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REVIEW ARTICLE

Development of DNA Biosensors Based on DNAzymes and Nucleases

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ABSTRACT

DNA biosensors play important roles in environmental, medical, industrial and agricultural analysis. Many DNA biosensors have been designed based on the enzyme catalytic reaction. Because of the importance of enzymes in biosensors, we present a review on this topic. In this review, the enzymes were divided into DNAzymes and nucleases according to their chemical nature. Firstly, we introduced the DNAzymes with different function inducing cleavage, metalation, peroxidase, ligation and allosterism. In this section, the G-quadruplex DNAzyme, as a hot topic in recent years, was described in detail. Then, the nucleases-assisted signal amplification method was also reviewed in three categories including exonucleases, endonucleases and other nucleases according to the digestion sites in DNA substrates. In exonucleases section, the Exo I and Exo III were selected as examples. Then, the DNase I, BamH I, nicking endonuclease, S1 nuclease, the duplex specific nuclease (DSN) and RNases were chosen to illustrate the application of endonucleases. In other nucleases section, DNA polymerases and DNA ligases were detailed. Last, the challenges and future perspectives in the field were discussed.

KEYWORDS

DNAzymes; DNA biosensor; enzyme catalytic reaction; nucleases

Introduction

Biosensors rely on biological molecules for target recognition, and they are widely used in environmental, medical, industrial and agricultural analysis.[1–3] The most successful example is the glucose meters that can utilize glucose oxidase to recognize glucose.[4] Each biosensor has two components for target recognition and signal transduction, respectively. Enzymes, antibodies, and DNA are often used for target recognition due to their outstanding molecular recognition characteristics.[5,6] Among them, DNA has been extensively researched because of its stable chemical properties, cost-effectiveness, and easy modification. In these DNA biosensors, many strategies have been used to improve the sensitivity or make the design convenient, such as hybridization chain reaction (HCR),[7] catalytic hairpin assembly (CHA),[8] and enzyme catalytic reaction.[9,10] In these strategies, the enzyme catalytic reactions are commonly used methods, because the enzymes usually have unique biological recognition capability and excellent catalytic activity.[11] The most common and successful example is Polymerase Chain Reaction (PCR). Recently, a lot of DNA biosensors have been designed based on the enzyme catalytic reaction.[12–14] Due to the importance of this strategy, several reviews have already been published on this topic.[15] These reviews mainly focused on the types of target molecules,[16] the modes of output signal,[17] and the types of enzymes.[18] However, there are few reviews on classification of enzyme according to their chemical nature. In this review, the enzymes were divided into DNAzymes and nucleases (Figure 1). In the DNAzymes section, DNAzymes with different function inducing cleavage, metalation, peroxidase, ligation and allosterism were discussed. The G-quadruplex DNAzyme, as a hot topic in recent research, was introduced in detail. In the nucleases section, the nucleases were divided into exonucleases, endonucleases and other nucleases according to the digestion sites in DNA substrates. This review might help readers better understand the applications of enzyme catalytic reaction strategy in the construction of DNA biosensors.

DNAzyme

DNAzyme is a special DNA with catalytic activity, which is formed by deoxyribonucleotides. In fact, the ribozyme (RNAzyme) was discovered earlier than DNAzyme. In 1982, the ribosomal RNA intervening sequence of Tetrahymena which could splice itself was revealed.[19] In 1983, the RNA part of ribonuclease P was found to have catalytic activity.[20] These discoveries changed the notion of enzyme that all enzymes were proteins. In 1994, the first DNAzyme was

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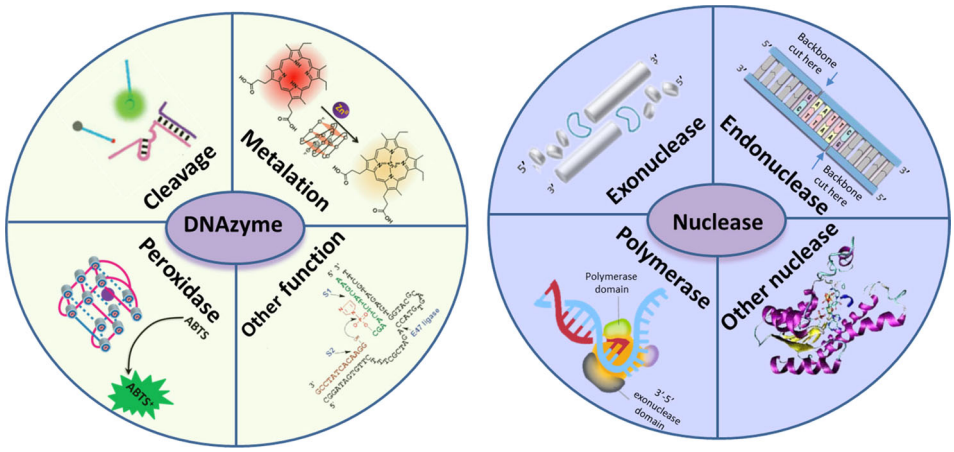

Figure 1. The enzymes were used in DNA biosensors.

isolated via *in vitro* selection by Breaker and Joyce.[21] Since then, several DNAzymes were screened to accelerate the biological reactions. Since DNA is more stable and cheaper than RNA, DNAzyme seems more attractive than ribozyme to be used in designing biosensors. The selected DNAzymes usually export the signal of biosensor by oxidizing substrate, cleaving DNA or RNA.

DNAzyme inducing cleavage reaction

Many studies have shown that a lot of DNAzymes need metal ions as cofactors to improve cleavage activity. When the first DNAzyme, named GR5, was found by Breaker and Joyce in 1994, the accompanying fact was that GR-5 needed Pb^{2+} as a cofactor to cleave the RNA substrate.[21] Thus, from then on, the cleavage reaction of DNAzymes provided a possibility for the detection of metal ions. As the first example, Li and Lu used 8-17 DNAzyme (named 17E) to detect Pb^{2+} in 2000.[22] At the beginning, the substrate strand 17DS was modified by fluorophore in its 5′-end and the quencher was modified at the 3′-end of 17E. In the absence of Pb^{2+}, no fluorescent signal was observed, because 17DS and 17E could form duplex structure to close the fluorophore with the quencher. In the present of Pb^{2+}, the 17DS was cleaved to release the fluorophore. Then, the fluorescent signal could be recorded. This biosensor showed the more than 80 fold selectivity for Pb^{2+} from other metal ions. Ten years later, the same group improved the selectivity for Pb^{2+} based on the first DNAzyme (GR-5) discovered in the field.[23] In this biosensor, to reduce the background signal, two quenchers were modified at the 3′-end of the substrate chain and the enzyme chain, respectively (Figure 2a). Similar to the previous 8-17 DNAzyme Pb^{2+} method, no fluorescence was generated without Pb^{2+}. In the present of Pb^{2+}, the fluorophore was released by the cleavage reaction. This biosensor showed much higher metal ion selectivity (about 40 000 fold) than the 8-17 DNAzyme Pb^{2+} method.

The 8-17 DNAzyme was also combined with other materials to develop novel Pb^{2+} biosensor. Wang et al. used 8-17 DANzyme and gold nanorods (GNRs) to design a Pb^{2+} biosensor.[24] In this biosensor, the fluorophore FAM was labeled on the substrate strand. In the absence of Pb^{2+}, the 8-17 DNAzyme with negative charge was adsorbed on the surface of GNRs (with positive charge) through strong electrostatic interactions. This resulted in the fluorescence of FAM was quenched by FRET between FAM and GNRs. When Pb^{2+} was added, the FAM-labeled substrate strand was cleaved to weaken the electrostatic interactions between the FAM-labeled DNA and GNRs. Then, the FAM-labeled DNA was released to emit the fluorescence signal (Figure 2b). This biosensor showed a high sensitivity for Pb^{2+} with a linear range from 0.1 nM to 100 nM and a detection limit of 61.8 pM.

