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REVIEW ARTICLE

DNA-modifying enzyme reaction-based biosensors for disease diagnostics: recent biotechnological advances and future perspectives

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ABSTRACT

Biosensor devices are important in clinical practice and environmental studies because they allow specific detection of target molecules from a variety of samples. However, challenges still remain when attempting to develop sensitive, selective, rapid, and cost-effective assays for biomolecule detection. Devices that use DNA-modifying enzymes to catalyze detection reactions have recently been developed. These devices show promise because they are often more sensitive and specific, have shorter assay times, are more cost-effective, and are easier to use than the currently used biosensors. Here, we review the current trends in DNA-modifying enzyme reaction-coupled biosensors, including devices using DNA polymerases, nicking endonuclease, exonucleases, and ligases. The molecular strategies underlying diverse DNA-modifying enzymes coupled to biomolecule detection platforms are reviewed. We also discuss the strengths and limitations of each strategy and suggest methods to overcome current limitations. Finally, the future prospects of DNA-modifying enzyme reaction-coupled biosensor development have been proposed.

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Introduction

Physical and stochiometric changes in intracellular biomolecules, including DNA, RNA, and proteins, can not only cause diseases but could also be the consequences of a disease. Since metabolic enzyme activity is critical for intracellular signaling, it is closely associated with human disease. Accordingly, biomolecule detection and activity monitoring are vital in clinical research, forensics, biodefense, food safety, and pathology [1]. For successful biomolecule detection, the assay system must meet the benchmark for several criteria, namely sensitivity, selectivity, speed, multiplexing capacity, and cost-effectiveness [2–4]. The major challenge for biomolecule detection is the ability to identify the target with high specificity in a variety of settings, because biological samples have complex compositions in addition to the target molecule. Furthermore, although the target may be present, low quantities of the target in the samples impedes successful molecule detection. To overcome these challenges, efforts have been made to develop biomolecule-detecting methods with an enhanced performance. Biosensors integrated with nanotechnology offer several advantages over conventional methods, including high-throughput screening, low limit of detection (LOD), real-time analysis, label-free detection, and the need for a small sample volume [5,6]. Interest in biosensors has increased because of the development of nanomaterial-based platforms that allow further enhancement of sensing performance [7]. New bioreceptors with an improved detection ability have also been used to optimize biosensors for sensitive and specific molecule detection, leading to the development of various sensing strategies [8]. Furthermore, newly described DNA-modifying enzymes and the development of derivatives have remarkably enhanced biosensor performance. Combining the distinct catalytic activities of DNA-modifying enzymes with biosensors has several advantages. First, the combination of enzymatic activities enhances biosensor sensitivity and specificity *via* specific recognition capabilities and distinct functional mechanisms (Table 1). For example, enzymes such as nicking enzymes or restriction endonucleases recognize specific nucleotide sequences and subsequently initiate enzymatic reactions. Some enzymes exhibit activity in specific directions, that is, 5′→3′ or 3′→5′ (e.g. Klenow fragment polymerase, Vent (exo-) DNA polymerase), in only one strand (e.g. exonuclease III), in strands with specific

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functional groups such as 5′-phosphate (e.g. λ exonuclease), or in strands with specific regions such as the nick and loop (e.g. S1 endonuclease). Second, enzymatic activity also enhances the sensitivity of the biosensor *via* diverse signal amplification reactions such as rolling circle amplification (RCA), strand-displacement amplification (SDA), and target recycling reaction (TRR), wherein the quantity of the DNA involved in the assay increases or is reusable (Supplementary Tables 1–3). Third, unlike PCR-based assays that require reiterative reaction cycling under different temperatures to achieve amplification, assays using DNA-modifying enzymes can be performed at isothermal conditions. Such isothermal amplification strategies allow faster reactions and do not require special equipment, which allows its widespread use in clinical and environmental settings [9–12]. Therefore, DNA-modifying enzyme reaction-coupled sensing approaches have great potential for sensitive and specific biomolecule detection.

There are many good reviews that provide a detailed information on the principles and characteristics of diverse biosensors [13,14]. In this article, focus will be given to the biomolecule-detecting strategies and biotechnologies using DNA-modifying enzymes in biosensors. This article will uniquely present a comprehensive overview of the most recent developments of DNA-modifying enzyme reaction-coupled sensor in clinical and environmental settings. Discussion of the strengths and drawbacks of these techniques and suggestions of steps to overcome their limitations will be helpful to all researchers in academia and industry who are interested in the development of biosensors for disease diagnostics. An approach to improve these biosensors is described to help achieve the long-term goal of its use in environmental and clinical settings.

Biomolecule-detecting strategies integrated with DNA-modifying enzyme reactions

DNA-modifying enzymes

The DNA-modifying enzyme is an enzyme that is involved in the degradation, synthesis and alternation of nucleic acids. DNA-modifying enzymes are generally used in biosensors that can be largely classified into three groups: cleavage, ligation, and synthesis (polymerization) enzymes (Table 1). Depending on where they digest target DNAs, DNA cleavage-associated enzymes are classified as endonucleases and exonucleases. Endonucleases cut the phosphodiester bond within a DNA chain. For example, S1 nuclease, nicking endonuclease, and restriction endonucleases all cleave phosphodiester bonds. Among the endonuclease, S1 endonuclease exhibit the distinct feature for specific recognition and the cleavage of single-stranded regions in duplexes. The enzyme removes single-stranded overhangs at the 5′-and/or 3′-ends of double-stranded nucleic acids, which makes blunt-end duplexes. It also recognizes and digests internal nicks, gaps, or duplex mismatches, leading to the release of DNA fragments from duplexes and enabling released fragment reuse in the assay. Furthermore, this enzyme removes even hairpin loops in duplexes. Excessive amounts of the enzyme and long incubation times can cause double-stranded DNA (dsDNA) degradation, causing a low signal-to-noise ratio, so the optimal conditions should be considered [15–17]. Nicking enzyme such as Nt.BstNBI, Nt.BstmAI, etc. recognizes specific nucleotide sequences and cleaves only one strand of dsDNA, creating DNA molecules with nicks. DNA polymerase which binds at the nicked strand and then amplifies the strand downstream of the extended primers, thereby producing a new 5′-3′ strand. During this reaction, target DNA can liberate from the template and bind another probe, which is called a TRR. Also, new DNA strands can be extended by adding nicking site-containing trigger DNA as primers. Importantly, new DNA strands can be continuously produced because the extended DNA contains a specific nicking enzyme recognition site, producing a cascade amplification reaction. Exonucleases, such as λ exonuclease, exonuclease III, and exonuclease I, degrade nucleotides from the end of the DNA chain. Among them, λ exonuclease is 5′→3′ exonuclease that removes 5′ mononucleotides from duplex DNA. Its enzymatic reaction begins in the presence of 5′-phosphorylated dsDNA. However, λ exonuclease does not initiate digestion at nicks or gaps in dsDNA. Exonuclease III specifically digests blunt-end dsDNA from the 3′ to 5′ direction. Depending on the probe DNA design, the enzyme can cleave unidirectionally or bidirectionally specific duplex strands. Using these distinct characteristics, exonuclease III plays a key role in background signal removal, target or trigger DNA recycling, DNA walking, and signal DNA amplification, resulting in high biosensor specificity and sensitivity [18–29]. Ligation-associated enzymes, such as T4 ligase, catalyzes the joining of two fragments by forming a new chemical bond. This enzyme regulates advancement to the next reaction step depending on the presence or absence of cofactors or conditions necessary for its catalytic reaction. This process can yield a high signal-to-noise ratio. Synthesis (polymerization)-associated enzymes, such as Klenow fragment polymerase or phi29 polymerase, elongate DNA chains by adding free nucleotides to the 3′-end.

