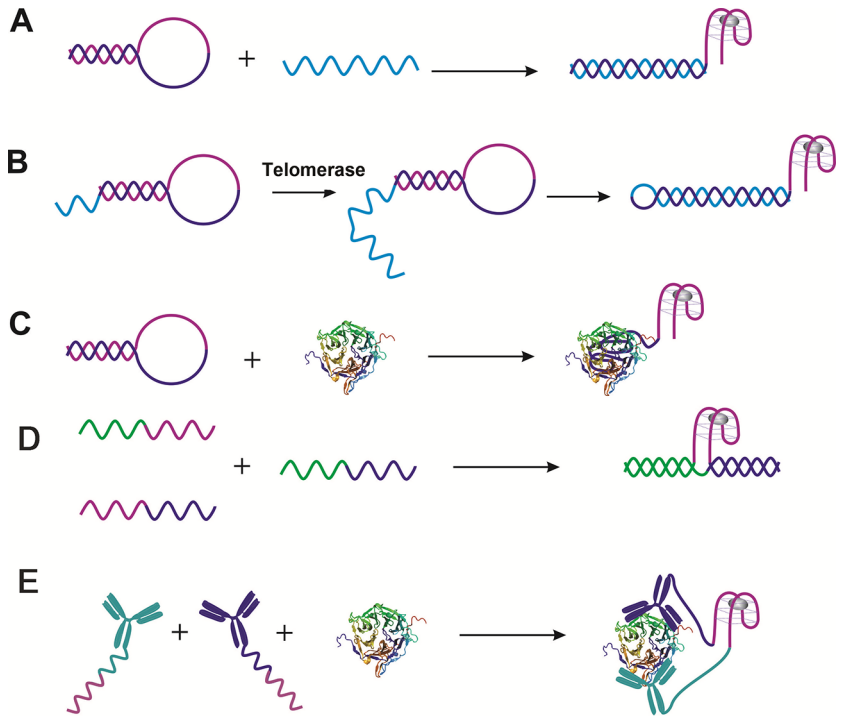


Figure 4. Catalytic beacons constructed with peroxidase-mimicking DNAzymes and assembly of two partial DNAzymes into a complete peroxidase-mimicking DNAzyme. (A) A typical example of catalytic beacon for detection of target DNA. (B) Release of a caged DNAzyme in hairpin structure through extension of a primer by telomerase.[54] Reproduced from Xiao, Y.; Pavlov, V.; Niazov, T.; Dishon, A.; Kotler, M.; Willner, I. *J. Am. Chem. Soc.* **2004**, *126*, 7430−7431 (ref 54). Copyright 2004 American Chemical Society. (C) An aptamer-based catalytic beacon for detecting a cytokine protein.[78] Reproduced from Zhang, H. X.; Jiang, B. Y.; Xiang, Y.; Chai, Y. Q.; Yuan, R. *Analyst* **2012**, *137*, 1020−1023 (ref 78), with permission of The Royal Society of Chemistry. (D) Two partial peroxidase-mimicking DNAzymes bind (hybridize) to the target molecule (DNA), inducing assembly of a complete peroxidase-mimicking DNAzyme.[53] Reproduced from Li, T.; Dong, S. J.; Wang, E. K. *Chem. Commun.* **2007**, 4209−4211 (ref 53), with permission of The Royal Society of Chemistry. (E) Binding of the target protein (CEA) to two antibody-conjugated partial DNAzymes resulted in the formation of the complete DNAzyme for detection of CEA.[84] Reproduced from Zong, C.; Wu, J.; Liu, M. M.; Yang, L. L.; Yan, F.; Ju, H. X. *Anal. Chem.* **2014**, *86*, 9939−9944 (ref 84). Copyright 2014 American Chemical Society.

essential for the catalytic activity of peroxidase-mimicking DNAzymes, and disruption of the G-quadruplex can lead to loss of DNAzyme activity. This feature has been used to construct catalytic beacons in which hairpins were used to cage a portion of the DNAzyme to prevent the formation of the G-quadruplex, rendering the DNAzyme inactive (Figure 4A).[72] In the presence of the target molecules, the target binding opened the hairpin structure to allow for the formation of G-quadruplex and thus reactivation of the DNAzyme. Like molecular beacons, catalytic beacons can be used in homogeneous assays without the need for separation. However, catalytic beacons are advantageous over molecular beacons as they are capable of signal amplification.

The peroxidase-mimicking DNAzyme-based catalytic beacon was initially developed to detect nucleic acid targets (Figure 4B).[54] The beacon was designed to have a hairpin structure, where the hairpin loop was designed to hybridize to the target DNA. The DNAzyme was caged in the stem of the hairpin, preventing the formation of G-quadruplex and deactivating DNAzyme. The hybridization of the target DNA with the loop opened the hairpin, releasing the DNAzyme strand. The catalytic activity of the DNAzyme was therefore restored, generating detection signals.

The hairpin structure of catalytic beacons can be opened through primer extension.[54,73−75] Xiao et al.[54] tethered a telomerase primer to one end of a hairpin caging a DNAzyme. The sequence, elongated by the telomerase, hybridized to the loop of the hairpin and opened the hairpin. Consequently, the caged DNAzyme was released to generate detection signals. This assay was used to detect telomerase activity. Catalytic beacons were also applied to the detection of T4 polynucleotide kinase (PNKP).[76] A DNA primer with a 3′-phosphate group was designed to hybridize to the loop of the hairpin but was insufficient to open it. In the presence of T4 PNKP, the 3′-phosphate group was dephosphorylated into a 3′-hydroxyl and initiated a primer extension reaction to open the hairpin.

Aptamers have been combined with DNAzymes to construct catalytic beacons, enabling detection of proteins.[77,78] Zhang et al.[78] designed an aptamer-based catalytic beacon for the detection of a cytokine protein. A peroxidase-mimicking DNAzyme was extended with an aptamer sequence that was partially complementary to the DNAzyme. In the absence of the target, a hairpin was formed, caging the DNAzyme in the stem of the hairpin (Figure 4C). Upon target binding, the hairpin was opened and thus exposed the DNAzyme sequence to form catalytically active DNAzyme.

Aptamer−target interactions can also inhibit the activity of an adjacent peroxidase-mimicking DNAzyme within a single DNA strand.[79] For example, a "turn off" assay was designed by linking a DNAzyme strand with an aptamer for carcinoembryonic antigen (CEA). In this design, binding of the target CEA molecule to the aptamer prevented the DNAzyme from forming a G-quadruplex. The consequent inhibition of the DNAzyme activity was a measure of the target CEA.

Assembly of Two Partial DNAzymes into a Complete DNAzyme. A peroxidase-mimicking DNAzyme can be split

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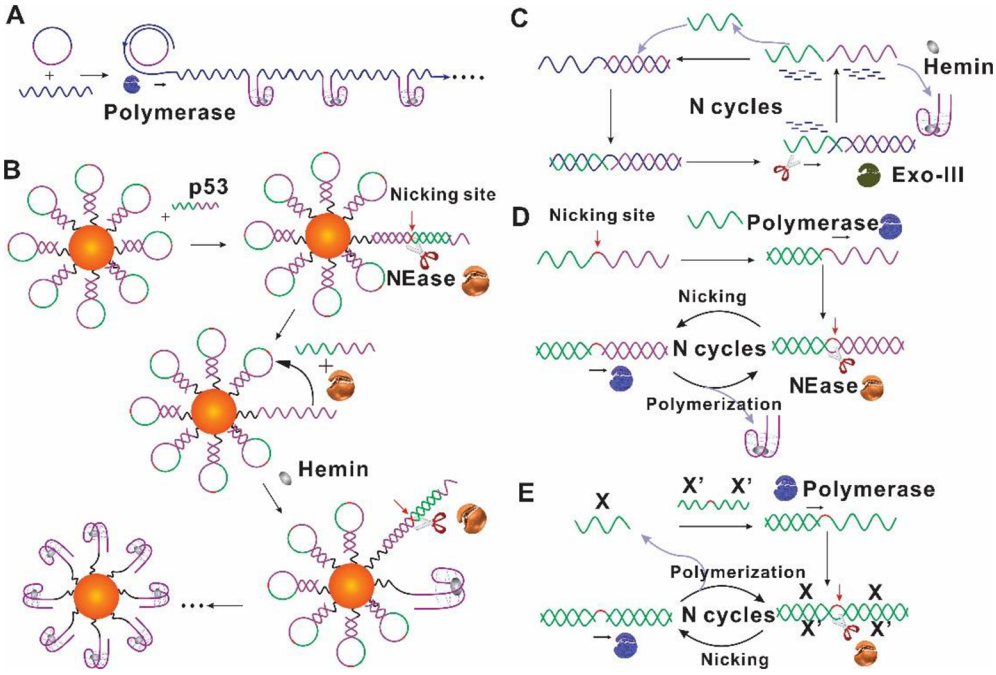