Since DNAzymes can be screened rapidly *in vitro*, many target DNAzymes have been further discovered. For example, Liu group obtained an EtNa-C5T DNAzyme which was highly selective for calcium by systematic mutating the original EtNa DNAzyme. The selectivity of EtNa-C5T for Ca^{2+} was about 20-fold than Na^{+}. Subsequently, a highly sensitive and selective biosensor was designed for the detection of calcium in milk based on EtNa-C5T DNAzyme.[27] The DNAzyme 39E that used UO_2^{2+} as cofactor was screened. 39E exhibited over 1-million-fold higher selectivity for UO_2^{2+} over other metal ions. Based on this DNAzyme, UO_2^{2+} biosensor was developed with a detection limit of 45 pM.[28] In 2020, the DNAzyme Ni03I, which was specific to Ni^{2+}, was screened for Ni^{2+} detection.[29] Except for metal ions, the DNAzyme targeting for bacteria was also screened. Gu et al. used the crude extracellular matrix (CEM) of *V. Anguillarum* as the target to select DNAzyme. Through seven rounds of screening, VAE-2 was obtained with high specificity and activity. Based on VAE-2, the *V. anguillarum* biosensor was developed with a detection limit of 4000 cfu/mL.[30] In addition to detecting corresponding cofactors through specific DNAzymes, targets can also be detected by forming of specific DNAzymes. For example, Qi et al. reported an Hg^{2+} biosensor based on Mg^{2+}- specific DNAzymes.[25] It is difficult to obtain Hg^{2+}-specific DNAzyme due to the weak interaction between Hg^{2+} and phosphate of DNA.[31] Meanwhile, the Hg^{2+} could specifically bind to thymine (T) to form a T-Hg-T mismatch.[32] In this biosensor, a successful Mg^{2+}-dependent DNAzyme was utilized with a highly catalytic activity.[33] The Mg^{2+}-

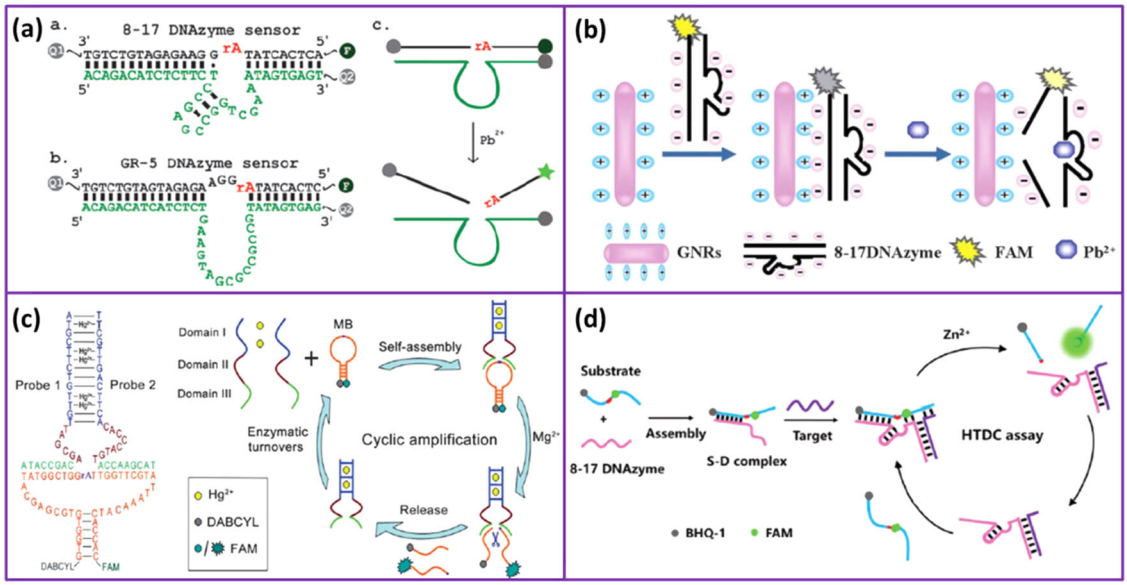

Figure 2. (a) A scheme of Pb^{2+} biosensor based on GR-5 DNAzyme. Reprinted with permission from Lan et al.[23] Copyright 2010 Royal Society of Chemistry. (b) A scheme of Pb^{2+} biosensor based on 8-17 DANzyme and gold nanorods (GNRs). Reprinted with permission from Wang et al.[24] Copyright 2011 Royal Society of Chemistry. (c) A scheme of Hg^{2+} biosensor based on forming of Mg^{2+}- specific DNAzymes. Reprinted with permission from Qi et al.[25] Copyright 2012 Royal Society of Chemistry. (d) A scheme of DNA biosensor based on forming of Zn^{2+}- specific DNAzymes. Reprinted with permission from Wang et al.[26] Copyright 2018 Royal Society of Chemistry.

dependent DNAzyme was split into two fragments (probe1 and probe 2). In the present of Hg^{2+}, the probe1 and probe 2 can form a complete active DNAzyme which can hybridize with a molecular beacon (MB) substrate. With the Mg^{2+}, the MB substrate was cut to release fluorophore. As a result, the DNAzyme became free and could bind another MB substrate to release more fluorophore (Figure 2c). At last, the intensity of fluorescent signal could be observed. The highly sensitive biosensor was realized with the detection limit down to 0.2 nM.

In 2019, Zn^{2+}- dependent DNAzyme was used to detect nucleic acids by Wang group.[26] In this biosensor, the substrate DNA was labeled by fluorophore (FAM) and quencher (BHQ-1). Without target DNA, the substrate DNA and DNAzyme could hybridize to form S-D complex with an unmatched short binding arm. In this S-D complex, the DNAzyme was inactive due to the unstably binding. In the present of target DNA, it could hybridize with DNAzyme and substrate to stabilize short arm. Then, the DNAzyme was activated to digest substrate DNA. In this process, the FAM, BHQ-1 and DNAzyme@target were separated. The free DNAzyme@target entered next cycle to bind another substrate and release more FAM (Figure 2d). At last, the detectable minimum target concentration (1 pM) of this biosensor was defined with an amplified fluorescent signal.

G-quadruplex DNAzyme catalyzing porphyrin metalation

A guanine-rich DNA sequence could form a special cage structure by non-canonical Hoogsteen-type base pairing which was named G-quadruplex structure.[34,35] This structure could be stabilized with metal ions such as K^+, Na^+, Pb^{2+} by coordinating with the oxygen of guanines.[36–39] In 1996, a guanine-rich DNA with 33 nucleotides was obtained through *in vitro* screening by Sen and colleagues.[40] This DNA was named PS5.ST1, which could catalyze the insertion of Cu^{2+} and Zn^{2+} into mesoporphyrin IX (MPIX) like ferrochelatase. Subsequently, much of the G-quadruplex DNA was screened for porphyrin metallization, such as PS5.M,[41] 18-mer,[42] and Nm2.[43] After metalation, the fluorescence and absorption spectra of porphyrin could be changed.[44,45] Therefore, G-quadruplex DNAzyme catalytic porphyrin metalation can be used to design biosensor to detect metal ions.

In 2013, Wang group reported a label-free fluorescent biosensor to detect Cu^{2+} based on the G-quadruplex DNA (PS5.M) accelerating Cu^{2+} insertion into protoporphyrin IX (PPIX).[46] In aqueous solution, the fluorescence intensity of PPIX was very low with micellar morphology.[47,48] In the absence of Cu^{2+}, the fluorescence intensity PPIX was enhanced by binding PS5.M. When Cu^{2+} was added, the PS5.M could catalyze the insertion of Cu^{2+} into PPIX to form CuPPIX which could not emit fluorescence even if binding with G-quadruplex. With the increase of Cu^{2+}, the fluorescence of PPIX decreased gradually. Thus, the highly sensitive Cu^{2+} biosensor was developed under the catalysis of G-quadruplex DNAzyme with a detection limit of 3.0 nM and a linear range from 8 nM to 2 mM.

In 2019, Liu group found that the G-quadruplex DNA T30695 could catalyze Cu^{2+} and Zn^{2+} insertion into mesoporphyrin IX (MPIX) with Pb^{2+} as a cofactor by screening a few metals and G-rich DNA sequences.[49] After the porphyrin metalation, the fluorescence of MPIX was quenched by forming CuMPIX. But for Zn^{2+}, a new peak appeared at 583 nm and the original peak at 623 nm decreased with forming ZnMPIX. Subsequently, the ZnMPIX was chosen to design biosensor for the detection of Pb^{2+} with the fluorescence ratio (583 nm/623 nm) as a signal reporter (Figure 3a). In the present of Pb^{2+}, Pb^{2+} could induce T30695 to form Pb^{2+}-G4 structure which could further catalyze the Zn^{2+}