Table 1. DNA-modifying enzymes commonly used in biosensors and their mechanisms.

Enzymes		Working principles				
Classification	Name	Substrate	Recognition site	Reaction direction	Specificity	Remarks
Cleavage	S1 endonuclease	ssDNA/single-stranded region in dsDNA	Nick, gap, mismatch or loop	5′→3′/3′→5′	–	Exhibits 3′-phosphomonoesterase activity
	Nt.BstNBI endonuclease	one strand of dsDNA	5′-GAGTCNNNN*N-3′	–	+	Exhibits nicking activity without degradation
	Nt.BbvCI endonuclease	one strand of dsDNA	5′-CC*TCAGC-3′	–	+	Exhibits nicking activity without degradation
	Nt.CviPII	one strand of dsDNA	5′-*CCD-3′	–	+	Exhibits nicking activity without degradation. D indicates A or G or T
	Restriction endonuclease	dsDNA	Specific nucleotides	–	+	Cleaves DNA into fragments at or near specific recognition sites: e.g. KpnI (5′-GGTAC*C-3′ and 3′-C*CATGG-5′)
	λ exonuclease	dsDNA	5′-phosphorylated strand	5′→3′	–	Reduced activity on ssDNA and non-phosphorylated DNA, no activity at nicks, limited activity at gaps in DNA
	Exonuclease III	dsDNA	3′-end in duplex	3′→5′	+	3′-overhang ends of DNA having at least 4-bases long is resistant
	Exonuclease I	ssDNA		3′→5′	–	dsDNA and DNA having 3′-phosphate or acetyl group are resistant
	DNase I	ssDNA/dsDNA		5′→3′/3′→5′	–	Nonspecific endonuclease
Ligation	T4 ligase	dsDNA	Single-strand nicks in duplex DNA or DNA/RNA hybrids	5′→3′	–	No activity on ssDNA. Requires ATP as a cofactor
Synthesis	Klenow fragment polymerase	dsDNA	Single-strand fragment in duplex DNA	5′→3′	–	Exhibits 5′→3′ polymerase activity and 3′→5′ exonuclease activity, but lacks 5′→3′ exonuclease activity of DNA polymerase I.
	Klenow fragment polymerase, (3′-5′ exo-)	dsDNA	Single-strand fragment in duplex DNA	5′→3′	–	Exhibits 5′→3′ polymerase activity, but lacks the 3′→5′ and 5′→3′ exonuclease activities of DNA polymerase I.
	Phi29 polymerase	dsDNA	Single-stranded fragment in duplex DNA	5′→3′	–	Exhibits the highest processivity and 3′→5′ exonuclease activity.
	Vent (exo-) DNA polymerase			5′→3′		Exhibits 5′→3′ polymerase activity but lacks the 3′→ 5′ proofreading exonuclease activity. Ideal for GC-rich or looped sequences. High thermostability.

Asterisk (*) indicates the cleavage site of enzyme.
ssDNA: single-stranded DNA; dsDNA: double-stranded DNA.

Biomolecule-detecting strategies using catalytic DNA-modifying enzyme reactions

DNA-modifying enzyme-assisted biosensors are mainly composed of enzymes and DNAs. The basic sensing principle of this biosensor is that when a probe DNA binds to the target DNA in samples, an enzymatic reaction begins to reuse target DNA or increase the quantity of the target DNA, which subsequently emits signals through conformational or stochiometric changes to probe DNA (Figure 1). For more sensitive and specific biomolecule detection, several DNA fragments, such as trigger DNA, signal DNA, and probe DNA, can be designed and added to the reaction mixture with multiple DNA-modifying enzymes (Figure 1). In the presence of target DNA, it first binds to probe DNA, where the subsequently added enzyme(s) acts on probe:target duplexes, resulting in the formation and amplification of trigger DNA for the next enzymatic reaction [30–34]. Another mechanism is to free target DNA from the probe:target duplexes for reuse [18,34,35]. Trigger DNA can be produced from probe:target duplexes *via* enzymatic reactions [31–34] but can also be sequentially added to the reaction mixture [32,36]. Such trigger DNA initiates another enzymatic reaction that directly involves the signaling reaction or generates signal DNA [30,31,34]. Like trigger DNA, signal DNA is created and amplified by trigger DNA-mediated reactions [30,34] or is sequentially [36,37] added to the mixture, involving another enzymatic reaction. Conformational changes (e.g. G-quadruplex structure) of DNA by its interactions with chemicals (e.g. SYBR green I) and stochiometric change of DNA by its interaction with circular (e.g. padlock DNA) induce diverse sensing signals, such as colorimetric, fluorescent, or electrochemical signals, enabling quantification analysis and the detection of the presence/absence of the target molecule in samples. During an enzyme reaction, target DNA can also be exponentially amplified [18], thereby increasing its sensitivity.