Figure 5. Enzyme-mediated amplification of peroxidase-mimicking DNAzymes. (A) A target DNA initiates the RCA amplification, producing repeat sequences of peroxidase-mimicking DNAzyme.[89] Reproduced from Cascade signal amplification for DNA detection, Tian, Y.; He, Y.; Mao, C. D. *Chembiochem*, Vol. 7, Issue 12 (ref 89). Copyright 2006 Wiley. (B) Initiated by hybridization of the target *p53* gene to the signaling probes on an AuNP, the NEase-catalyzed cyclic cleave of the signaling probes produces multiple copies of peroxidase-mimicking DNAzymes.[102] Reproduced from *Biosens. Bioelectron.*, Vol. 77, Wang, Z. H.; Xia, J. F.; Song, D. M.; Zhang, F. F.; Yang, M.; Gui, R. J.; Xia, L.; Bi, S.; Xia, Y. Z. Lable-free quadruple signal amplification strategy for sensitive electrochemical p53 gene biosensing, pp 157−163 (ref 102) Copyright 2016 with permission from Elsevier. (C) Stepwise removal of the ssDNA template from the duplex DNA by Exo-III releases the blocked peroxidase-mimicking DNAzyme.[104] Reproduced from Zhao, C. Q.; Wu, L.; Ren, J. S.; Qu, X. G. *Chem. Commun.* **2011**, *47*, 5461−5463 (ref 104), with permission of The Royal Society of Chemistry. (D) Concerted operations of a DNA polymerase with strand displacement activity and a NEase result in amplification of peroxidase-mimicking DNAzymes.[107] Reproduced from Weizmann, Y.; Cheglakov, Z.; Willner, I. *J. Am. Chem. Soc.* **2008**, *130*, 17224−17225 (ref 107). Copyright 2008 American Chemical Society. (E) EXPAR exponentially amplifies ssDNA, including replication of peroxidase-mimicking DNAzymes.[111] Reproduced from *Biosens. Bioelectron.*, Vol. *56*, Nie, J.; Zhang, D. W.; Tie, C.; Zhou, Y. L.; Zhang, X. X. G-quadruplex based two-stage isothermal exponential amplification reaction for label-free DNA colorimetric detection, pp 237−242 (ref 111) Copyright 2014 with permission from Elsevier.

into two partial DNAzymes that are inactive, and can be reassembled to a complete DNAzyme upon target binding.[27,53,58,80−87] Each partial DNAzyme is typically extended with a target-binding domain. When two partial DNAzymes bind to the same target molecule, the two partial DNAzymes assemble into a complete peroxidase-mimicking DNAzyme, restoring the catalytic activity of the DNAzyme. Note that two partial DNAzymes can potentially assemble into a complete DNAzyme without the trigger of target binding. Such target-independent assembly can generate background signal and must be minimized during method development.

Li et al.[53] split a DNAzyme into two symmetric parts and extended each partial DNAzyme with a target-binding sequence for DNA detection (Figure 4D). They found that in the absence of the target DNA, two partial DNAzymes could bind to hemin and form an unstable G-quadruplex. The addition of the target DNA at a low concentration significantly enhanced the stability of the G-quadruplex, therefore generating an increased detection signal. However, when the target DNA was in excess, the complete hybridization of the target DNA to the two partial DNAzymes disrupted the formation of the G-quadruplex, a finding consistent with the previous report.[87] A possible reason is that the rigidity of the duplex distorts two partial DNAzymes into a different configuration instead of the G-quadruplex. Kolpashchikov et al.[86] added a triethylene glycol linker between the target-binding sequence and the partial DNAzyme, allowing the formation of a stable G-quadruplex upon the hybridization of two partial DNAzymes with the target DNA. The target-independent formation of the G-quadruplex was observed in these studies. Concentrations of partial DNAzymes and buffer conditions were optimized to reduce this problem of background formation of the G-quadruplex.

Splitting the DNAzyme into two asymmetric parts is an option to address the issue of target-independent formation of G-quadruplex. Deng et al. split a DNAzyme into two asymmetric parts, one having three GGG repeats and the other only containing one GGG.[83] The target-independent assembly of these two partial DNAzymes was significantly reduced, leading to a large decrease in the background.

Partial DNAzymes can be used for protein detection by attaching an antibody or aptamer molecule to each partial DNAzyme. Zong et al.[84] conjugated an antibody molecule to each partial DNAzyme and used these antibody-conjugated partial DNAzymes to develop an assay for CEA (Figure 4E). The binding of two antibody molecules to the same CEA molecule brought the two partial DNAzymes into close proximity, enabling the formation of the complete peroxidase-mimicking DNAzyme. In their design, one partial DNAzyme was conjugated to the electrode surface. Thus, the complete peroxidase-mimicking DNAzyme was formed near the electrode surface. The electrochemical detection of CEA

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resulted in a detection limit of 0.5 pM. This method was further applied to the detection of thrombin by using aptamers to bind with the target thrombin.[88]

Amplification of Peroxidase-Mimicking DNAzymes Using DNA Amplification Techniques. As single-stranded DNA (ssDNA), DNAzymes can be amplified by various DNA amplification techniques. The detection sensitivity can be enhanced by combining DNA amplification with signal generation of peroxidase-mimicking DNAzymes. A general strategy is to use target binding to initiate DNA amplification that in turn produces abundant peroxidase-mimicking DNAzyme sequences. Assays using peroxidase-mimicking DNAzymes have been combined with a variety of DNA amplification techniques, including rolling circle amplification (RCA), nicking endonuclease (NEase)-assisted signal amplification (NESA), exonuclease III (Exo-III)-assisted signal amplification, strand displacement amplification (SDA), and exponential amplification reaction (EXPAR).

RCA replicates a circular DNA template to produce a long ssDNA containing multiple copies of the same DNA sequences linked in tandem. When the complementary sequence of the peroxidase-mimicking DNAzyme was included in the circular DNA template, the products of RCA contained multiple copies of the DNAzyme.[89−99] Tian et al.[89] designed a circular template containing two segments: the complementary sequences of a DNA target and a peroxidase-mimicking DNAzyme (Figure 5A). Target DNA, serving as a primer, hybridized to the circular template, initiating RCA amplification. Multiple copies of the DNAzyme in the long ssDNA product generated amplified detection signals. A detection limit of 1 pM was achieved.

To detect M13 phage circular ssDNA, Cheglakov et al. designed a hairpin probe to bind to the target DNA and then trigger RCA amplification.[100] The stem sequence at the 3′-end of the hairpin probe was designed to hybridize to the circular template and the loop was designed to hybridize to the target DNA. In the absence of the target DNA, the stable hairpin structure prevented the stem sequence from hybridizing to the circular template. The hybridization of the hairpin probe to the target DNA opened the hairpin, allowing the stem sequence to bind to the circular template and initiated RCA amplification.

RCA was also applied to the detection of proteins through affinity binding to their aptamers.[92,96] Bi et al. used a structure-switching aptamer and RCA for the detection of thrombin.[92] The structure switching aptamer, prepared by hybridizing the thrombin-binding aptamer with the RCA circular template, was immobilized on magnetic beads. The binding of thrombin to the aptamer released the circular template to the solution. After removing the magnetic beads from the solution, a primer was added to trigger RCA, producing a long ssDNA containing multiple DNAzymes that were then used to generate detection signals.