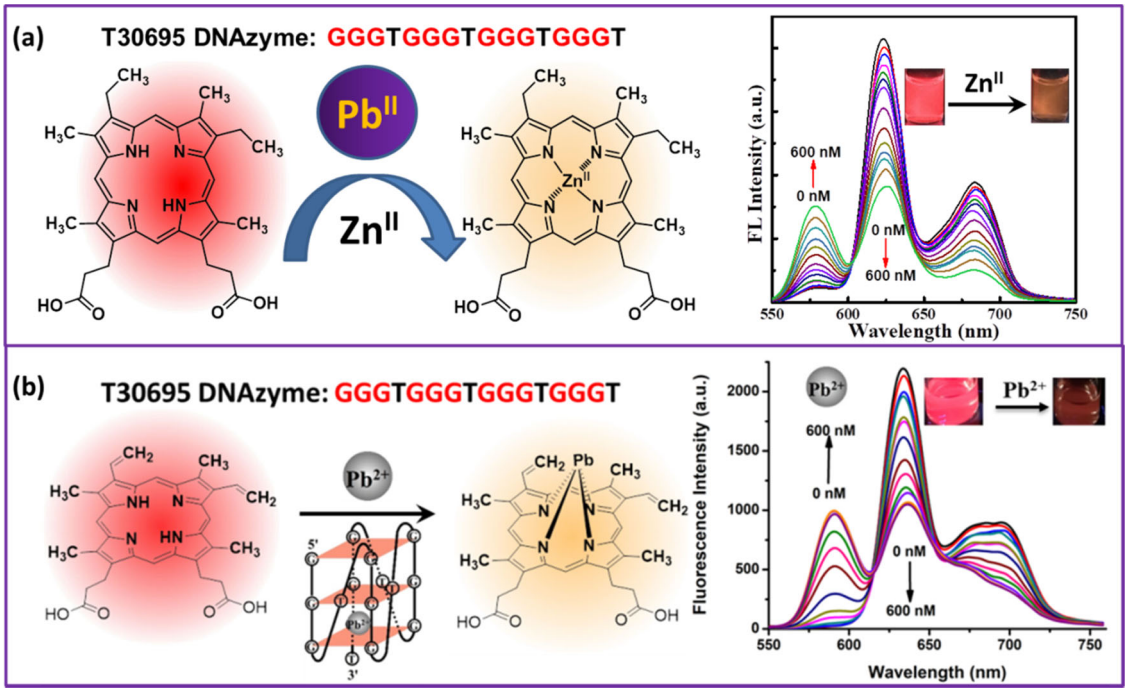

Figure 3. (a) A scheme of Pb^{2+} biosensor based on T30695 catalyzed Zn^{2+} insertion into MPIX with Pb^{2+} as a cofactor. Reprinted with permission from Peng et al.[49] Copyright 2019 American Chemical Society. (b) A scheme of Pb^{2+} biosensor based on T30695 catalyzed Pb^{2+} insertion into PPIX with another Pb^{2+} as a cofactor. Reprinted with permission from Yang et al.[50] Copyright 2021 Wiley-VCH.

insertion into MPIX to produce a fluorescence ratio signal. When the concentrations of T30695, Zn^{2+} and MPIX were all fixed, the fluorescence ratio signal increased along with the increase of Pb^{2+} concentration. Thus, Pb^{2+} could be detected by this biosensor with the detection limit of 23.5 nM.

Subsequently, through studying different porphyrin substrates, we found that T30695 could catalyze Pb^{2+} insertion into PPIX with another Pb^{2+} as a cofactor.[50] But for MPIX, the reaction did not happen. The structures of PPIX and MPIX were very similar, except that PPIX had two more double bonds than MPIX. The possible reason might be that the slightly larger conjugated structure of PPIX was more favorable for accepting the larger Pb^{2+}. When the Pb^{2+} was inserted into PPIX, the fluorescence spectrum of PPIX was blue shifted from 635 nm to 590 nm. Thus, the fluorescence ratio (590 nm/635 nm) could be used as a signal reporter to detect Pb^{2+} (Figure 3b). Interference experiments showed that the biosensor was highly selective to Pb^{2+}. Meanwhile, titration experiments showed that the biosensor was very sensitive with a detection limit of 1.19 nM Pb^{2+}.

G-quadruplex DNAzyme as peroxidase

In 1996, when Sen and colleagues studied porphyrin metallization catalyzed by G-quadruplex, they found that hemin could inhibit this reaction as a competitive inhibitor with high affinity for G-quadruplex.[40] Subsequently, in 1998, Sen group found that the G-quadruplex was similar with protein peroxidases (such as horseradish peroxidase), which could enhance the peroxidase activity of hemin. The catalytic rate of the G-quadruplex/hemin complex was 250 fold higher than that of hemin alone.[51] In addition, some research reported that parallel G-quadruplex/hemin indicated higher peroxidase activity than antiparallel G-quadruplex/hemin. The reason might be that the protruding ring on the outer antiparallel G-quadruplex reduced the binding of hemin, while the parallel structure was not affected by this steric hindrance.[52] Like protein peroxidases, G-quadruplex/hemin could oxidize some substrate to produce signal changes, such as color signal,[53,54] chemiluminescence signal,[55] and electrochemistry signal.[56] Since G-quadruplex was more stable, economical and easy to modify than protein peroxidases, it could be more often used in biosensors to amplify signal instead of the traditional protein peroxidase.[57]

Zhou et al. reported an Ag^{+} biosensor based on G-quadruplex/hemin DNAzyme amplified colorimetric method.[58] In this biosensor, one unlabeled oligonucleotide DNA, which contained G-quadruplex sequence at the 3′-end, was used. In the absence of Ag^{+}, the oligonucleotide DNA could spontaneously form a double stranded structure by classical base complementation (Form 1 in Figure 4a). In this double stranded structure, the G-quadruplex sequence was covered and could not form G-quadruplex structure. Accordingly, the peroxidase activity of hemin could be improved and no signal was observed. In the present of Ag^{+}, the sequence of 5′-end could form another duplex structure by C-Ag(I)-C mismatches (Form 2 in Figure 4a). This structure caused G-quadruplex sequence to be released. The free G-quadruplex could bind hemin to form G-quadruplex/hemin DNAzyme which could oxidize colorless substrate 2,2′-azinobis (3-ethylbenzothiozoline)-6-sulfonic acid (ABTS) to become green $ABTS^{+}$ which could be observed by naked eyes (Figure 4a). Thus, the simple colorimetric biosensor was developed with detection limit of 6.3 nM.

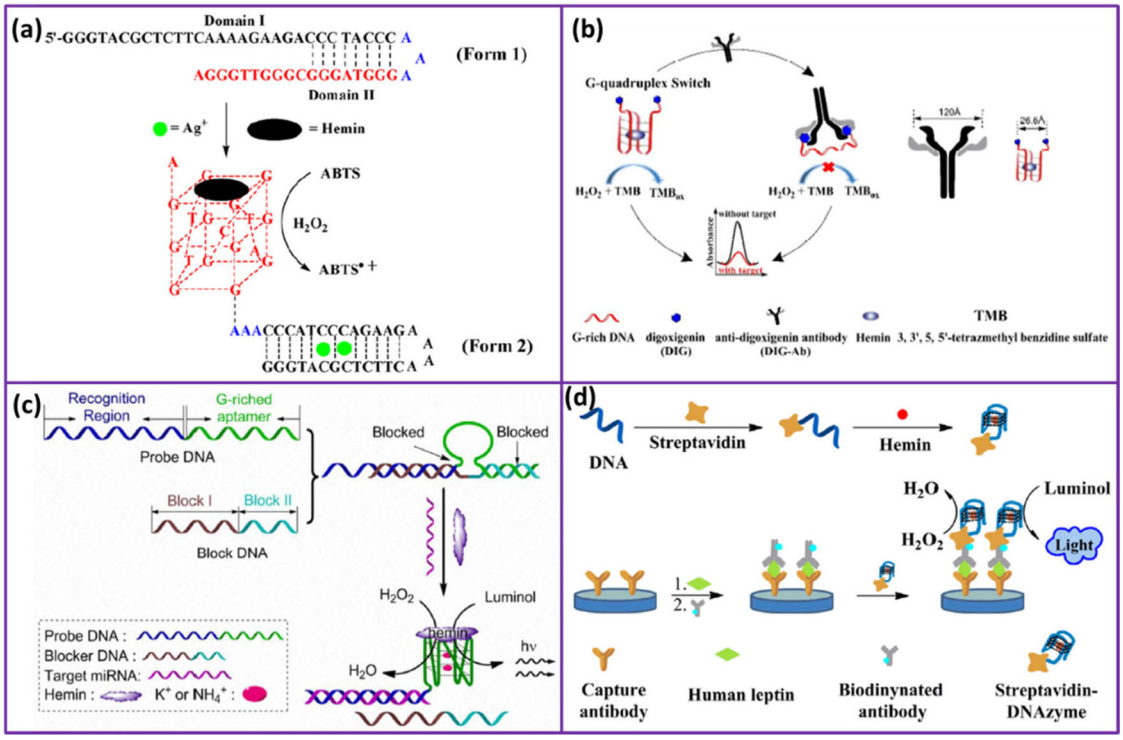

Figure 4. (a) A scheme of Ag^{+} biosensor based on G-quadruplex/hemin DNAzyme amplified colorimetric method. Reprinted with permission from Zhou et al.[58] Copyright 2010 Elsevier. (b) A scheme of DIG-Ab detection based on G-quadruplex/hemin DNAzyme. Reprinted with permission from Hu et al.[59] Copyright 2017 Royal Society of Chemistry (c) A scheme of miRNA biosensor based on G-quadruplex DNAzyme. Reprinted with permission from Li et al.[60] Copyright 2017 American Chemical Society (d) Analytical procedure of DNAzymes-catalyzed chemiluminescent immunoassay for the detection of human leptin. Reprinted with permission from He et al.[61] Copyright 2013 Elsevier.

In addition to ABTS, G-quadruplex/hemin DNAzyme could also oxidize other substrates such as 3,3,5,5′- tetramethylbenzidine (TMB). Li group designed a colorimetric biosensor to detect antibody based on protein-induced unfolding of G-quadruplex switch.[59] In this biosensor, two digoxigenin (DIG) molecules (antigen) were modified on both ends of the G-quadruplex DNA. In the beginning, the G-quadruplex DNA could form G-quadruplex structure very well with a size of 26.5 Å in reaction buffer. This structure could bind hemin to form hemin/G-quadruplex DNAzyme. Afterwards, the colorless TMB was oxidized to produce blue color. When the anti-digoxigenin antibody (DIG-Ab) was added, it could bind two digoxigenin (DIG) molecules. Because the distance of these two binding domains was about 120 Å, the G-quadruplex structure was stretched to result in a decrease of catalytic activity (Figure 4b). Under the optimal conditions, the simple, visual biosensor showed a detection limit of 39 pM and a linear range of 0.5 nM − 1 μM.