Importantly, the DNA fragments should be designed such that they are suitable for the enzymatic reaction. For example, DNA fragments participating in ligation-mediated reactions should include the nick region [19,38]. DNA strands that remain after exonuclease III attack need 3′-overhangs after annealing to complementary strands [20,21]. To induce nicking-trigged SDA, DNA fragments must have a nicking endonuclease recognition site [35,36,39]. The resulting DNA fragments can be used as a target or trigger DNA for subsequent recycling or amplification processes.

Signal DNA can be modified to use a low-cost fluorescent signal. One example is the interaction of signal DNA with target (or trigger) DNA resulting in the formation of a G-quadruplex structure; the subsequent interaction between DNA and N-methylmesoporphyrin (NMM) emits fluorescence [34]. Another example is signal DNA:target (or trigger) DNA duplexes binding to hemin, which forms DNAzyme and acts as a peroxidase mimic to catalyze the $ABTS^{2-}$-H_2O_2 system

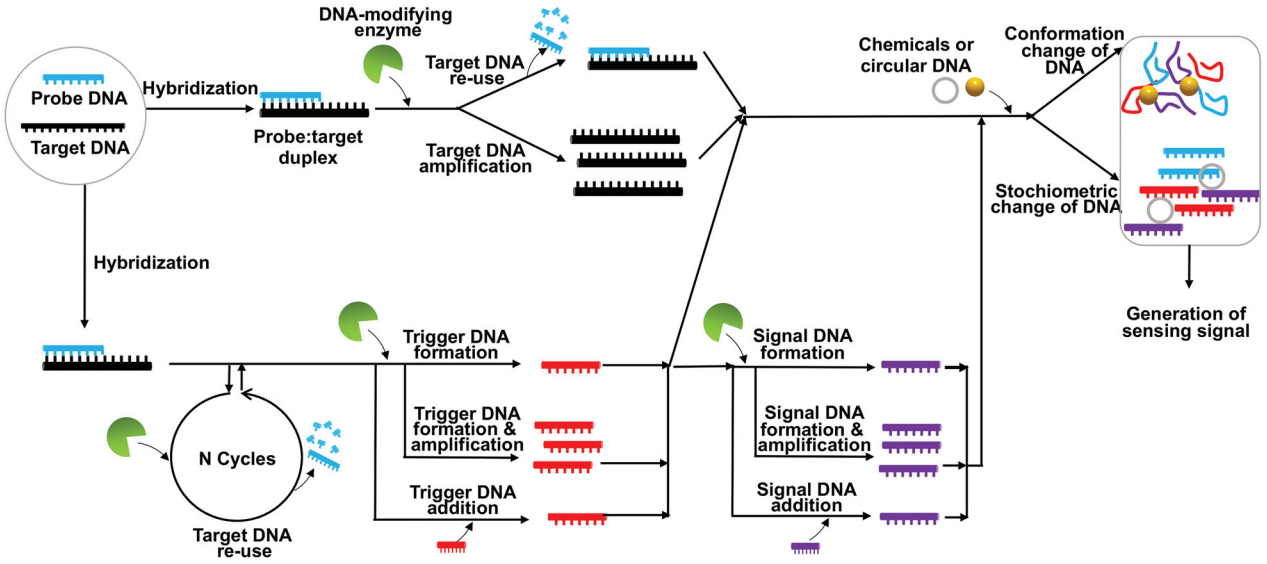

Figure 1. Biomolecule-detecting strategies using DNA-modifying enzyme reactions in a biosensor. When a probe DNA binds to the target DNA, the target DNA can be reused or amplify through an enzymatic reaction. Upon the formation of a probe:target duplex, multiple enzymatic reactions can produce and amplify trigger DNA and signal DNA, which directly involved the next enzymatic reaction or the signaling reactions. Trigger DNA and signal DNA can be also added to the reaction mixture sequentially. After that, the produced or added DNAs undergoes the conformational changes *via* interaction with chemicals or the stochiometric changes via interaction with circular DNA, leading to the generation of diverse sensing signals.

Table 2. Challenges and strategies to improve the performance of DNA-modifying enzyme reaction-coupled biosensors.

Challenges	Strategies to improve the sensing performance	References
High specificity	Consideration of coaxial stacking hybridization	[67]
	Use of invading stacking primer	[52,68]
High sensitivity	Use of SDA	[35,73,74]
	Use of ICSDP	[75,76]
	Use of RCA	[77]
	Use of EXPAR	[78–82]
	Use of nanoparticle-based amplification	[83]
	Use of two-stage isothermal strand-displacement polymerase reaction	[33]
	Integration of SDA and TMSD	[31]
	Use of nanomaterials such as magnetic nanoparticles, graphene oxide and gold nanoparticles as quenchers for high signal-to-noise	[84,85–92]
	Use of the DNA walking system for moving autonomously and stepwise along programed nucleic acid tracks	[93–98]
	Combination of DNA walking machines for nucleic acid detection with hyperbranched RCA and large surface area of nanoparticles for signal amplification	[72]
	Combination of target recycling reaction with hyperbranched RCA	[99,100]
	Use of copper nanoclusters as luminophore and TiO_2 as co-reaction accelerator in combination with exonuclease III-assisted amplification	[101]
	The development of enzymes with high activity, high processivity, and heat/chemical stability	[59–64]
Short assay time	Integration of SDA and TMSD	[31,46–49,84,102,103]
	Use of invading stacking primer	[25,68]
	Use of TMSD	[25,21]
	Identification and development of enzymes with improved processivity and catalytic activity	[60–63]
Low cost	No modification of exogenous DNAs	[43]
	Use of NMM	[34,57,104,105]
	Use of enzyme mimic such DNAzyme with hemin	[40,106–109]
	Use of metal nanoclusters as signal reporting molecules	[100,110]
	Use of a paper-based platform	[111]
	Use of probe DNA with palindromic sequences for primer-free amplification	[109]
Multiplexed sensing	Attachment of diverse probe DNA to magnetic beads	[37]
	Use of various electroactive labels with distinct voltammetric peak potentials	[71]
	Use of various fluorescence labels	[72]
	Use of nanowires functionalized with different probe DNAs	[15]

SDA: strand displacement amplification; TMSD: toehold-mediated strand displacement; ICSDP: isothermal circular strand-displacement polymerization; RCA: rolling circle amplification; EXPAR: exponential amplification reaction; NMM: N-methylmesoporphyrin IX.