NEases recognize specific double-stranded DNA (dsDNA) sequences and catalyze the cleavage of only one strand of dsDNA at specific sites relative to the recognition sequences. NESA uses targets to trigger NEase-catalyzed cyclic cleavage of signaling probes.[101] Incorporating peroxidase-mimicking DNAzymes into signaling probes can result in NESA of the DNAzymes. Wang et al.[102] designed a signal DNA with a hairpin structure in which a peroxidase-mimicking DNAzyme was caged (Figure 5B). The hairpin also consisted of a target-binding domain containing a NEase-recognition sequence. The hairpins were conjugated to AuNPs that were attached to the electrode surface. In the presence of the *p53* gene target, the hybridization of the target DNA with the target-binding domain of the hairpin formed the complete recognition sequence of the NEase, initiating NEase-catalyzed cleavage of the hairpin. The cleavage released the target DNA for the next cycle of reaction and restored the activity of the DNAzyme. This repeating process resulted in linear amplification of the amount of active peroxidase-mimicking DNAzymes that subsequently generated electrochemical signals for detection. One limitation of this method is that the target DNA must include a NEase recognition sequence. To overcome this limitation, Li et al. designed a DNA hairpin to enable a microRNA (miRNA) target to initiate NESA.[103] The stem of the hairpin caged a DNA domain that contained a NEase recognition sequence. The binding of miRNA target to the hairpin DNA exposed the DNA domain for hybridization of the signaling DNA containing peroxidase-mimicking DNAzyme.

Exo-III, a 3′-5′ exonuclease, catalyzes stepwise removal of mononucleotides from the 3′-end hydroxyl of duplex DNA with a blunt or a recessed 3′-end. Exo-III-assisted signal amplification uses targets to initiate cyclic digestion of signal DNA strands catalyzed by Exo-III. The incorporation of peroxidase-mimicking DNAzymes into the signaling probes enables signal amplification through both Exo-III assisted signal amplification and the use of peroxidase-mimicking DNAzymes.[104−106] Zhao et al.[104] designed a signaling duplex DNA in which the DNAzyme was hybridized to a blocking sequence (Figure 5C). The signaling duplex DNA contained a single-stranded overhang at the 3′-ends such that the construct could not be cleaved by Exo-III. When the target DNA was hybridized to this overhang of the duplex, the Exo-III was able to catalyze the cleavage of the blocking sequence. The cleavage reduced strand stability of this construct and allowed the dissociation of the DNAzyme and the target DNA. The released DNAzyme catalyzed the subsequent reaction for signal generation and the target DNA was recycled by hybridizing to additional duplex overhangs in the next cycle of reactions, resulting in amplification. A similar strategy was designed to achieve cascade signal amplification using tandem Exo-III assisted signal amplifications, further improving sensitivity of the DNA detection.[106]

SDA can generate multiple copies of a ssDNA through the co-operation of strand displacement activity of a DNA polymerase and nicking activity of a NEase.[72,107−110] A target DNA, acted as the primer, and hybridized to a ssDNA template. This hybridization initiated the extension of the primer by the DNA polymerase. The extension formed a complete recognition sequence of the NEase, enabling the NEase to generate a nick within one strand of the dsDNA. DNA polymerase then extended the 3′-end of the nick, simultaneously displacing the newly synthesized strand. Repeating cycles of this reaction generated multiple copies of the ssDNA. Weizmann et al.[107] used SDA to replicate peroxidase-mimicking DNAzyme for amplified detection of target DNA (Figure 5D). A SDA template was designed to consist of three domains: a target-binding domain, a NEase recognition sequence, and the complementary sequence of the DNAzyme. The addition of the target DNA initiated SDA amplification and produced multiple copies of the DNAzyme that generated a detection signal. A detection limit of 10 fM was obtained. This assay, with a modified hairpin probe and RCA, was used to detect M13 phage circular ssDNA. The replication of peroxidase-mimicking DNAzymes using SDA was also applied to the detection of DNA ligase. The activity of the DNA ligase was detected via the

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ligase-catalyzed joining of the SDA template and primer duplex.[108]

EXPAR uses the same reaction mechanism as SDA, with the exception of the template design, to achieve exponential amplification of a target sequence (Figure 5E).[111−115] An EXPAR template usually contains two repeated X′ regions, commonly referred to as X′-X′, which are complementary to the target sequence (X) and are separated by a NEase recognition sequence. The ssDNA products generated from each cycle can serve as the primer to trigger the next cycle of reactions, achieving exponential amplification. Nie et al.[111] used a two-template system of EXPAR to replicate peroxidase-mimicking DNAzymes. The replicated DNAzymes were used to generate either colorimetric or electrochemical signals for DNA detection. The use of EXPAR to replicate peroxidase-mimicking DNAzyme was also applied to the detection of telomerase and transcription factors. In these assays, the activities of telomerase and transcription factors were used to generate the primers of EXPAR.[113,114]

The peroxidase-mimicking DNAzyme signals can be further amplified by combining several amplification techniques.[91,114,116−118] For example, Wen et al. developed a cascade amplification assay that combined RCA and NESA for the amplification of DNAzymes.[91] Briefly, hybridization of the target miRNA with a circular DNA template initiated RCA. Then, each repeated sequence in the long ssDNA products of RCA further triggered the NESA amplification and produced multiple peroxidase-mimicking DNAzymes for signal generation.

Amplification of Peroxidase-Mimicking DNAzymes Using Noncovalent DNA Catalytic Reactions. Noncovalent DNA catalytic reactions involve multiple strand-displacement reaction cycles that generate many output DNA strands from a specific input DNA strand.[119,120] These reactions rely on toehold-mediated strand displacement processes that are initiated by the binding of a displacing DNA strand to a toehold on a dsDNA duplex, followed by replacing the protecting DNA from the duplex via branch migration. These noncovalent catalytic reactions can be used for the amplified detection of the input DNA without the need for protein enzymes. Three main types of noncovalent DNA catalytic reactions have been developed: hybridization chain reaction (HCR),[121] catalytic hairpin assembly (CHA),[122] and entropy-driven catalysis (EDC).[123] Because these reactions are enzyme-free and are thus not restricted to protein-enzyme storage requirements, they have potential applications in point-of-care testing and on-site analysis. Peroxidase-mimicking DNAzymes have been incorporated into these noncovalent DNA catalytic reactions to increase detection sensitivity.

HCR is a hybridization cascade which forms a long dsDNA by using hairpins that are stable in solution but undergo continuous assembly when triggered by a target DNA.[124−141] A typical HCR uses two hairpins (H1 and H2) whose potential energy is stored in short loops protected by long stems. The two hairpins are kinetically trapped, preventing the interaction between the two hairpins from rapidly equilibrating. The hybridization of the initiator DNA to one hairpin triggers a chain reaction involving hybridization of the two hairpins, forming a long dsDNA that contains multiple repeat units. Amplification of peroxidase-mimicking DNAzymes by HCR can be achieved by including two partial DNAzymes, one at each end of the two hairpins. The partial DNAzymes are assembled into complete DNAzymes by the formation of the long, target-initiated HCR dsDNA. Thus, each repeat unit of HCR creates a complete DNAzyme. These DNAzymes generate detection signals for amplified detection of target nucleic acids and proteins.