In 2017, Li et al. reported a both-end blocked chemiluminescent biosensor to detect miRNA based on the luminol-H_2O_2 chemiluminescence (CL) reaction system.[60] In this biosensor, probe DNA and block DNA were designed. The probe DNA contained a target recognition region and a G-quadruplex sequence. In the absence of target miRNA, the both-end of probe DNA was blocked by block DNA. Thus, the G-quadruplex/hemin DNAzyme could not be formed. The luminol could not be oxidized to generate CL signals. In the present of miRNA, it hybridized with recognition region of probe DNA to replace block DNA. Then, the block DNA was separated from probe DNA and G-quadruplex sequence was released to form G-quadruplex structure (Figure 4c). Last, luminol was oxidized to generate a strong CL signals. The both-end blocked strategy provided low background signaling. This low-background chemiluminescent biosensor had a detection limit of 8 pM and a linear concentration range of 25 pM to 1 nM.

The G-quadruplex DNAzyme could be combined with other materials to exploit more flexible biosensor designs, such as antibody. He et al. reported an ultrasensitive chemiluminescent immunosensor for the detection of human leptin based on G-quadruplex/hemin DNAzyme signal amplifier.[61] In this biosensor, a G-quadruplex DNA labeled with streptavidin was designed to oxidize luminol thus to give off light. At the beginning, capture antibody (the primary antibody) was bound to the 96-well plates which could bind antigen (human leptin) specifically. In the presence of human leptin, the biotinylated antibody (the secondary antibody) was combined to form the sandwich-type complex by binding another antigen epitope. Then, the streptavidin-DNAzyme was assembled to the sandwich complex by the interactions between the streptavidin and biotin. Finally, the signal was obtained by oxidizing the chemiluminescent reaction of luminol (Figure 4d). This chemiluminescent immunosensor showed high sensitivity and selectivity with a linear range of 10-1000 pg/mL and a detection limit of 1.9 pg/mL.

In addition, G-quadruplex binding with Cu^{2+} also showed peroxidase activity, like that with hemin.[62,63] G-quadruplex -Cu^{2+} DNAzyme has also been used in the

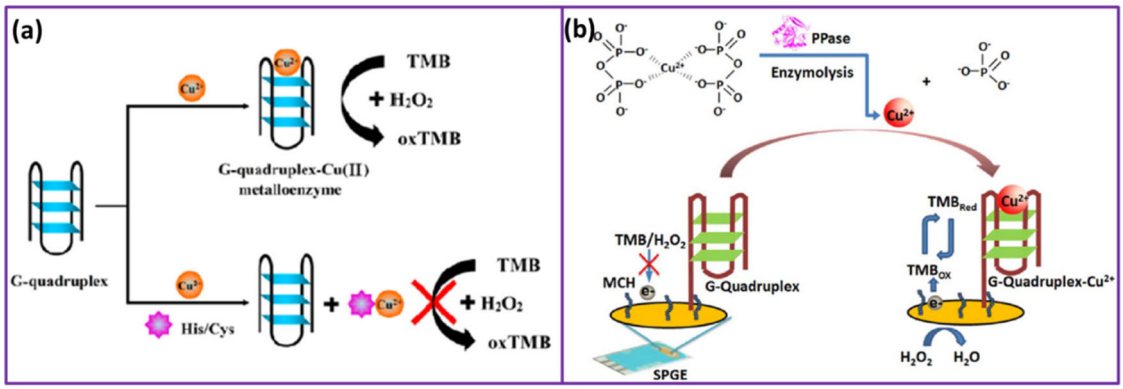

Figure 5. (a) A scheme of His/Cys biosensor based on G-quadruplex -Cu^{2+} DNAzyme. Reprinted with permission from Wu et al.[64] Copyright 2016 American Chemical Society (b) A scheme of pyrophosphatase electrochemical biosensor based on G-quadruplex -Cu^{2+} metalloenzyme. Reprinted with permission from Wang et al.[65] Copyright 2018 Elsevier.

design of biosensors. Wu et al. developed a colorimetric method to detect histidine (His) and cysteine (Cys) based on G-quadruplex -Cu^{2+} DNAzyme (Figure 5a).[64] In the absence of His and Cys, Cu^{2+} could bind with G-quadruplex to form metalloenzyme which could oxidize TMB to produce blue color. When both His and Cys were added, Cu^{2+} was chelated and the metalloenzyme was then destroyed. Accordingly, the TMB could not be oxidized. This simple biosensor showed a detection limit of 10 nM for His and 5 nM for Cys. Based on this DNAzyme, Wang and coworkers designed an electrochemical biosensor to detect pyrophosphatase.[65] At the beginning, the Cu^{2+} coordinated with pyrophosphate (PPi) to form PPi-Cu^{2+} complex which hindered the Cu^{2+} from binding with G-quadruplex. When pyrophosphatase was added, it can trigger the hydrolysis of PPi-Cu^{2+} to release Cu^{2+}. Then, the free Cu^{2+} bound with G-quadruplex to form G-quadruplex -Cu^{2+} DNAzyme which could further oxidize TMB to emit electrical signal (Figure 5b). This electrochemical biosensor suggested a linear range of 1.0-50.0 mU/mL and a detection limit of 0.6 mU/mL.

DNAzyme with other function

In addition to cleavage, metalation, peroxidase function, DNAzyme has other characteristics such as ligation and allosterism. These characteristics also can be used to design biosensors. The function of ligation DNAzyme is the opposite of that of cleavage DNAzyme. In 2005, Ellington group used a deoxyribozyme ligase and rolling circle amplification (RCA) to detect ATP.[66] In this biosensor, ATP could help DNAzyme form an active structure which could connect the substrate DNA end to end to form a circular template. Then, the RCA reaction was triggered and the signal was recorded. In 2007, Cu^{2+}-dependent DNA ligation DNAzyme (E47) was utilized to detect Cu^{2+} by Juewen Liu and Yi Lu.[67] This biosensor was a colorimetric method with gold nanoparticles as a reporter. The results showed that the sensitivity of this ligation DNAzyme-based biosensor with a detection limit of 5 μM was higher than that of cleavage DNAzyme biosensor. Subsequently, the detection limit had been increased to 1 nM by Wang et al.[68] They used hemin/G-quadruplex to catalyze the oxidation of ABTS instead of the aggregation of Au nanoparticles (Figure 6a). In the presence of Cu^{2+}, E47 was activated to catalyze the connection of the substrate DNA. As a result, the DNA became an intact structure including DNAzyme, substrate and G-quadruplex. This intact structure could withstand damage from low salt. Thus, the G-quadruplex was not destroyed. Another example was Zn^{2+}-dependent ligation DNAzyme. In 2012, Lu et al. reported a new method to detect DNA based on this ligation DNAzyme.[69] In this method, the Zn^{2+}-dependent ligation DNAzyme was split (Figure 6b). With the help of the target DNA, the divided DNAzyme could form a complete and active enzyme. Then, they could catalyze the ligation of the substrate DNA. Subsequently, the substrate DNA could hybridize with molecular beacons to emit fluorescence. And the DNAzyme entered next cycle to link more substrate DNA. This biosensor showed high sensitivity with a detection limit of 20 nM.

There were many examples of allosteric responses for proteases and gene regulatory proteins. It had similar properties for DNAzyme.[72] For example, the DNAzyme could be inactivated by hybridization of complementary chain. When the complementary chain was removed, the DNAzyme could fold properly again. In addition, some cofactors could also promote or inhibit the folding of DNAzyme to achieve allosterism.[73] Li et al. designed a Pb^{2+} biosensor based on Pb^{2+}-induced allosteric G-quadruplex DNAzyme.[70] In the absence of Pb^{2+}, the G-quadruplex was stabilized by K^+. This G-quadruplex could bind hemin to form hemin/G-quadruplex complex which could catalyze the oxidation of ABTS or luminol. When the Pb^{2+} was added, the Pb^{2+}-quadruplex was formed (Figure 6c). The Pb^{2+}-G-quadruplex indicated a more compact and smaller quadruplex structure than that of K^+, and Pb^{2+}-G-quadruplex could not bind hemin.[39] Thus, no signal was observed. The detection limit of this highly sensitive biosensor was 32 nM for colorimetry and 1 nM for chemiluminescence. Another example was that allosteric of UO_2^{2+}-specific DNAzyme was utilized to detect Hg^{2+} by Juewen Liu and Yi Lu.[71] In this biosensor, the stem loop part of UO_2^{2+}-specific DNAzyme was replaced by PolyT (Figure 6d). In the help of Hg^{2+}, the stem loop part could be