($ABTS^{2-} + H_2O_2 \rightarrow ABTS + H_2O$) or H_2O_2-mediated oxidation of 3-aminophthalhydrazide (luminol) [22,30,39,40].

Current DNA-modifying enzyme reaction-coupled biosensors for biomolecule detection

Examples of DNA-modifying enzyme-assisted biosensors, including those described in this paper and others, that have been developed and employed for diverse biomolecule detection are summarized in Supplementary Tables 1–3. Challenges and strategies to improve the performance of DNA-modifying enzyme reaction-coupled biosensors, and the related parameters (LOD, assay time, etc.) are summarized in Table 2 and Supplementary Tables 1–3 (see the column of features of strategy, LOD, and linear range), respectively.

Nicking endonuclease and DNA polymerase

Enzymatic reaction for cascade amplification

One example of the cascade amplification biosensor strategy is the biosensor developed to detect K-Ras gene mutations (Figure 2(A)) [39]. In this study, probe DNA that detects K-Ras mutations has two hairpins at both ends like dumbbell shape. The presence of mutation-containing target DNA makes a hairpin at one end uncoil by forming probe:target duplexes. The subsequently added trigger DNA (PPT in Figure 2(A)) acts as a primer for Klenow fragment polymerase-mediated polymerization, producing an extended strand containing a Nt.BbvCI-specific region and a G-rich region. Nt.BbvCI nicking enzymes recognize and digest the specific region in the extended strand where Klenow fragment polymerization occurs, leading to TRR and SDA. Since the amplified strands contain a G-rich region, they form the horseradish peroxidase (HRP)-mimicking DNAzyme after hemin addition, which catalyzes the $ABTS^{2}$-H_2O_2 system. Trigger DNA-mediated TRR and SDA and nick-mediated signal DNA amplification allows an LOD of 4 pM point mutations and can be visualized by the naked eye.

In another strategy, for another cascade RCA, signal DNA produced by nicking site-triggered amplification acts as an RCA primer and the added padlock DNA acts

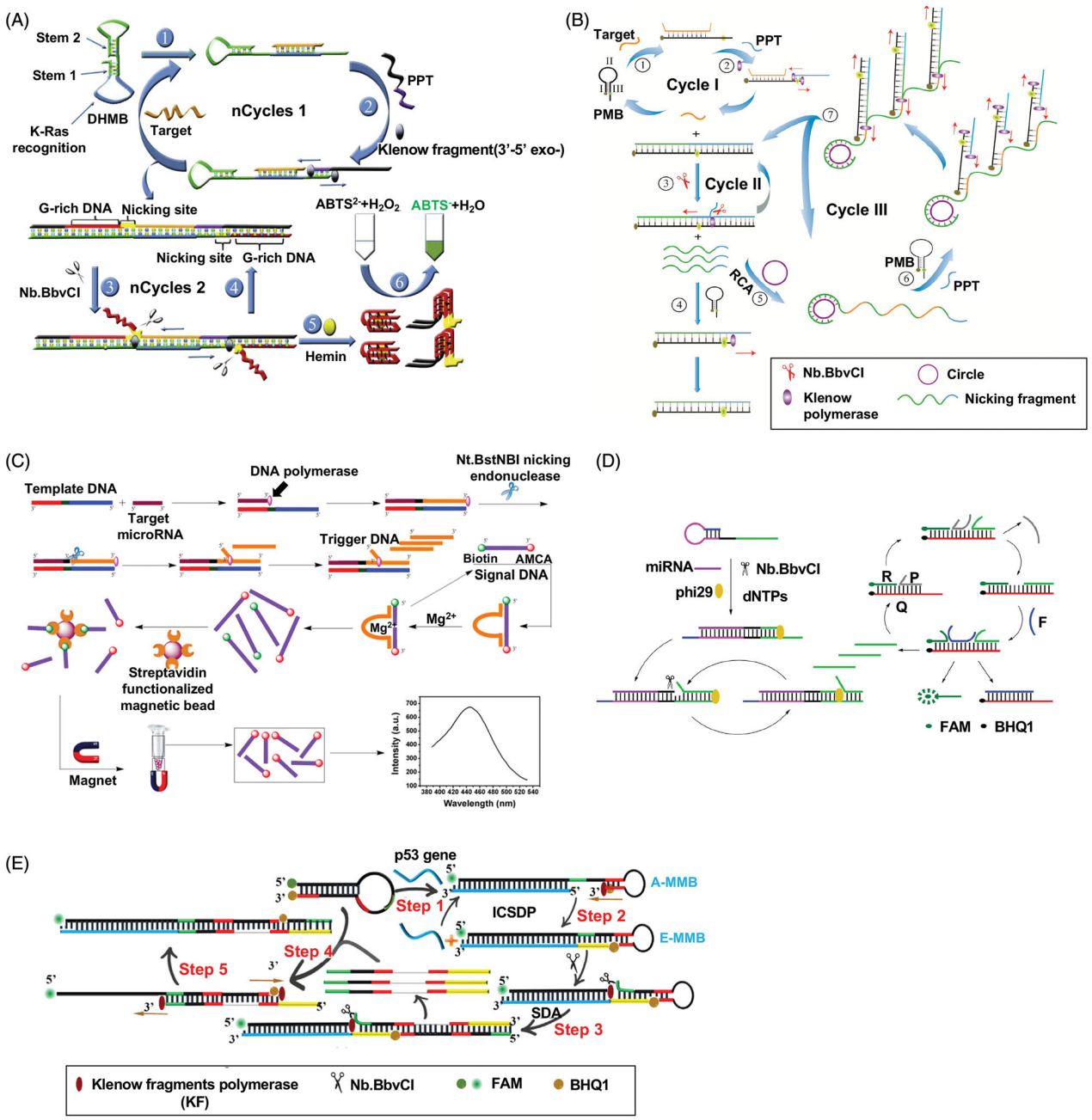

Figure 2. Biomolecule-detecting strategies of nicking enzyme and DNA polymerase-assisted biosensors. (A) Schematic illustration of fluorescence detection of a K-Ras mutation by a target recycling reaction (TRR) and a strand-displacement reaction (SDR). Reproduced with permission from reference [39]; (B) Schematic illustration of fluorescence detection of a p53 gene mutation by TRR, SDR, and rolling-circle amplification (RCA). Reproduced with permission from reference [35]; (C) Schematic illustration of a fluorescent miRNA biosensor using SDR and DNAzyme mimic-based amplification. Reproduced with permission from reference [36]; (D) Schematic illustration of a fluorescent miRNA biosensor using toehold-mediated SDR. Reproduced with permission from reference [31]; (E) Schematic illustration of fluorescence detection of a p53 gene mutation using isothermal circular strand-displacement polymerization (ICSDP) and reverse SDA. Reproduced with permission from reference [52].