Shimron et al. split the peroxidase-mimicking DNAzyme sequence into two partial DNAzymes, one with three-fourths and the other with one-fourth of the complete sequence. They added the former sequence to the 5′-end of H1 and 3′-end of H2, and the latter sequence to the 3′-end of H1 and 5′-end of H2 (Figure 6A).[125] H1 was designed to contain a recognition

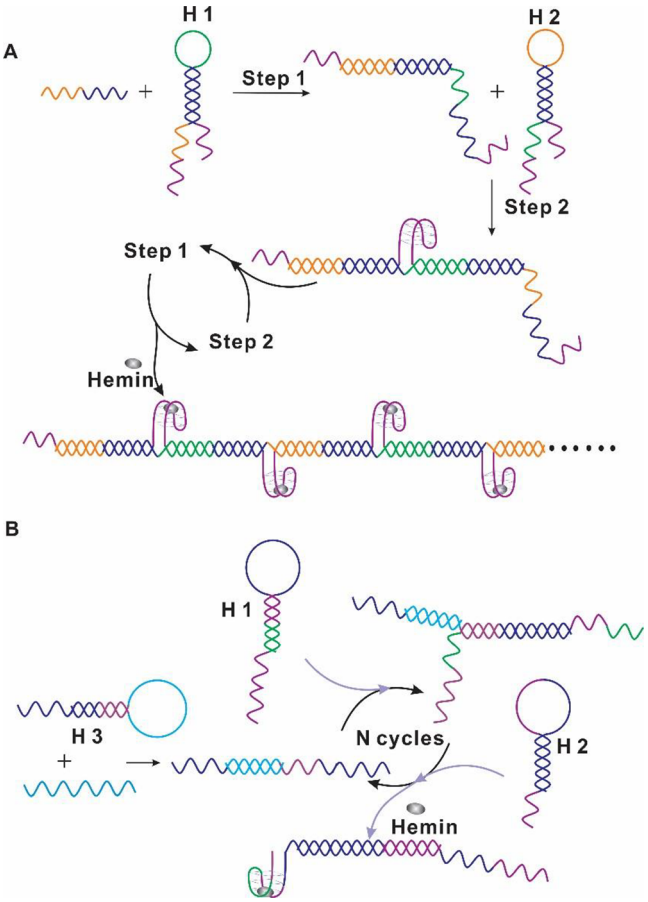


Figure 6. Amplification of peroxidase-mimicking DNAzymes using noncovalent DNA catalytic reactions. (A) Hybridization chain reaction (HCR) using two hairpin probes (H1 and H2), with each end containing a partial peroxidase-mimicking DNAzyme seqeunce.[125] Reproduced from Shimron, S.; Wang, F.; Orbach, R.; Willner, I. *Anal. Chem.* **2012**, *84*, 1042−1048 (ref 125). Copyright 2012 American Chemical Society. (B) Catalytic hairpin assembly (CHA) achieving amplification and liberation of caged DNAzymes.[143] Reproduced from *Biosens. Bioelectron.*, Vol. *64*, Xu, Y. Y.; Zhou, W. J.; Zhou, M.; Xiang, Y.; Yuan, R.; Chai, Y. Q. Toehold strand displacement-driven assembly of G-quadruplex DNA for enzyme-free and nonlabel sensitive fluorescent detection of thrombin, pp 306−310 (ref 192) Copyright 2015 with permission from Elsevier.

sequence for the target DNA. HCR was initiated by the target DNA to form a long dsDNA on which multiple complete DNAzymes were assembled. These peroxidase-mimicking DNAzymes catalyzed oxidation of ABTS or luminol, generating the colorimetric or chemiluminescence signals for amplified detection of the target DNA. A detection limit of 0.1 pM was achieved for the detection of the *BRCA1* oncogene. A similar

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strategy was also applied to the electrochemical detection of miRNA.[124,138,142]

The use of HCR for the amplified detection of proteins was demonstrated using two main approaches: (1) labeling of detection antibodies with nanoparticles onto which the initiator DNA of HCR was conjugated and (2) binding of the target proteins to aptamers to release caged initiator DNA of HCR. The first approach used a general sandwich format for protein detection. AuNPs,[132,134,135] carbon nanotubes,[136] and C_{60}@Pt–Pd nanoparticles[137] were used as scaffolds to carry multiple initiator DNA strands. After the target proteins were bound by the capture antibodies, the detection antibodies conjugated with initiator-functionalized nanoparticles were used to bind to the captured target molecules. After removal of the unbound detection antibodies by washing, two hairpins were added to initiate HCR, which induced the assembly of the complete DNAzymes to generate detection signals. The second approach was primarily demonstrated for the detection of thrombin.[131,133,134,139,143] A structure-switching aptamer was used to cage the initiator DNA. The binding of thrombin to the aptamer released the caged initiator DNA, triggering HCR for the amplification of peroxidase-mimicking DNAzymes.

Similar to HCR, CHA typically uses two hairpins (H1 and H2) designed to be complementary. Caging the complementary domains within the stems of the hairpins kinetically deters the spontaneous hybridization between H1 and H2. In the presence of an initiator DNA, the hybridization of the initiator DNA with H1 via the toehold-mediated strand displacement exposes the caged complementary domain for hybridization with H2. The hybridization between H1 and H2 consequently recycles the target DNA for the next cycle of reaction, resulting in amplification.

The incorporation of peroxidase-mimicking DNAzymes into CHA has been achieved by either releasing caged DNAzymes or assembling partial DNAzymes through CHA.[143−146] Chen et al.[147] used CHA to amplify and release caged DNAzymes (Figure 6B). A peroxidase-mimicking DNAzyme was caged in the stem of H2. In the absence of the target DNA, the caged DNAzymes were inactive. Hairpin H3 was designed to contain a target binding domain. In the presence of the target DNA, the hybridization of the target DNA with H3 released the initiator DNA. The initiator DNA triggered CHA to sequentially open H1 and H2, releasing the caged DNAzymes for signal generation. One variant of CHA used more than two hairpins to generate DNA junctions containing multiple arms. Three and four hairpins have been used to produce three-arm and four-arm DNA junctions, respectively, through CHA.[60,145] As partial DNAzymes were included at the ends of hairpins, the formation of DNA junctions placed the ends of different hairpins into close proximity, enabling the formation of the complete DNAzymes.

DNAzymes have been incorporated into CHA for protein detection. Xu et al.[143] designed a structure-switching aptamer that caged the initiator DNA required for CHA. Upon binding of the aptamer to the target thrombin, the structural change of the aptamer released the caged initiator DNA that then triggered CHA. The detection of uracil-DNA glycosylase (UDG) was also demonstrated using the activity of UDG to release the precaged initiator DNA.[146]

CHA has been combined with other nucleic acid amplification techniques, including RCA,[117] SDA,[148] and HCR,[124,149] to further improve the sensitivity. The combination of RCA and SDA with CHA was achieved by using ssDNA products of RCA and SDA as the initiator DNA for the subsequent CHA. CHA was coupled to HCR by using CHA as the first amplification step to generate the initiator DNA for HCR.

AMPLIFIED DETECTION OF NUCLEIC ACIDS AND PROTEINS USING RNA-CLEAVING DNAZYMES

Three main types of RNA-cleaving DNAzymes, 8-17, 10-23, and Mg^{2+}-dependent DNAzymes, have been used to develop DNAzyme-mediated assays for the detection of nucleic acids and proteins. These specific RNA-cleaving DNAzymes are frequently chosen because of their small catalytic cores, high activities, and cleavage capability independent of the arm sequence. As both RNA-cleaving DNAzymes and peroxidase-mimicking DNAzymes are made from DNA, they share similar advantages over enzymes made from protein, which was previously discussed. Thus, the strategies used for developing assays based on peroxidase-mimicking DNAzymes have also been used to develop assays using RNA-cleaving DNAzymes. In this section, we address the unique properties of RNA-cleaving DNAzymes, which have been used advantageously in the construction of assays for the detection of nucleic acids and proteins.

Labeling of Affinity Ligands with Nanoparticles of Cofactor Metals. The general requirement of specific metal ions as cofactors for RNA-cleaving DNAzymes to perform their catalytic activities inspired the development of various DNAzyme-based sensors for metal ions.[7,10,22,23] For example, Lu and co-workers[41] developed a set of sensors for the highly sensitive detection of Pb^{2+} by using a lead-dependent RNA-cleaving DNAzyme, 8-17E.

Strategies that convert the detection of other types of targets (e.g., nucleic acids and proteins) into the detection of metal ions can expand the applicability of these DNAzyme sensors to beyond that of metal ions.[150−152] One strategy is to use metallic nanoparticles as a signal tag, as this offers two main benefits: (1) the detection of the target can be further amplified by releasing thousands of metal ions from a single metallic nanoparticle and (2) the use of metallic nanoparticles facilitates the labeling of affinity ligands with metal ions.