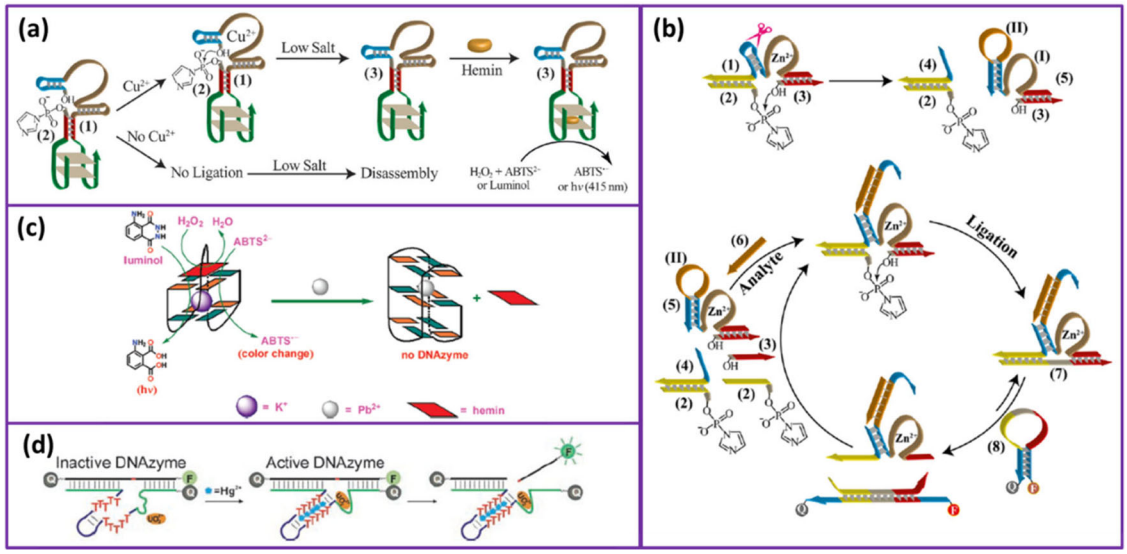

Figure 6. (a) A scheme of Cu^{2+} biosensor based on Cu^{2+}-dependent ligation DNAzyme. Reprinted with permission from Wang et al.[68] Copyright 2012 Wiley. (b) A scheme of DNA biosensor based on Zn^{2+}-dependent ligation DNAzyme. Reprinted with permission from Lu et al.[69] Copyright 2012 American Chemical Society. (c) A scheme of Pb^{2+} biosensor utilizing Pb^{2+}-induced allosteric G-quadruplex DNAzyme. Reprinted with permission from Li et al.[70] Copyright 2010 American Chemical Society. (d) A scheme of Hg^{2+} biosensor utilizing Hg^{2+}-induced allosteric UO_2^{2+}-ion-specific DNAzyme. Reprinted with permission from Liu et al.[71] Copyright 2007 Wiley.

stabilized by T-Hg(II)-T mismatch to form a valid conformation. The active DNAzyme could cleave DNA substrate labeled by fluorescence and quencher to emit signal. This allosteric DNAzyme biosensor was ultrahigh sensitivity and selectivity with the detection limit 2.4 nM.

Nuclease

In addition to DNAzyme, the substrate of some protein is also DNA and these proteins are called nucleases. Nucleases are important analytical enzymes in DNA biosensor. The restriction sites of some nucleases are specific. Some nucleases are not very specific in sequence, but they can recognize particular structural features of their DNA substrates.[74] Since the generation and disappearance of restriction enzyme sites can be caused by the addition of targets, these nucleases can be employed in biosensors to amplify signal. According to the digestion sites in DNA substrates, nucleases can be divided into exonucleases, endonucleases and other nucleases. In this section, we illustrated the applications of these nucleases in biosensors.

DNA exonuclease

Until now, many DNA exonucleases have been discovered, such as Exo I, Exo III, T7 Exo VI, Exo VII and λExo. These exonucleases have specific preferences and functions. For example, the substrate of Exo I and Exo VII is single strands DNA, and the substrate of Exo III, T7 Exo VI and λExo is duplex DNA. Since Exo I and Exo III were economical and commercial, in this review, they were selected as examples to illustrate the applications of nucleases in biosensors.

Exo I commonly removes the nucleotides from single-stranded DNA according to the 3′ to 5′ direction, but it does not work on double strands DNA or RNA.[75] Many studies have applied Exo I to design signal conversion strategies to achieve ultra-sensitive detection of nucleic acids, proteins, antibiotics and other biological molecules.[76] Chen et al. developed a microchip electrophoresis biosensor to detect kanamycin based on Exo I -assisted signal amplification.[77] In this biosensor, an aptamer of kanamycin (S-DNA) was used as a recognizer which could form duplex structure (R-DNA) with the hairpin DNA (H-DNA) (Figure 7a). In the presence of kanamycin, it could bind with S-DNA to form S-DNA- kanamycin complex, and the S-DNA was released. The formed R-DNA then became the original H-DNA. Thus, the size of DNA became smaller. The size signal could be recorded by microchip electrophoresis. The S-DNA- kanamycin complex could be further digested to release kanamycin by Exo I. Then, the kanamycin entered the next circle reaction to produce more small size H-DNA. As the reaction progressed, the fluorescence intensity of H-DNA increased and the fluorescence intensity of R-DNA decreased. The fluorescence intensity ratio of H-DNA and R-DNA was directly proportional to the kanamycin concentration. In this biosensor, the Exo I -assisted signal amplification and targeted recovery were successfully achieved. The ratio metric biosensor indicated the high sensitivity with a linear range from 0.5 pg/mL to 10 ng/mL and a detection limit of 150 fg/mL.

In 2012, Yuan et al. designed a sensitive label-free biosensor to detect Hg^{2+} by using Exo I.[78] In the absence of Hg^{2+}, single-stranded T-rich DNA was digested by Exo I. Even if the DNA dye was added, no fluorescence signal was observed. When the Hg^{2+} was added, the T-rich DNA became duplex structure through T- Hg^{2+}-T mismatches which were more difficult to be digested by Exo I. Subsequently, the duplex structure could bind with DNA dye to emit an intense fluorescence signal (Figure 7b). In this biosensor, two single-stranded DNAs (ssDNA) TT-21 and TT-44 were compared. The results showed that the TT-

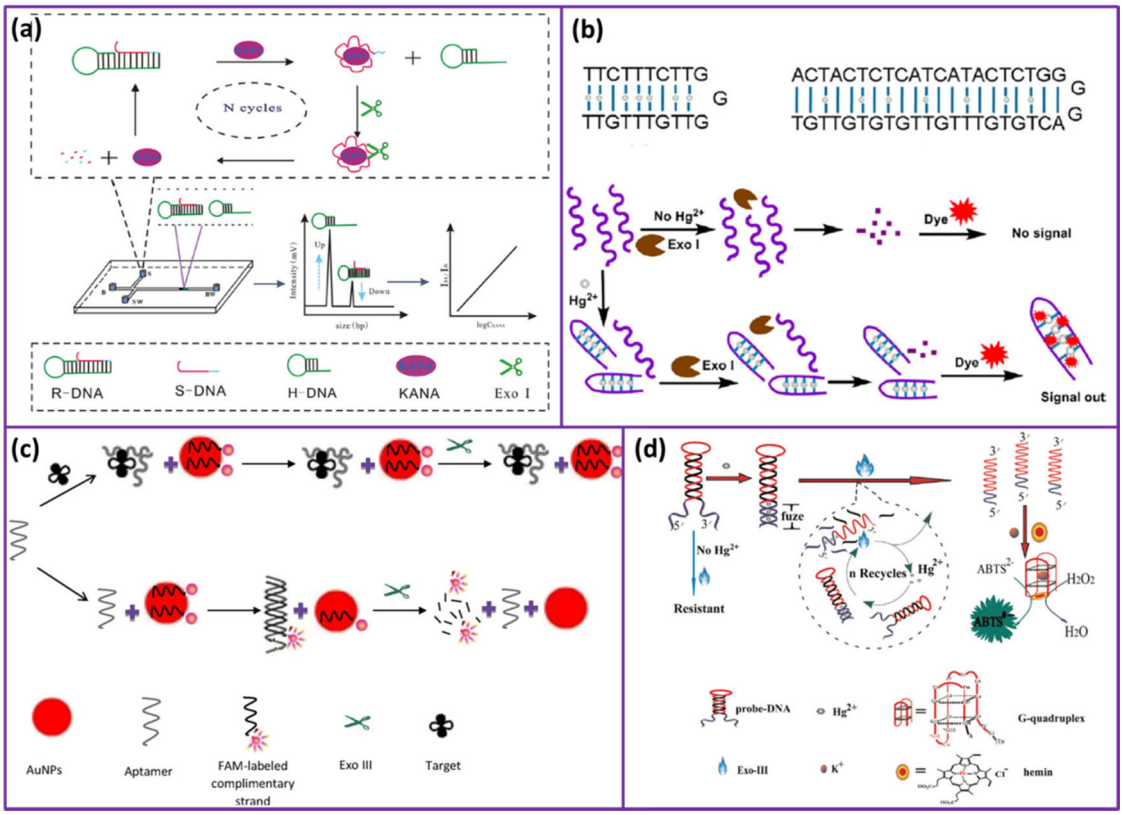

Figure 7. (a) A scheme of microchip electrophoresis biosensor to detect kanamycin based on Exo I -assisted signal amplification. Reprinted with permission from Chen et al.[77] Copyright 2018 Elsevier. (b) A scheme of Hg^{2+} biosensor by using Exo I. Reprinted with permission from Yuan et al.[78] Copyright 2012 Elsevier. (c) A scheme of kanamycin biosensor based on catalytic recycling activity of exonuclease III and gold nanoparticles. Reprinted with permission from Ramezani et al.[79] Copyright 2016 Elsevier (d) A scheme of Hg^{2+} biosensor based on Exo III-assisted target recycling and G-quadruplex DNAzyme amplification. Reprinted with permission from Ren et al.[80] Copyright 2015 Elsevier.