as an RCA template (Figure 2(B)) [35]. Circular padlock DNA is complementary to the signal and probe DNA (PMB in Figure 2(B)), so the resulting RCA products can bind again to probe DNA regions, causing continuous nicking site-triggered amplification. This strategy yielded an LOD of 1 pM p53 mutation with high specificity by introducing three different cycling reactions: polymerase-assisted TRR (Cycle I in Figure 2(B)), trigger

DNA-mediated SDA (Cycle II in Figure 2(B)), and signal DNA-triggered RCA (Cycle III in Figure 2(B)).

Enzymatic reactions combined with DNA amplification based on metal ion-mediated enzyme mimic

DNA polymerase and nicking enzyme-assisted cascade amplification has also been coupled to another DNA amplification method using metal ion-mediated enzyme mimics. For example, in the presence of target microRNA (miRNA), linear probe DNA binds to target DNA, producing an extended strand containing an Nt.BstNBI-recognition site and trigger DNA (Figure 2(C)) [38]. Nt.BstNBI recognizes and cleaves an enzyme-specific sequence on the extended strand. A phi29 polymerase-mediated SDA reaction occurs at this nick, resulting in trigger DNA amplification. The amplified trigger DNA binds to 5′-bioin and 3′-AMCA-labeled signal DNA, forming DNAzyme in the presence of Mg^{2+} and digesting signal DNA to two fragments – biotin-modified and dye-labeled fragments. The biotin-modified fragment is isolated using streptavidin-coated magnetic beads, while the dye-labeled fragment is measured *via* fluorescence. This strategy combines two amplification steps, phi29 polymerase-assisted SDA and Mg^{2+}-dependent DNAyzme catalysis-mediated amplification, allowing a low detection limit (0.27 fM miRNA).

Enzymatic reaction combined with toehold-mediated strand displacement

Cascade amplification strategies imply excellent flexibility, easy construction, and enhanced sensitivity. Despite their advantages, these strategies have some limitations, namely a complicated workflow, the need for fragment purification, low signal-to-noise ratio, and long assay time, because several DNA fragments and enzymes are involved in each step and they must be present at the appropriate location and the appropriate time for the reaction to occur [32,41–43]. The slow dissociation of the extended strand from the template also reduces the number of amplification events and lengthens the reaction time [44]. Therefore, toehold-mediated strand displacement (TMSD) was used to decrease the reaction time [31,45–49]. Toehold is a short-strand stretching region that initiates strand-displacement reaction (SDR) hybridization and enhances the SDR rate by 10^6-fold [50,51]. In TMSD-coupled cascade amplification, after trigger DNA amplification using the nicking enzyme and DNA polymerase-assisted SDR, the amplified trigger DNA cyclically generates signal DNA (Figure 2(D)) [31]. TMSD starts with a three-strand DNA complex consisting of a FAM-labeled reporter strand (R), a BHQ1-labeled quencher strand (Q), and a non-fluorescent by-product strand (P). The amplified trigger DNA binds to the three-strand DNA complex and releases the P. The subsequently added a nonfluorescent new strand (F) binds to the R-Q-trigger DNA complex, releasing the R and trigger DNA and leading to R-Q dissociation and fluorescence emission. The incorporation of SDR and TMSD reduces the assay time to 30 min with a low, 0.18 fM miRNA LOD.

Enzymatic reaction combined with isothermal circular strand-displacement polymerization

Such multi-stage amplification requires the sequential addition of many exogenous DNA fragments or enzymes and rigorous operations, which limits its practical use in a point-of-care (POC) sensor. To address this, isothermal circular strand-displacement polymerization (ICSDP) was developed, which required only one probe DNA and no DNA addition (Figure 2(E)) [52]. ICSDP begins at hairpin probe DNA labeled with a dye and quencher at opposite ends. When probe DNA binds to target DNA, hairpin structures open (A-MMB) and Klenow fragment polymerase releases the target from the probe:target duplex by producing an extended strand (E-MMB; Steps 1 and 2 in Figure 2(E)). Then, Nt.BbvCI recognizes and generates a nick on E-MMB. Next, the Klenow fragment polymerase binds at the nick site and opens the E-MMB hairpin by reverse SDA, where polymerization occurs in the direction opposite to that of ICSDP (Step 3 in Figure 2(E)). The amplified and released strands then bind to the initial probe DNA, which then becomes a fluorescence-emitting reporter DNA (Steps 4 and 5 in Figure 2(E)). This strategy enables simple and rapid biomolecule detection with a short assay time (>30 min).

S1 endonuclease

Enzymatic reaction for mismatch discrimination

Some biosensors take advantage of the enzyme mismatch-cleaving ability, thus successfully detecting disease-relevant mutations, including single point mutations. For example, the combination of S1 nuclease enzymatic activity with surface-enhanced Raman scattering (SERS) successfully detects point mutations associated with two different genetic disorders, Wilson disease and Avellino corneal dystrophy (Figure 3(A)) [15]. To accurately detect these disease-relevant point mutations, four different probe DNAs were designed with four different sequences in the middle region and same sequences in the resting region. The probe DNA was then immobilized on gold nanowires and hybridized with target DNA. When the sample contains