Zhang et al.[150] developed a sandwich immunoassay to achieve indirect detection of a protein target, prostate specific antigen (PSA), through the direct detection of Pb^{2+} by an electrochemical DNAzyme sensor (Figure 7A). A primary antibody affixed to a solid support first captured the target PSA. The captured PSA was sandwiched by a second antibody that was labeled with a PbS nanoparticle. After removal of the unbound antibody, the PbS nanoparticles on the solid support were released and digested using nitric acid to form lead ions. A DNAzyme sensor conjugated to a gold electrode was used to detect these lead ions. The authors achieved a detection limit 220-fold lower than that of conventional enzyme-linked immunosorbent assay (ELISA). A similar strategy was used to detect human immunoglobulin G (IgG), in which Pb^{2+} released from PbS nanoparticles was detected by a fluorescent DNAzyme sensor.[151] It is conceivable that other metallic nanoparticles can be used for signal tags and that DNAzyme sensors with different signal readouts can detect these released metal ions.

Catalytic Beacons Made from RNA-Cleaving DNAzymes. Catalytic beacons use hairpins to cage and deactivate DNAzymes, which become active in the presence of a target.[153−161] Either the binding arms or the catalytic core

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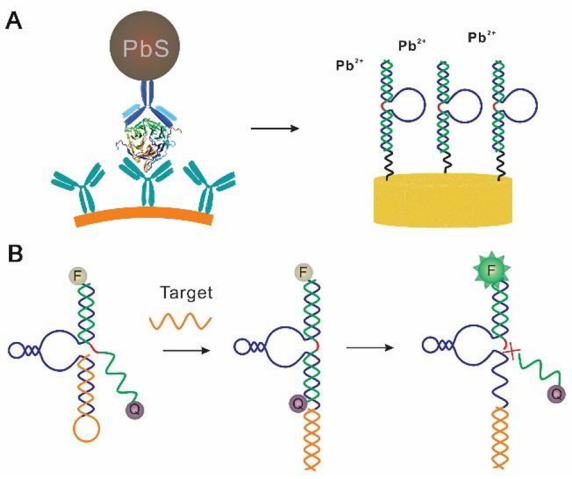


Figure 7. Two examples of assay using RNA-cleaving DNAzymes. (A) Sandwich immunoassay for prostate specific antigen using an antibody labeled with PbS nanoparticles and the subsequent detection of Pb^{2+} using a DNAzyme.[150] Reproduced from Zhang, B.; Liu, B. Q.; Zhuang, J. Y.; Tang, D. P. *Bioconjugate Chem.* **2013**, *24*, 678−683 (ref 150). Copyright 2011 American Chemical Society. (B) A catalytic beacon designed for the detection of a target DNA.[162] Reproduced from Catalytic molecular beacons, Stojanovic, M. N.; de Prada, P.; Landry, D. W. *Chembiochem*, Vol. *2*, Issue 6, 411−415 (ref 162). Copyright 2001 Wiley.

can be caged in the stem of the hairpin structure to deactivate RNA-cleaving DNAzymes. Stojanovic et al. reported a catalytic beacon constructed using an E6 RNA-cleaving DNAzyme.[162] The 5′-end of the DNAzyme was extended by an additional sequence that formed a hairpin structure, blocking one binding arm of the DNAzyme (Figure 7B). The DNAzyme was inactive because only one binding arm could hybridize to the substrate strand. The loop of the hairpin was designed to hybridize to the target DNA, so that the presence of the target DNA could open the hairpin. Opening of the hairpin exposed the binding arm sequence and allowed it to bind to the substrate, which activated the DNAzyme to cleave the substrate. The substrate was dually labeled with a pair of fluorophore and quencher at its ends. The catalytic cleavage of the substrate separated the fluorophore and the quencher, turning on the fluorescence. Repeating actions of DNA binding and cleavage of the substrate by the DNAzyme resulted in signal amplification. This strategy was also used to design catalytic beacons for logic gates.[12,163−169]

One potential issue of the above design is that although the exposed stem sequence no longer blocks the binding arm of the same DNAzyme upon target hybridization, it can compete with the substrate in hybridizing with other DNAzymes. To address this issue, Tian and Mao[170] added a toehold to the end of the stem sequence that blocked the binding arm of the 10-23 DNAzyme. The target DNA hybridized to the stem sequence through the toehold-mediated strand displacement reaction, which released the binding arm to hybridize to the substrate. Because the stem sequence was bound by the target DNA, it could not hybridize to the DNAzyme and thus overcame the issue of dimer formation. This design is feasible because the RNA-cleaving activities of these DNAzymes are independent of their arm sequence. Thus, appropriate stem sequences can be designed based on the target DNA.

Target-Induced Assembly of Multiple Partial DNAzymes. Catalytic beacons can produce background signal if DNAzymes are not completely caged by the hairpin structure. One solution to this problem is to deactivate the RNA-cleaving DNAzyme by splitting it within the catalytic core into partial DNAzymes. Because the full catalytic core is indispensable for the activity of the DNAzyme, partial DNAzymes do not have any catalytic activity and can thus eliminate background. Additionally, unlike partial peroxidase-mimicking DNAzymes, partial DNAzymes split from RNA-cleaving DNAzymes do not spontaneously assemble together. In order to detect target molecules, these partial DNAzymes are extended to include target-binding domains so that the target induces the assembly of partial DNAzymes into the active conformation. A systematic study is required to examine the split sites for the best restored catalytic activity since remarkable differences in catalytic activities have been observed from DNAzymes assembled by partial DNAzymes that were split at different sites.

Todd and co-workers pioneered the concept of so-called multicomponent nucleic acid enzymes (MNAzymes).[171−174] They split the catalytic cores of 10-23 and 8-17 DNAzymes at different sites to generate various pairs of partial DNAzymes. They extended two partial DNAzymes with a target-binding domain at the split ends and evaluated them for the construction of MNAzymes. Each partial DNAzyme consisted of a portion of the catalytic core, a binding arm, and a target-binding domain. Upon addition of the target DNA, hybridization of the target DNA with two target-binding domains assembled a pair of partial DNAzymes together, forming the whole catalytic beacon (Figure 8A). Therefore, the activity of the DNAzyme was restored for substrate cleavage. They demonstrated that MNAzymes generated by splitting the catalytic core at different sites exhibited a large variation in their catalytic activities. They found that splitting the catalytic core of 10-23 DNAzyme between the eighth and ninth nucleotides numbered from the 5′-end resulted in the MNAzyme with the highest catalytic activity (k_{obs} = 1.2 min^{-1}) which was about 100-fold higher than that of the MNAzyme with the lowest activity. On the other hand, compared to the intact DNAzyme (k_{obs} = 4.0 min^{-1}), the activity of the reassembled MNAzymes was more than 3-fold lower.

MNAzymes have been used to detect genetic material of infectious pathogens, microRNA, and PCR products.[172,175−180] Several signal readout approaches have been applied to detection of substrate cleavage by MNAzymes.[181,182] Fluorescence detection usually involved dual labeling of the substrate strand with a fluorophore and quencher pair. Colorimetric and electrochemical detection have been also used for signal readout.[183,184]

Wang et al.[185] showed an alternative approach for the assembly of two partial DNAzymes (Figure 8B). Rather than a target-binding domain being added to each partial DNAzyme, the first partial DNAzyme was extended to include a hairpin structure whose loop was designed to bind to the target DNA, while the second partial DNAzyme was added with a domain that was complementary to one stem of the hairpin. In the absence of the target, the stable hairpin inhibited the hybridization of two partial DNAzymes to each other. When present, the target DNA hybridized to the loop of the hairpin to open the hairpin structure. The stem sequence was therefore exposed, which allowed the two partial DNAzymes to hybridize together. Consequently, the complete catalytic core was formed, restoring the activity of the DNAzyme. The assembled DNAzyme acted on a molecular beacon as substrate to improve