44 DNA was higher sensitivity than TT-21 DNA. Under optimal conditions, this biosensor had a high sensitivity with the limit of detection 15 nM.

Unlike Exo I, Exo III is specific for double-stranded DNA which also removes nucleotides from 3′ to 5′ direction. It cannot digest single-stranded DNA or 3′ -overhang ends of DNA with at least four-bases long.[81,82] In 2016, Ramezani et al. designed a sensitive fluorescent biosensor to detect kanamycin based on Exo III -assisted signal amplification.[79] In this biosensor, the aptamer of kanamycin was used to recognize kanamycin. The complementary strand labeled fluorophore FAM was designed to emit fluorescence signal. At the beginning, the FAM-labeled DNA was absorbed on the gold nanoparticle (AuNPs) to cause the quenching of fluorescence. In the absence of kanamycin, the free aptamer could compete to adsorb its complementary strands from gold nanoparticles to form duplex structure. When Exo III was added, the complementary strands in the duplex structure were digested. Subsequently, the aptamer and FAM were released. On one hand, the aptamer could enter the next cycle to release more FAM. On the other hand, the fluorescence of FAM was recovered due to the longer distance from AuNPs. When kanamycin was added, the free aptamer was bonded by kanamycin, which resulted in the aptamer lost the ability to combine complementary strands. Thus, the fluorescence of FAM was not recovered, even if Exo III was added (Figure 7c). This turn-off fluorescent biosensor showed high selectivity and sensitivity with a detection limit of 321 pM.

In addition to a single enzyme, multiple enzymes combination can also be used to design biosensors. For example, Ren et al. reported a label-free colorimetric biosensor for the detection of Hg^{2+} based on the Exo III-assisted target recycling and G-quadruplex DNAzyme amplification.[80] In this biosensor, a hairpin DNA probe containing a G-quadruplex DNA was designed. Meanwhile, the poly T was added at both the 5′- and 3′- end of this DNA probe. Without Hg^{2+}, the DNA probe formed DNA duplex with unpaired ends which could prevent Exo III digestion. And the G-quadruplex DNA was covered by its complementary strand to hinder G-quadruplex structure forming. Thus the ABTS could not be oxidized and no signal was observed. When Hg^{2+} was added, the unpaired poly T ends could be fused by thymine- Hg^{2+}- thymine (T- Hg^{2+}-T) mismatches, and then could be digested by Exo III from the blunt 3′ to 5′. After the duplex stem was fully consumed, the G-quadruplex DNA and Hg^{2+} were released. The G-quadruplex DNA could form G-quadruplex structure which bound hemin to oxidize ABTS. And the released Hg^{2+} could bind with another DNA probe to release more G-quadruplex DNA (Figure 7d). In the help of Exo III, the numerous G-quadruplexes were produced which bound hemin to form G-quadruplex DNAzymes. Then, the signal was further amplified by catalyzing ABTS to produce color. The ultra-sensitive

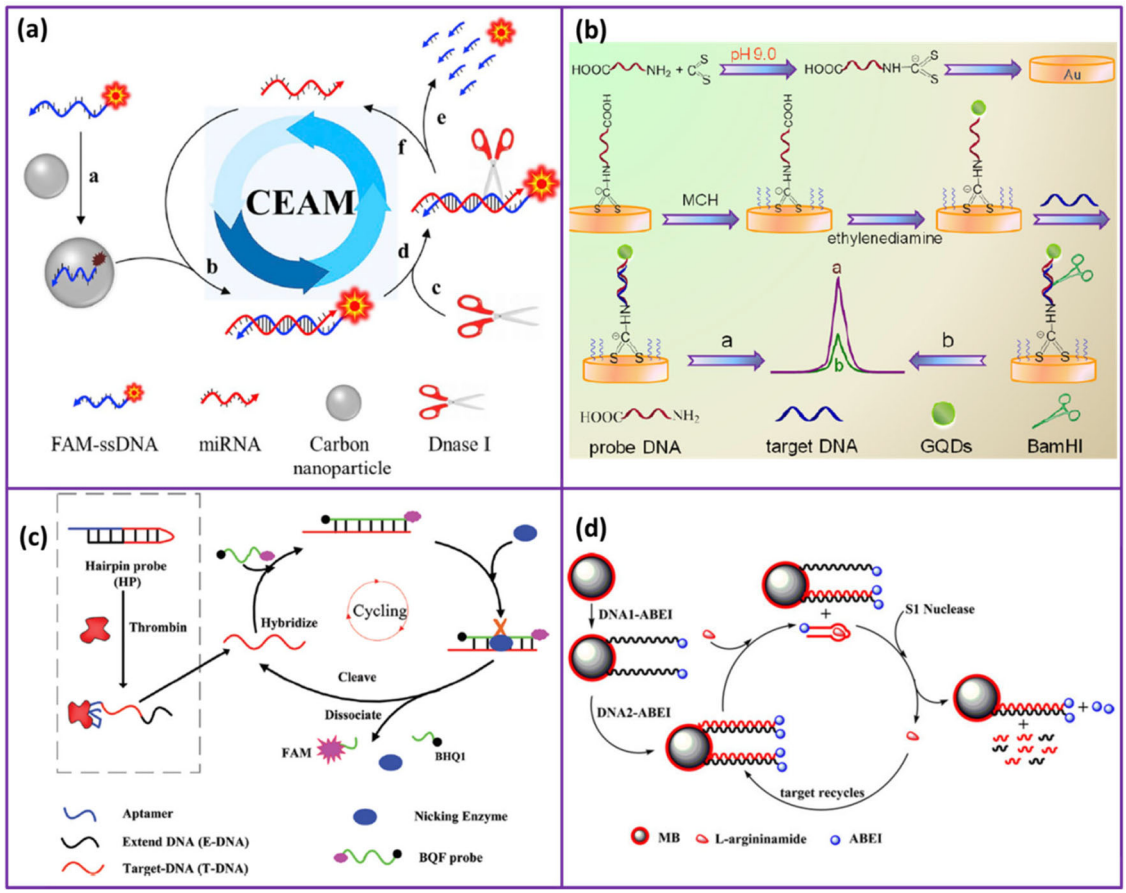

Figure 8. (a) A scheme of a DNA biosensor for miRNA detection based on DNase I-assisted amplified strategy. Reprinted with permission from Li et al.[87] Copyright 2020 Elsevier. (b) A schematic diagram of an ECL biosensor for DNA detection with BamH I- assisted signal output. Reprinted with permission from Lou et al.[88] Copyright 2015 American Chemical Society. (c) A scheme of a thrombin biosensor based on aptamer and nicking enzyme assisted fluorescence signal amplification. Reprinted with permission from Xue et al.[89] Copyright 2012 American Chemical Society (d) A scheme of an L-argininamide biosensor based on S1 nuclease hydrolysis signal amplification. Reprinted with permission from Hun and Wang.[90] Copyright 2012 Springer Nature.

biosensor had a linear range from 50.0 pM to 20.0 nM with a detection limit of 10.0 pM.

DNA endonuclease

DNA endonuclease is a kind of nuclease which cleaves phosphodiester bonds in the middle of DNA. Many Endonucleases were discovered and used to design biosensor. The involved ones were mainly DNase enzyme families, restriction endonuclease, nicking endonuclease and S1 nuclease. Different types of endonuclease had different requirements of DNA sequences. According to the characteristics of endonuclease, the DNA digestion can be controlled to achieve the unique signal amplification.

DNase I is one of the most typical DNase enzyme. Its substrate is ssDNA and dsDNA.[83] Free DNA is easily digested by DNase I.[84,85] DNase I has been extensively used to remove DNA in RNA sample preparation, RT-PCR and DNase footprint analysis.[86] Li and his colleagues[87] designed a DNA biosensor for miRNA detection based on DNase I-assisted amplified strategy. In this biosensor, a carbon nanoparticle was used to protect DNA from DNase I digestion and to quench the fluorescence signal of FAM. At the beginning, the DNA (complementary strand of the target miRNA) was labeled with FAM to form a DNA probe. Without the target miRNA, the DNA probe was absorbed on the carbon nanoparticle to cause the disappearance of the fluorescence. When the target miRNA was added, the DNA probe was separated from the surface of carbon nanoparticle by forming DNA/RNA hybrid duplex. Meanwhile, the fluorescence signal of DNA probe was restored. It was important that the DNA probe in the DNA/RNA hybrid became a substrate of DNase I. After the addition of DNase I, the DNA probe could be cut into fragments which led to the release of the target miRNA. The released target miRNA could then combine with another DNA probe on the carbon nanoparticle and start the second cycle, thus to recover stronger fluorescence (Figure 8a). Thus, the sensitivity of the method was improved with a detection limit down to 3.2 pM.