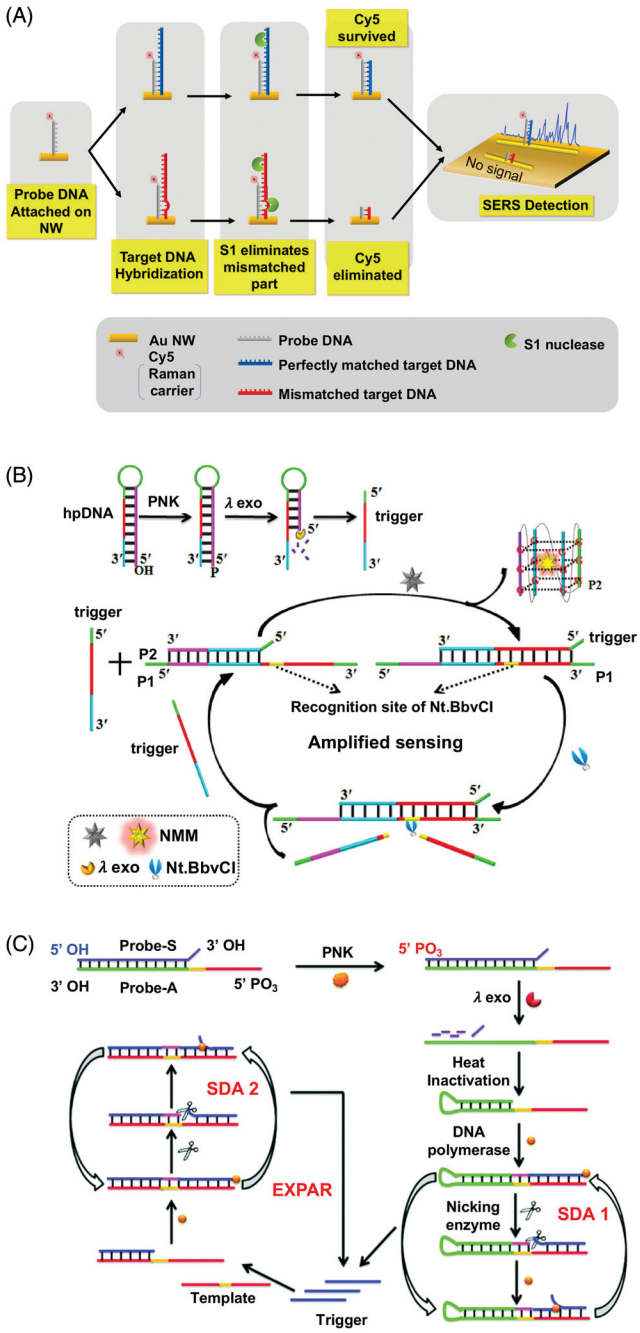

Figure 3. Biomolecule-detecting strategies of S1 endonuclease- or λ exonuclease-assisted biosensors. (A) Schematic illustration of a surface-enhanced Raman scattering sensor combined with an S1 nuclease reaction to detect multiple genetic disease-associated mutations. Reproduced with permission from reference [15]; (B) Schematic illustration of a fluorescent sensor that can perform a λ exonuclease reaction to detect T4 polynucleotide kinase (PNK). Reproduced with permission from reference [34]; (C) Schematic illustration of fluorescence detection of PNK using λ exonuclease. Reproduced with permission from reference [33], Reproduced by permission of The Royal Society of Chemistry.

perfectly matched target DNA complementary to the Raman dye-labeled probe DNA, the target and probe DNAs form DNA:DNA duplexes without nicks, gaps, or loops. The perfect duplex resists S1 nuclease attack, and a SERS signal appears from the Raman-dyed probe DNA near the gold nanowire. However, if there is a mismatch between the target DNA and the probe DNA, the probe and target DNAs form a duplex containing a single-stranded region. The S1 nuclease recognizes and digests this region in the probe:target DNA duplex, causing the Raman dye-labeled probe DNA to be released from the duplex, which eliminates the SERS signal. S1 nuclease activity is critical for increasing the selectivity of the mutation-sensing assay. Furthermore,

only one enzyme treatment step enables multiplexed mutation or single nucleotide polymorphism (SNP) detection by arranging multiple probes on a sensor. The possibility of high throughput DNA detection was expanded by using microarray platforms [16].

λ Exonuclease

Enzymatic reaction for cascade amplification

Using its distinct characteristic, λ exonuclease has been used to find and measure T4 polynucleotide kinase (PNK) activity (Figure 3(B)) [34]. PNK phosphorylates the 5′-terminal of stem-loop probes. Exposure to λ exonuclease leads to stem-loop probe digestion in the 5′ to 3′ direction until the enzyme encounters a single-stranded region of the stem-loop probe, typically the loop region. The remaining DNA fragment acts as trigger DNA in the next round. The trigger DNA then separates both strands of other duplex DNA (P1:P2 in Figure 3(B)). One strand (P1) of the P1:P2 duplex has a Nt.BbvCI recognition site and binds to trigger DNA, which is designed to be more stable than P2. Upon Nt.BbvCI treatment, P1 is partially digested, releasing the trigger DNA from the new duplex, which separates another P1:P2 duplex, causing trigger DNA-mediated signal amplification. The other strand (P2) forms a G-quadruplex structure and the NMM fluorescent dye intercalates into this structure. In this study, strand displacement and trigger DNA release occurs by the difference of binding affinities among the trigger DNA, P1 and P2. Trigger DNA generation is limited by stem-loop probe quantity, but this strategy can reduce the assay time and cost.

Enzymatic reaction combined with two-stage isothermal exponential amplification

Improved sensitivity for PNK activity measurement is achieved using two-stage isothermal exponential amplification (Figure 3(C)) [33]. As a probe DNA for detecting PNK, dsDNA with a single-stranded region at one end is phosphorylated at the other blunt end. The λ exonuclease degrades the 5′-phosphorylated blunt-ended dsDNA. The resulting single-stranded region folds into a stem-loop structure with a 5′-protruding termini. DNA polymerase then extends the strand, after which Nt.BbvCI catalyzes a nicking-mediated polymerization cycling reaction, resulting in the repeated generation of trigger DNA by SDA (SDA1 in Figure 3(C)). The generated trigger DNA then binds to another template DNA, allowing DNA polymerase and Nt.BbvCI to amplify more trigger DNA (SDA2 in Figure 3(C)). This two-stage amplification method lowers the PNK activity detection limit to 0.0002 U/mL, allowing endogenous PNK activity measurement even at the single-cell level.