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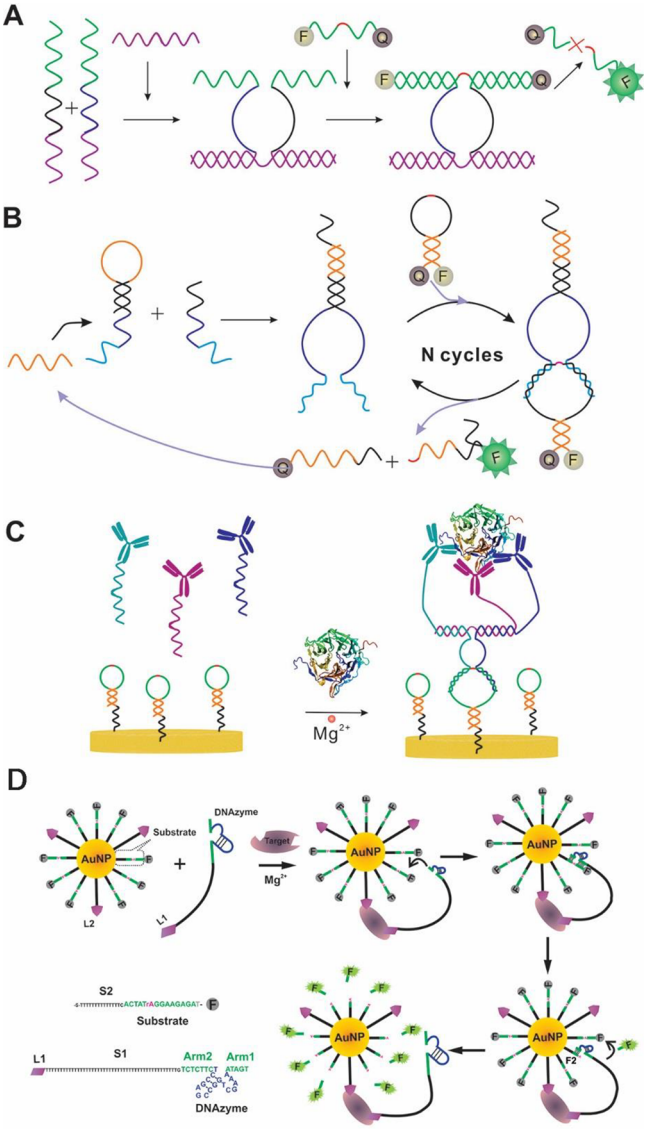


Figure 8. Target-induced assembly of multiple partial DNAzymes. (A) Target-induced assembly of multicomponent nucleic acid enzymes (MNAzymes).[171] Reproduced from Mokany, E.; Bone, S. M.; Young, P. E.; Doan, T. B.; Todd, A. V. *J. Am. Chem. Soc.* **2010**, *132*, 1051−1059 (ref 171). Copyright 2011 American Chemical Society. (B) Target-initiated MNAzyme for the cleavage of a molecular beacon.[185] Reproduced from Amplified detection of DNA through an autocatalytic and catabolic DNAzyme-mediated process, Wang, F.; Elbaz, J.; Teller, C.; Willner, I. *Angew. Chem. Int. Ed.*, Vol. *50*, Issue 1 (ref 185). Copyright 2010 Wiley. (C) Assembly of a MNAzyme through triple proximity binding to a target protein molecule.[186] Reproduced from Ren, K. W.; Wu, J.; Ju, H. X.; Yan, F. *Anal. Chem.* **2015**, *87*, 1694−1700 (ref 186). Copyright 2015 American Chemical Society. (D) the target-triggered DNAzyme motor enabling homogeneous, amplified detection of proteins.[188] Reproduced from Chen, J.; Zuehlke, A.; Deng, B.; Peng, H.; Hou, X.; Zhang, H. *Anal. Chem.* **2017**, DOI: 10.1021/acs.analchem.7b03529 (ref 188). Copyright 2017 American Chemical Society.

the sensitivity. The target sequence was also caged in the hairpin of the molecular beacon. When cleaved, the hairpin structure of the molecular beacon was disrupted, releasing the sequence to open the first hairpin. Thus, each cleavage of the substrate yielded the formation of a new assembled DNAzyme that in turn cleaved more substrates. Consequently, the reaction was propagated and thus achieved exponential signal amplification. Compared to the MNAzyme strategy, this approach could have higher background because incomplete blocking by the hairpin can result in target-independent hybridization.

The use of MNAzymes was further expanded to detect proteins through combination with proximity probes (Figure 8C). Ren et al.[186] achieved amplified detection of a protein biomarker by using binding of three proximity probes to a single protein molecule to trigger MNAzyme assembly. Two partial DNAzymes were each extended by a DNA spacer that was also conjugated to an antibody molecule, forming probes 1 and 2. Probe 3 was prepared by conjugating a DNA strand to the third antibody molecule. The DNA spacers of probes 1 and 2 contained a short DNA domain complementary to the DNA strand of probe 3. The complementary domains were designed to be so short that the three probes could not form a stable hybrid in the absence of the target protein. The binding of the three probes to the same protein molecule placed the complementary domains in close proximity, enabling the DNA strand of probe 3 to hybridize with the two short complementary domains of probes 1 and 2. As a consequence, the MNAzyme was formed and cleaved substrates conjugated to a gold electrode. An electrochemical signal was used for amplified detection of the protein target. However, the requirement of three binding events limits the application of this method because many target molecules lack the size to accommodate three simultaneous antibody-binding events. Zou et al.[187] used two proximity probes to assemble the MNAzyme, which simplified the method design and expanded the applicability of the MNAzyme for protein detection. Molecular beacons were used as substrates to produce the fluorescence signal for detection of α-fetoprotein.

Besides splitting DNAzymes within the catalytic core, an alternative to deactivating DNAzymes is to truncate their binding arms. Chen et al. truncated the binding arms of the 8-17E DNAzyme and used this truncated 8-17E DNAzyme to construct a target-triggered DNAzyme motor for protein detection (Figure 8D).[188] The motor system consisted of two main components: a DNAzyme linked to an affinity ligand and an AuNP decorated with a second affinity ligand and hundreds of substrate strands. Arm 1 and arm 2 of the 8-17E DNAzyme were shortened from 9 bases to 5 and 7 bases, respectively, so that the truncated DNAzyme was unable to spontaneously hybridize with the substrate strands on the AuNPs. Binding of a protein molecule to the two ligands anchored the DNAzyme onto the AuNP. Therefore, the local effective concentrations of the DNAzyme and substrate were dramatically increased, enabling hybridization of the DNAzyme with its substrate on the AuNP. The hybridization of DNAzyme with the substrate initiated the autonomous cleavage of the substrate on the AuNP. Each substrate strand was labeled with a fluorophore molecule that was quenched by the AuNP before cleavage. Therefore, cleavage of each substrate strand restored the fluorescence of the fluorophore molecule, which was used for protein detection.

Incorporation of RNA-Cleaving DNAzymes into HCR and CHA. Like peroxidase-mimicking DNAzymes, RNA-cleaving DNAzymes have been incorporated into HCR and CHA for amplified detection of nucleic acids. Willner and co-workers[189] demonstrated the incorporation of MNAzymes into HCR for DNA detection (Figure 9A). Two partial DNAzymes of one MNAzyme were added to the 5′ and 3′ ends of both

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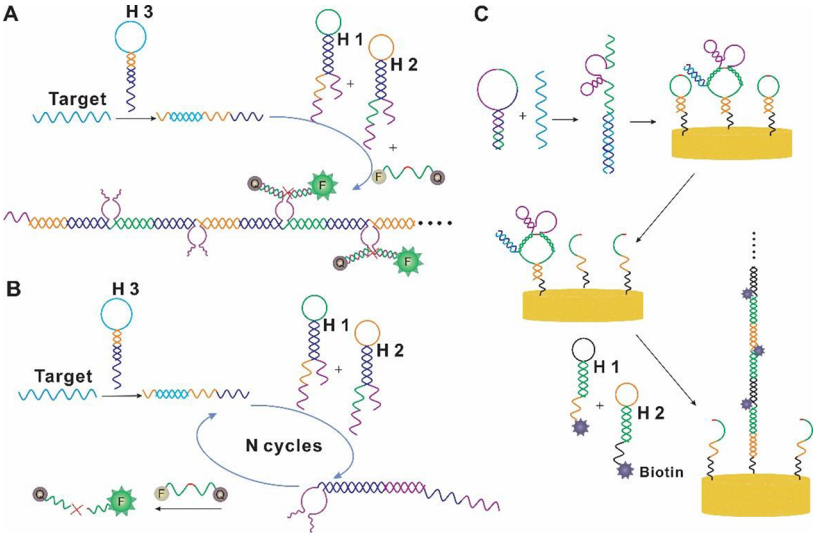