Restriction endonucleases are a large group of endonucleases that recognize short palindrome with a length of 4-8 bp and cut double-stranded DNA in or near the recognition site.[91] According to the different sequence of recognition, these enzymes have different names such as BamH I, EcoR I, Hind III. Restriction endonucleases are realized as "molecular scalpel" and often used for molecular cloning. Due to their specificity for sequences, they have also been used in the design of biosensors in recent years. For example, Luo and his colleagues[88] proposed an

electrochemiluminescent (ECL) strategy to detect DNA with the BamH I- assisted signal output. In this DNA biosensor, a bidentate chelation of dithiocarbamate DNA (DTC-DNA) probe was attached onto the gold surface by bidentate anchoring (S-Au-S bonds). At the other end of the DTC-DNA, the graphene quantum dots (GQDs) were labeled. Due to the excellent ECL activity of GQDs, the ECL signal was high enough. When the target DNA (hepatitis C virus-1b genotype complementary DNA) was added, it could form double-strand DNA with DTC-DNA. This structure could be digested by BamH I, which induced the GQDs to break off from the electrode surface (Figure 8b). Afterwards, the ECL signal was decreased. This DNA bisosensor was highly sensitive with a detection limit of 0.45 fM and a linear range from 5 fM to 100 pM.

Nicking endonucleases are similar to the restriction endonucleases by recognizing specific sequences to cut DNA. However, the way they cut is quite different. Restriction endonucleases cut double chains while nicking endonucleases only cut single chain. Because of this feature, the other chain can be protected to conduct a further reaction. Thus, these endonucleases can be used to amplify signal. Many nicking endonuclease-based amplification strategies have been developed for detection of different analysis.[92] Generally speaking, nicking endonucleases have attracted wide attention because of its high specificity, convenient operation, low cost and high selectivity.[93,94] For example, Hong et al. developed an ultrasensitive and selective electrochemical biosensor to detect Hg^{2+} by the nicking endonuclease (named Nt.AlWI)-assisted target recycling.[95] Liu et al. used another nicking endonuclease-Nt.BstNBI to design Hg^{2+} DNA biosensor.[96] The protein target could also be detected by the nicking enzyme assisted fluorescence signal amplification. Xue et al. developed a thrombin biosensor based on the Nb.BbvC nicking enzyme and thrombin aptamer.[89] In this biosensor, the BQF probe contained the fluorescent group (FAM), quencher and a short fragment DNA. Such DNA carried the cleavage site and recognition sequence. Meanwhile, the hairpin probe contained the aptamer, extend DNA and Target-DNA (T-DNA). At the beginning, the T-DNA was covered in the hairpin probe. In the present thrombin, it could bind the aptamer to release T-DNA. Then the T-DNA hybridized with the BQF probe to form a double chain structure which could be recognized by Nb.BbvC. Under the action of Nb.BbvC, the BQF probe was digested to release FAM and T-DNA. Then FAM emitted fluorescence signal and T-DNA entered the next cycle to increase sensitivity (Figure 8c). This sensitive biosensor had a detection limit as low as 100 pM.

S1 nuclease is another endonuclease that can digest certain single strands of DNA and RNA. It can also cut single-stranded regions in double-stranded DNA or RNA such as loops and gaps. Meanwhile, this enzyme has no directivity and sequence specificity. It is often used to remove the single strands of DNA or single-stranded regions. Hun and Wang designed a sensitive biosensor to detect L-argininamide based on this property of S1 nuclease.[90] In this biosensor, a chemiluminescence (CL) reagent *N*-(4-aminobutyl)-*N*-ethylisoluminol (ABEI) was labled on the L-argininamide aptamer (DNA2-ABEI) and its complementary strand (DNA1-ABEI). And the DNA1-ABEI was modified on magnetic beads (MBs). In the absence of L-argininamide, the DNA1-ABEI and DNA2-ABEI could hybridize to form duplex which could prevent the digestion of S1. When L-argininamide was added, it could bind with DNA2-ABEI to destroy the above mentioned duplex. Then the single-stranded DNA1-ABEI and DNA2-ABEI were digested, with the release of L-argininamide and ABEI. The free L-argininamide entered the next cycle to interact with another duplex structure (Figure 8d). Ultimately, a lot of ABEI was generated to emit strong CL signal. The detection limit of this sensitive biosensor was 1.0×10^{-7} M.

Duplex specific nuclease (DSN) is another member of the endonuclease family which was isolated from the digestive glands or hepatopancreas of shrimps and crabs by Shagin and his colleagues in 2002.[74,97] It can specifically cleave DNA in double-stranded DNA or DNA-RNA heteroduplex, but has no activity toward single-stranded oligonucleotides or double-stranded RNA.[98,99] In addition, DSN is stable within a wide range of pH and temperature, and is not sensitive to protease digestion. These characteristics indicate that DSN is a good candidate for biotechnology applications.[74] Wang et al. developed a biosensor for miRNA detection based on duplex-specific nuclease (DSN).[100] In this biosensor, the AuNPs modified with the complementary DNA was used. Without the target miRNA, the AuNPs-DNA was loaded on the surface of electrode to form a strong negative layer. This could inhibit the adsorption of AgNPs which was capped with citrate by electrostatic repulsion and steric hindrance effect. Thus, the electrochemical signal of AgNPs was very low. The added miRNA could hybridize with complementary DNA to form DNA/RNA duplex which was subsequently recognized by DSN. DSN could selectively cut DNA strand from DNA/RNA duplex. Then the target miRNA could be released to re-hybridize with the remaining AuNPs-DNA. As the DNA was digested, the AgNPs could be reloaded on the surface of electrode. Thus the electrochemical signal of AgNPs could be obtained (Figure 9a). With the DSN-assisted target recovery, a small amount of miRNAs was able to trigger the release of a considerable number of "signal molecules" to improve the sensitivity of biosensor. The limit of this biosensor was as low as 0.62 fM with a broad linear range from 1 fM to 1 pM.

RNases are also large categories of endonuclease family including RNase A, RNase T1, RNase H and so on which degrade RNA into smaller fragments. Their substrate is RNA, not DNA. For example, RNase H specifically degrades RNA strand in RNA/DNA hybrid double strands. And, it cannot digest single or double stranded RNA. Kim and Chung reported a highly sensitive biosensor for the detection of protease based on RNase H-assisted signal amplification.[101] Two kinds of gold nanoparticles (GNPa and GNPb) were designed in this assay. GNPa was modified by peptide-DNA. And GNPb was labeled by RNA-FITC. The fluorescence signal of FITC could be quenched by GNPb. In the presence of matrix metalloprotease-2 (MMP-2), the

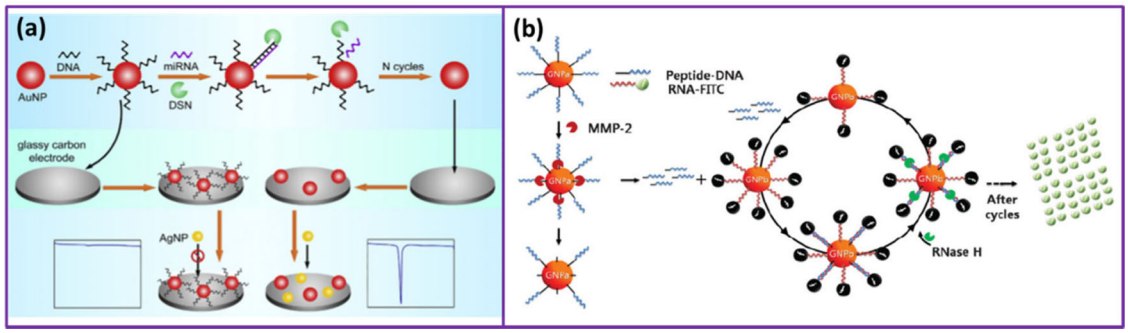

Figure 9. (a) A scheme of a biosensor for miRNA detection based on duplex-specific nuclease (DSN). Reprinted with permission from Wang et al.[100] Copyright 2020 Elsevier. (b) A scheme of a sensitive biosensor for detection protease (MMP-2) based on RNase H-assisted signal amplification. Reprinted with permission from Kim et al.[101] Copyright 2010 Wiley-VCH.

peptide was cleaved to release DNA from GNPa. The free DNA could hybridize with RNA of GNPb to from DNA/RNA double strands. The RNA of this structure could be digested by RNase H. Then, the FITC and DNA in DNA/RNA were both released. The free FITC regained the fluorescence signal and DNA entered the next cycle to hybridize with another RNA-FITC (Figure 9b). Finally, the signal was amplified with the help of RNase H. And the highly sensitive biosensor for detection protease was realized with a detection limit of 10 pM within 4 h.