Exonuclease III

Enzymatic reaction for background signal removal

The best way to eliminate the background signal is to specifically digest probe DNA that does not bind with target DNA, by exonuclease III reaction. To do this, as an example, probe DNA can be designed to have a 3′ double-stranded region that does not escape from enzymatic attack (Figure 4(A)) [20]. In more detail, when probe DNA is designed to have 5′-protruding termini complementary to the target DNA and a double-stranded 3′ stem, the presence of target DNA in the sample unravels the probe stem by forming a target:probe duplex, which prevents exonuclease III attack. The probe also contains the HRP-mimicking G-quadruplex/hemin DNAzyme, thus enabling ABTS-induced color change. Conversely, samples lacking target DNA are under enzymatic attack and cannot perform the subsequent colorimetric reaction. In this strategy, DNA recycling and amplification reactions do not occur. When the sample does not have target DNA, exonuclease III specifically digest probe DNA and background signal is removed, yielding eventually a high signal-to-noise ratio and an enhanced LOD. This strategy was further applied to detect diverse biomolecules by replacing the 5′-protruding terminal sequence with target molecule-specific sequences (for human alpha-thrombin, ref [22]; for p53 gene mutation, ref [21]; for human immunodeficiency virus (HIV) gene, ref [24]). The exonuclease III-assisted background signal removal strategy was also combined with the toehold-mediated SDR reaction (Figure 4(b)) [19]. Two different hairpins (HP1 and HP2 in Figure 4(B)) were used as probe DNA. Target DNA binding to HP1 triggers the opening of the hairpin structure and the sequential binding of HP2 to the HP1:target DNA duplex with the 3′-protruding ends resistant to exonuclease III digestion. Because the thermodynamic stability of HP1 coupled to HP2 is higher than that of HP1 coupled to target DNA, the target DNA is released from the duplex, where it can bind to another HP1 molecule (Figure 4(B)) [19]. Conversely, when there is no target in samples, the original structure of probe DNAs remains intact and so they break down by enzymatic reaction, resulting in a background signal removal.

Enzymatic reaction for target and trigger DNA recycling

The exonuclease III reaction also leads to TRR, which can be achieved by releasing target DNA from the probe:target duplex by degrading only probe DNAs. For

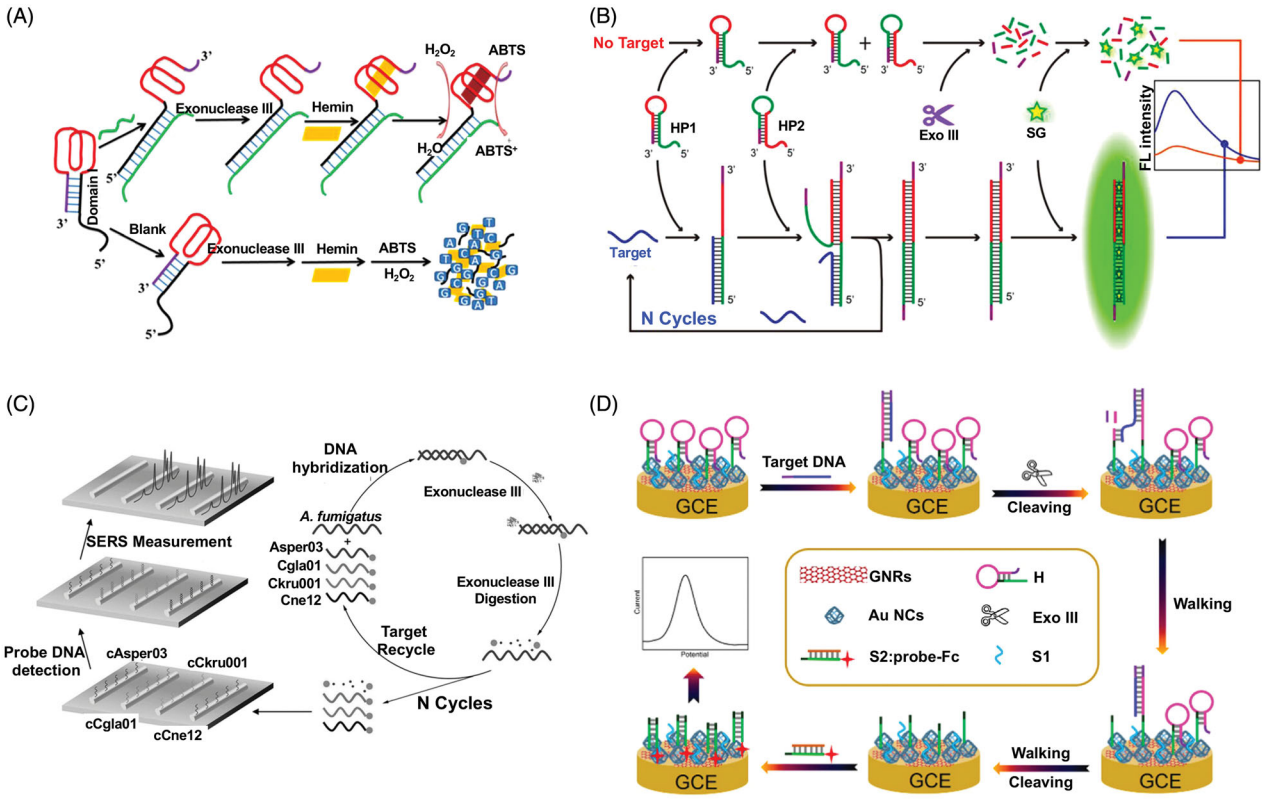

Figure 4. Biomolecule-detecting strategies of exonuclease III-assisted biosensors. (A) Schematic illustration of fluorescence detection of a p53 gene mutation using exonuclease III-aided background signal removal. Reproduced with permission from reference [20]; (B) Schematic illustration of fluorescence detection of a p53 gene mutation using toehold-mediated SDR and exonuclease III-aided background signal removal. Reproduced with permission from reference [21]; (C) Schematic illustration of a surface-enhanced Raman scattering (SERS) sensor using TRR for pathogen DNA detection. Reproduced with permission from reference [53]; (D) Schematic illustration of an electrochemical mismatched DNA biosensor using TRR and the DNA walking system. Reproduced with permission from reference [25].

example, exonuclease III specifically cleaves mononucleotides from the 3′-hydroxyl termini of duplex DNA, digesting only probe DNA in target-probe DNA hybrids, which allows the released target DNA to bind to another probe DNA (Figure 4(C)) [53]. This system could have increased sensitivity for multiple fungal DNA fragments, with an LOD as low as 100 fM (3 amol). Similarly, using an exonuclease III reaction-assisted sensor decreased the LOD for *E. coli*-specific DNA to 40 CFU/mL within 3.5 h. This strategy is a facile approach for signal amplification, despite low levels of target molecules in some samples such as bacterial DNA (4.0×10 CFU/mL LOD for Enterobacteriaceae, [54]; 8 CFU/mL LOD for *Salmonella typhimurium*, [55]), heavy metals (50 pM LOD for Pb^{2+}, [56]), and viral DNA (52 fM LOD for HIV DNA, 65 fM LOD for hepatitis B virus DNA, [57]).