Figure 9. Incorporation of RNA-cleaving DNAzymes into HCR and CHA. (A) Incorporation of MNAzymes into HCR for DNA detection.[189] Reproduced from Wang, F.; Elbaz, J.; Orbach, R.; Magen, N.; Willner, I. *J. Am. Chem. Soc.* **2011**, *133*, 17149−17151 (ref 189). Copyright 2011 American Chemical Society. (B) Assembly of a MNAzyme through the hybridization between H1 and H2 via CHA.[191] Reproduced from Liu, S. F.; Cheng, C. B.; Gong, H. W.; Wang, L. *Chem. Commun.* **2015**, *51*, 7364−7367 (ref 191), with permission of The Royal Society of Chemistry. (C) HCR initiated by target binding and incorporated with the DNAzyme-mediated cleavage.[192] Reproduced from *Biosens. Bioelectron.*, Vol. *63*, Qian, Y.; Wang, C. Y.; Gao, F. L. Ultrasensitive electrochemical detection of DNA based on Zn^{2+} assistant DNA recycling followed with hybridization chain reaction dual amplification, pp 425−431 (ref 192). Copyright 2015 with permission from Elsevier.

hairpins (H1 and H2). In the hairpin format, the partial DNAzymes were too far from each other to retain MNAzyme catalytic activity. H1 was designed to contain a target binding sequence. In the presence of the target DNA, HCR was initiated to form a long dsDNA on which multiple MNAzymes were formed. These MNAzymes then cleaved substrates that were dually labeled with a pair of fluorophore and quencher, generating a fluorescent signal for target detection. As well as using the target DNA as the initiator of HCR, a hairpin was designed to cage the initiator strand in such a way that it was liberated upon the addition of target DNA. This method was applied to the detection of the BRCA1 oncogene and a detection limit of 10 fM was achieved. One disadvantage of this method is that it requires a long reaction time, more than 10 h.

MNAzymes have also been combined with CHA for the amplified detection of DNA targets.[190,191] Liu et al.[191] included two partial DNAzymes into two hairpins (H1 and H2) of CHA (Figure 9B). The third hairpin was designed to cage the toehold domain of the initiator strand in the stem. Therefore, in the absence of the target DNA, the two hairpins of CHA did not interact with one another. The hybridization of the target DNA with the loop of the third hairpin opened the hairpin, releasing the initiator strand for CHA. The hybridization between H1 and H2 via CHA induced the formation of MNAzymes that then cleave the substrate, generating a fluorescence signal for detection.

Another approach for the incorporation of RNA-cleaving DNAzyme into HCR is to use target-activated DNAzymes to cleave hairpins that cage the initiator strands of HCR.[192−195] Qian et al.[192] designed a hairpin to cage a Zn^{2+}-dependent DNAzyme (Figure 9C). The hybridization of the target DNA to this hairpin released the caged DNAzyme. The DNAzyme then cleaved the substrate which had a hairpin structure and was conjugated onto an Au electrode. The hairpin of the substrate caged the initiator strand of HCR. Therefore, the cleavage of the hairpin released the initiator strand for triggering HCR. Because H1 and H2 of HCR were biotinylated, the HCR product, a long dsDNA polymer, was detected by adding streptavidin-conjugated alkaline phosphatase (SA-ALP) which catalyzed the reduction of silver ions and produced an electrochemical signal output.

Detection of microRNA in Live Cells. miRNA are small (19−23 nt), noncoding single stranded RNA that act in RNA silencing and gene expression.[196−198] Aberrant expressions of miRNA have been associated with human diseases, including cancer. The detection of miRNA in live cells is critical for understanding the biological functions of miRNA and providing insights into its dynamic processes within cells.[199−201] However, intracellular levels of miRNA are often low, requiring *in situ* signal amplification for their detection. RNA-cleaving DNAzymes have been demonstrated to be effective tools for *in situ* amplified detection of miRNA in live cells,[202−206] because they can be engineered to be activated in response to specific intracellular miRNA and generate fluorescent signals for *in vivo* fluorescence imaging. The use of RNA-cleaving DNAzymes to detect miRNA in live cells is focused on three major aspects: effectively delivering DNAzymes and substrates into live cells, engineering DNAzymes to be initially inactive and then activated in response to specific intracellular miRNA and efficiently quenching fluorophore molecules used to label substrates.

Taking advantage of their efficient cellular uptake and fluorescent quenching, nanomaterials have been used as the scaffold in the construction of nanosensors for intracellular miRNA. AuNPs are appealing for construction of DNAzyme-based nanosensors because of their ease of DNA conjugation and powerful fluorescence quenching efficiency. Peng et al. constructed a miRNA-initiated DNAzyme motor by using AuNPs as the scaffold and applied it to amplified detection of miRNA miR-10b in live cells (Figure 10A).[202] The whole motor system was constructed on an AuNP conjugated with hundreds of substrate strands serving as DNA tracks and dozens of DNAzyme molecules each silenced by a locking strand. The free end of each substrate strand was conjugated

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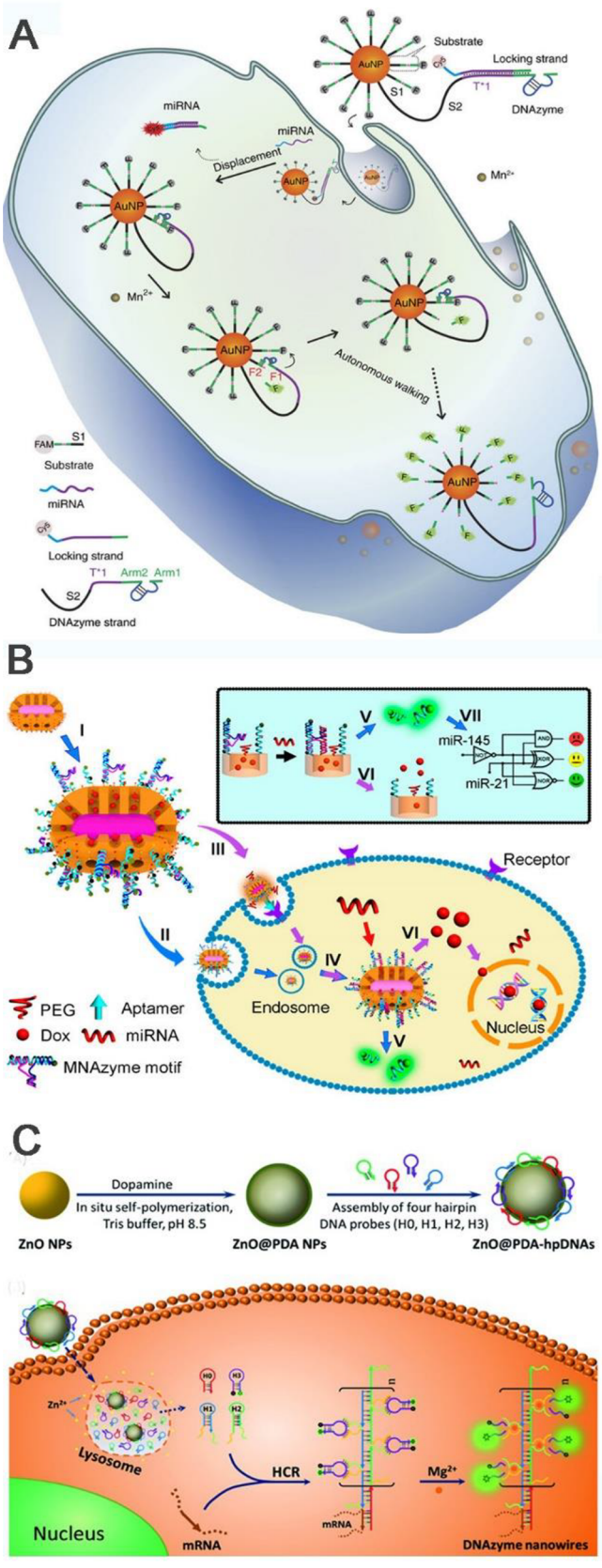