Other nuclease

DNA ligases can connect two pieces of DNA by catalyzing the formation of phosphodiester bonds. The substrate of DNA ligases is also DNA. It should be noted that the *E. coli* DNA ligase requires nicotinamide adenine dinucleotide (NAD^+) as a cofactor and the phage T4 DNA ligase needs ATP as a cofactor.[102] Yu group reported a highly sensitive and selective biosensor to detect NAD^+ based on *E. coli* DNA ligase and DNA polymerase (Klenow).[103] In this biosensor, a DNA probe was designed with two loop structure. One loop structure contained G-quadruplex sequence at the 5′-end. And the DNA probe could form self-complementary structure. In the absence of NAD^+, the *E. coli* DNA ligase was inactive and the 3′-end could be used as a primer. Thus, the extension reaction could be carried on with the help of DNA polymerase to make the G-quadruplex sequence covered. When NAD^+ was added, the *E. coli* DNA ligase could connect the 5′-end and 3′-end of DNA probe. The 3′-end could not be used as a primer to stop the extension reaction. Thus, the G-quadruplex sequence could be reserved and form G-quadruplex structure. Then, the NMM was bound to emit a great fluorescence signal (Figure 10a). This biosensor was realized by two enzymes with a detection limit of 0.5 nM NAD^+.

DNA polymerases are a large family of nucleases that extend DNA. One of the most classic is the Thermus Aquaticus (Taq) polymerase, which is commonly used to amplify nucleic acid signals in PCR reactions. PCR technique is a commonly-used and well-recognized technology in nucleic acid detection with very high sensitivity. However, it requires a PCR apparatus to perform the process of denaturation, renaturation and extension. Recently, DNA polymerases (such as phi29, Bst) with chain replacement functions have been identified.[105,106] They can be used to achieve isothermal polymerization amplification which does not require a PCR apparatus.[107] Huang et al. designed a sensitive aptasensor to detect gastric cancer exosomes based on phi29 DNA polymerase- assisted signal amplification strategy.[104] In this biosensor, anti-CD63 antibody which could bind with different kinds of exosomes was modified on the gold electrode. The specific aptamer of gastric cancer exosome with a primer sequence was used to recognize gastric cancer exosome. In the presence of gastric cancer exosome, the electrode-anti-CD63-aptamer-primer structure could be formed. Then, the primer might trigger rolling circle amplification (RCA) to produce many G-quadruplex DNA with the help of phi29 DNA polymerase. The generated G-quadruplex could bind NMM to improve the activity of peroxidase which could oxidize substrate to generate electrochemical signal (Figure 10b). Thus, the high selectivity and sensitivity of biosensor for detecting gastric cancer exosome could be obtained with a detection limit of $9.54 \times 10^2\ mL^{-1}$ and a linear response range from 4.8×10^3 to $4.8 \times 10^6\ mL^{-1}$.

Summary and future perspectives

In this article, we reviewed the role of enzymes to design DNA biosensor. According to chemical nature, the enzymes are divided into DNAzymes and nucleases. The components of DNAzymes are nucleic acids. Besides, the nucleases are essentially proteins. However, their substrates are all nucleic acids (DNA or RNA). They all have excellent catalytic activity and unique biological recognition capability, so they can be used for signal amplification in DNA biosensor. DNAzymes have the advantages of stable chemical properties, cost-effectiveness, and easy modification. And they can be easily synthesized in vitro. Usually, they need cofactors to increase their activity. Therefore, some cofactors can be detected as targets based on DNAzymes. In addition, some targets can help partial DNAzymes to form entire DNAzymes. This also can be used to design DNA biosensor. G-quadruplex, as a new DNAzyme, can catalyze porphyrin metalation, and improve the peroxidase activity of hemin. Meanwhile, it can also increase the fluorescence signals of porphyrins and dyes.[108] These are all very useful in

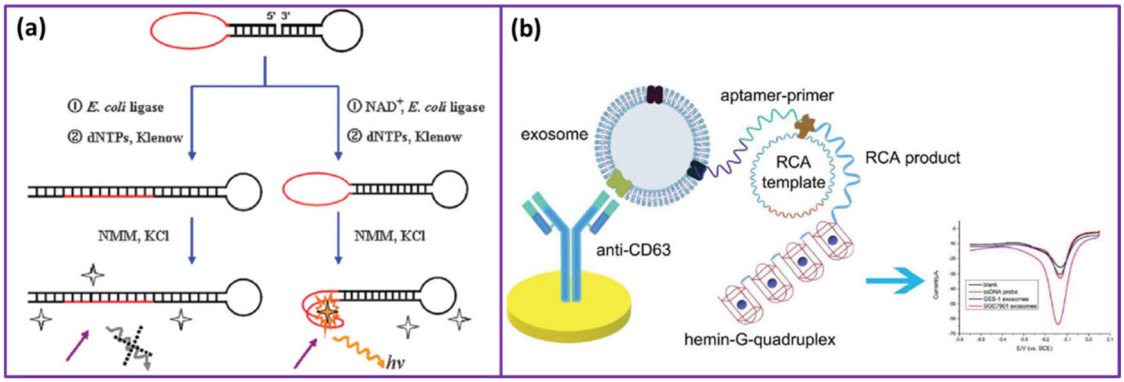

Figure 10. (a) A scheme of a label-free fluorescence biosensor to detect NAD^+ based on *E. coli* DNA ligase and DNA polymerase. Reprinted with permission from Zhao et al.[103] Copyright 2012 Royal Society of Chemistry. (b) A scheme of a sensitive aptasensor to detect gastric cancer exosomes based on phi29 DNA polymerase- assisted signal amplification strategy. Reprinted with permission from Huang et al.[104] Copyright 2019 Wiley-VCH.

Table 1. Comparison of different enzyme-based DNA biosensors.

Enzymes	Function	Target	Analytical technique	Detection limit	Ref.
DNAzyme 8-17	Cleavage	Pb^{2+}	Fluorescence	61.8 pM	[24]
DNAzyme T30695	Metalation	Pb^{2+}	Fluorescence	23.5 nM	[49]
DNAzyme G-rich DNA	Peroxidase	miRNA	Chemiluminescence	8 pM	[60]
DNAzyme E47	Ligation	Cu^{2+}	Absorbance	1 nM	[68]
DNAzyme PS2.M	Allosterism	Pb^{2+}	Colorimetry	32 nM	[70]
Exo I	Degradation of ssDNA	Hg^{2+}	Fluorescence	15 nM	[78]
Exo III	Degradation of dsDNA	Hg^{2+}	Absorbance	10 pM	[80]
DNase I	Degradation of DNA	miRNA	Fluorescence	3.2 pM	[87]
BamH I	Cleavage particular DNA sequence	DNA	Electrochemiluminescence	0.45 fM	[88]
Nb.BbvC	Cleavage particular DNA sequence	Thrombin	Fluorescence	100 pM	[89]
S1 nuclease	Degradation of ssDNA	L-argininamide	Chemiluminescence	100 nM	[90]
DSN	Degradation of dsDNA	miRNA	Electrochemistry	0.62 fM	[100]
RNase H	Degradation of RNA	Protease	Fluorescence	10 pM	[101]
DNA ligase	Ligation	NAD^+	Fluorescence	0.5 nM	[103]

designing DNA biosensors. Controlling the formation or disappearance of G-quadruplex structure is an important way to design DNA biosensor. Nucleases include a lot of types with many different functions such as Exo I, Exo III, DNase I, BamH I, S1 nuclease, DSN, DNA polymerases and so on. The advantages of nucleases are variety and versatility. These provide us a lot of options for designing DNA biosensors. Formation or disappearance of nucleases digestion sites is the heart of these approach. Moreover, a brief comparison was made between DNAzymes and nucleases in development of biosensors (Table 1). The nucleases were more variety and functions than DNAzymes. However, DNAzymes were more stable than nucleases. These enzyme-based DNA biosensors could be used to detect multiple targets such as heavy metal ions, nucleic acids and proteins. A variety of analytical technique could be chosen for these biosensors such as fluorescence, chemiluminescence, colorimetry and electrochemistry. Therefore, enzymes should be chosen according to actual requirements.

Future work can be focused on solving a few key challenges in the field. Firstly, the discovery of new enzymes is still worth studying to achieve new functions. For example, the cofactor of new DNAzyme should not be limited to metal ions. Gu et al. obtained a new DNAzyme using protein VAE-2 as a factor.[30] Second, co-catalysis of two or more enzymes is a strategy to improve the sensitivity of DNA biosensors. For example, Exo III and nicking endonucleases were used to amplify signal to detect Hg^{2+} with a ultra-low detection limit 6 pM.[109] Finally, the combination of enzymes with other materials (such as gold nanorods, nano-magnetic beads) may be a method to improve the stability and reaction efficiency of enzymes. In the near future, we will have a deeper understanding in the enzyme catalytic reaction strategy for development of DNA biosensors.

Disclosure statement

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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