Figure 10. Examples of imaging microRNA in live cells. (A) A microRNA-initiated DNAzyme motor operating in living cells.[202] Reprinted by permission from Macmillan Publishers Ltd.: NATURE, Peng, H.; Li, X. F.; Zhang, H.; Le, X. C. *Nat. Commun.* **2017**, *8*, 14378 (ref 202). Copyright 2012. (B) MNAzymes assembled on silica-coated mesoporous gold nanorods for microRNA imaging in live cell.[203] Reproduced from Zhang, P.; He, Z.; Wang, C.; Chen, J.; Zhao, J.; Zhu, X.; Li, C. Z.; Min, Q.; Zhu, J. J. *ACS Nano* **2015**, *9*, 789−798 (ref 203). Copyright 2015 American Chemical Society. (C) Delivery of components of a HCR mediated MNAzyme assembly system into live cell through polydopamine ZnO nanoparticles.[204] Reproduced from He, D. G.; He, X.; Yang, X.; Li, H. W. *Chem. Sci.* **2017**, *8*, 2832−2840 (ref 204), with permission of The Royal Society of Chemistry.

with a fluorophore molecule. The fluorescence of all fluorophore molecules was quenched by the AuNP. The locking strand was designed to respond to the target, an intracellular miR-10b. Direct incubation of functionalized AuNPs with cells allowed cellular uptake of the DNAzyme motor system. Within the cell, the intracellular miR-10b hybridized with the locking strand through a toehold exchange reaction, releasing the DNAzyme. The unlocked DNAzyme then began to hybridize to and cleave its substrate on the AuNP. Each substrate cleavage released a fluorescently labeled segment from AuNP, which restored the fluorescence. Multiple substrates were cleaved in response to a single miRNA strand, achieving *in situ* signal amplification. Monitoring intracellular fluorescence enabled amplified detection of the miRNA target. In addition to using a locking strand to silence the DNAzyme, MNAzymes were used to construct nanosensors using AuNPs as the scaffold.[205] In the presence of intracellular miRNA target, MNAzymes were formed to cleave fluorescently labeled substrates on AuNPs.

Gold nanorods have been used to construct a DNAzyme-based nanosensor capable of tuning the reaction temperature. Zhang et al.[203] constructed a nanosensor in which complexes between a MNAzyme and its substrate were conjugated to silica-coated mesoporous gold nanorods (Figure 10B). After cellular uptake, the miRNA targets, miR-21 and miR-145, hybridized to the binding arms of the MNAzyme, activating the MNAzyme to cleave the fluorescently labeled substrate and generated a fluorescence signal for imaging. Besides serving as the scaffold for the MNAzyme sensor, the use of gold nanorods allowed fine-tuning of the reaction temperature for the MNAzyme, because gold nanorods can convert near-infrared (NIR) irradiation into heat. Therefore, MNAzyme catalytic activity was regulated and optimized through intermittent NIR exposure.

ZnO nanoparticles have been used as carriers to deliver hairpins of HCR and the hairpin substrate of the DNAzyme into target cells. He et al.[204] used ZnO nanoparticles to adsorb four hairpins: one target hairpin containing the miRNA binding domain and caging initiator strand of HCR, two hairpins of HCR, and a molecular beacon containing the cleavage site within its loop (Figure 10C). When ZnO nanoparticles were endocytosed into the cells, the acidic environment of the endosome caused the breakdown of the nanoparticles and release of the hairpins. The binding of the mRNA target with the target hairpin released the caged initiator to trigger HCR. The HCR produced a long dsDNA containing multiple MNAzymes that subsequently cleaved a molecular beacon substrate to generate fluorescence for imaging.

■ CONCLUSIONS AND PERSPECTIVES

DNAzymes have been increasingly used in the development of a variety of assays for amplified detection of nucleic acids and

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proteins. DNAzymes have several advantageous features: good chemical and thermal stability, ease of production and modification, and compatibility with DNA amplification techniques. Two types of DNAzymes, peroxidase-mimicking DNAzymes and RNA-cleaving DNAzymes, are extensively used for analytical purposes. Peroxidase-mimicking DNAzymes can adopt well-developed substrates of HRP for signal amplification. RNA-cleaving DNAzymes, especially the 8-17 and 10-23 DNAzymes, are well-recognized for their higher catalytic activity over all other DNAzymes. In this review, we summarized and discussed the major strategies used to develop DNAzyme-mediated assays for amplified detection of nucleic acids and proteins. Our discussions have highlighted the intrinsic properties of DNAzymes as enzymes (catalytic property) and ssDNA molecules (programmable and amplifiable properties).

As enzymes, DNAzymes have catalytic activity. Therefore, DNAzymes can be directly used for signal amplification, as HRP is used in ELISA. However, the catalytic activity of DNAzymes is lower than that of HRP, which impacts the detection sensitivity. Fortunately, the ease of conjugating DNAzymes onto nanoparticles facilitates the use of DNAzyme-conjugated nanoparticles for signal amplification, compensating for the lower sensitivity due to a lower catalytic activity. Various detection signals, such as electrochemical, colorimetric, chemiluminescence, and fluorescence, can be generated from the DNAzyme-catalyzed reactions.

The catalytic activity of DNAzymes requires the formation of well-defined secondary or tertiary structures. Disruption of such structures can deactivate DNAzymes, and this property has been used to construct catalytic beacons and split DNAzymes. Catalytic beacons generally use hairpins to cage one portion of the DNAzyme, preventing the sequence from forming desired secondary or tertiary structures. Upon target binding, the hairpins are opened, restoring the activity of DNAzymes for signal amplification. DNAzymes can be split into two inactive parts that can be reassembled into functional conformations upon target binding.

DNAzymes are ssDNA and can be amplified by various DNA amplification techniques; this is a main strategy to improve the detection sensitivity. Detection signals resulting from the amplified DNAzymes can be used for monitoring the amplification process. Isothermal DNA amplification techniques[207,208] are generally preferred for real-time monitoring and potential applications to point-of-care testing and on-site analysis. To date, RCA, SDA, NESA, EXPAR, and Exo-III-assisted signal amplification techniques have been used for amplification of DNAzymes.

As ssDNA strands, DNAzymes can be incorporated into noncovalent DNA catalytic reactions to achieve signal amplification. HCR and CHA are two DNA catalytic reactions commonly incorporated with the DNAzymes. Metastable hairpins are the key components designed for the HCR and CHA reactions. The incorporation of DNAzymes into HCR and CHA is usually accomplished by including partial DNAzymes to the ends of hairpins or caging DNAzymes into the stems of hairpins.

Although a variety of DNAzymes have been selected to achieve a broad scope of catalytic activities, peroxidase-mimicking DNAzymes and RNA-cleaving DNAzymes are mainly used for the amplified detection of nucleic acids and proteins. It is anticipated that exploration of other types of DNAzymes can introduce new venues for the development of assays for detection of nucleic acids and proteins. For instance, a Zn^{2+}-dependent ligation DNAzyme has been used to develop an autonomous ligation DNAzyme machinery for the amplified detection of a target DNA.[209] A key challenge of using DNAzymes for biosensing is their relatively low catalytic activities. Therefore, signal amplification of DNAzymes has been combined with other amplification techniques that rely on protein enzymes and noncovalent DNA catalytic reactions. However, such combinations either complicate the detection process or increase the detection time, which is not ideal for point-of-care testing. Although several studies have attempted to develop DNAzyme-only cascades for DNA detection with exponential amplification, high backgrounds limit their applications.[173,174] Thus, simple, rapid, specific, and sensitive DNAzyme-mediated assays are still required. Considering that DNAzymes have been effectively used for *in situ* amplified detection of miRNA in live cells, we envision the use of DNAzymes for amplified detection of proteins and other molecules in live cells and tissues in the near future.

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