Enzymatic reactions using cascade amplification

In another strategy, exonuclease III activity resulted in signal DNA amplification and target recycling over time [18,43]. As an example, probe DNA is designed with a hairpin structure that partially binds to target DNA [18]. Upon exonuclease III treatment, the partially bound part of the probe DNA duplexed with target DNA is digested by enzymatic attack, and the target DNA is released from the duplex. The hairpin section of the probe DNA survives enzymatic attack, which drives the recycling reaction. This occurs because the remaining probe DNA has the same sequences as the target DNA. Therefore, it behaves as target DNA. Such probe DNA design allows not only a TRR, but also increases the amount of target DNA, which lowers LOD to 6 pM [18]. Exonuclease III was used in a DNA walking system for target recycling and signal amplification (Figure 4(D)) [25]. For example, hairpins were bound to short oligos (S1 in Figure 4(D)) and immobilized on gold nanocages of graphene nanoribbons of glassy carbon electrode (GCE). Upon exposure to target DNA, TMSD occurred in the probe:S1 hairpin-forming duplex, resulting in the release of the S1 and probe-bound target DNA and the opening the hairpin structure. Treatment with exonuclease III specifically digests the probe DNA of the probe DNA:target DNA duplex, leaving the single-stranded probe portion. Next, the liberated target DNA

binds to another target DNA, resulting in exonuclease III-triggered DNA walking. Over time, DNA walking occurs repeatedly. Finally, ferrocene-labeled signal DNA is added, which binds to the surviving partial probe DNA. Like probe DNAs, ferrocene-labeled signal DNA is duplexed with a short oligo (S2 in Figure 4(D)) before addition to the reaction, which allows rapid-hybridization TMSD and yields a high signal-to-noise ratio.

Enzymatic reaction for regulating downstream reactions

A recent study exploited the property of ligase to use a NAD^+ as a cofactor (Figure 5(A)) [19]. A dumbbell hairpin with two stem-loop structures and nicking sites was used as probe DNA. In the presence of NAD^+, DNA ligase-NAD^+ binding induces nick healing in the dumbbell hairpin, which is resistant to both exonuclease I and III digestion. In the absence of NAD^+, the nick is not repaired, allowing exonuclease digestion. SYBR green I dye was then integrated into the ligated dumbbell hairpin stem, producing fluorescence.

Another DNA ligase reaction exploits the ability of the enzyme to repair nicks in both strands of the DNA duplex (Figure 5(B)) [58]. One study used a short capture probe (CP): long signal probe (SP) duplex immobilized on an electrode. The CP is first immobilized on the substrate, which then binds to a methylene blue-labeled SP. If Ag^+ is present, the linear CP:SP duplex undergoes a conformational change, yielding a folded structure *via* a cytosine-Ag^+-cytosine composite. Ligase then fills in a nick in the folded duplex, forming a stem-loop structure. Upon denaturation and removing unligated SP, fluorescence is detected from the methylene blue bound to the remaining ligated SP. If Ag^+ is absent, the conformational change in the CP:SP duplex does not occur. SP dissociates from the linear duplex after denaturation and washing, preventing detection of the fluorescent signal (Figure 5(B)).

Enzymatic reaction for initiating the cascade amplification

A ligase reaction can facilitate the next round of a subsequent enzymatic reaction. In the presence of target DNA, the open circular probe and target DNA are hybridized (Figure 5(C)) [59]. T4 DNA ligase-assisted ligation closes the circular probe, allowing a DNA polymerase-trigged RCA reaction of the closed circular DNA, thereby releasing and recycling the target DNA.

Another study used nicked dsDNA as a starting material, which binds to the DNA binding domain of an allosteric transcription factor (aTF) [38]. In the presence of a target, an effector-binding domain of aTF binds to and is then dissociated from nicked dsDNA, allowing nick repair by T4 ligase. In the absence of a target, aTF still binds to nicked dsDNA, but T4 ligase does not repair the nick because aTF binding prevents nick repair. Primer-triggered RCA is initiated only on ligated DNA, and nicking enzyme-triggered DNA amplification is also initiated at the nick site to generate G-quadruplexes and additional primers.

Conclusion and future perspectives

Over the past few decades, biomolecule detection has continuously advanced *via* the development of new assay systems that can detect and identify the biomolecules responsible for various diseases. This review has focused on recently developed biosensors combined with DNA-modifying enzyme reactions and describes recent examples of their sensing strategies. We have highlighted the role of DNA-modifying enzymes, such as DNA polymerase, nicking endonuclease, exonuclease, and DNA ligase, for biomolecule detection. There are many challenges when attempting to develop multiplexed, sensitive, selective, rapid, and cost-effective assays for POC biomolecule detection. Many strategies to improve the performance of biosensor have been and are being developed as discussed in the above sections (Table 2).

Together with the attempt mentioned in the above sections, one of the important researches focuses for improving DNA-modifying enzyme-coupled biosensors is the identification and development of enzymes with new functions and distinct characteristics. However, most biosensor-related research focuses on probe DNA design. The biosensors developed so far for biomolecule detection use limited types of DNA-modifying enzymes (Table 1). Thus, efforts need to be undertaken to identify new enzymes and develop engineered ones. Enzymes with high activity, high processivity, and heat/chemical-stability are actively being developed to build robust and reliable assays [59–64] (Table 2). It is also worth applying enzyme-based logic gates [65,66] to detect biomolecules.

Enzyme-assisted cascade reactions may require complex probe design and the addition of extra enzymes, which may lead to nonspecific reactions, an increased risk of false-positive results, and low portability. Enhancing the thermodynamic stability of the nucleic acid hybridization between target and probe DNAs can enhance assay specificity and sensitivity, since the assay employs enzymes working at a constant low temperature for simple and rapid sensing. Recently, the thermodynamic stability of coaxial stacking hybridization has

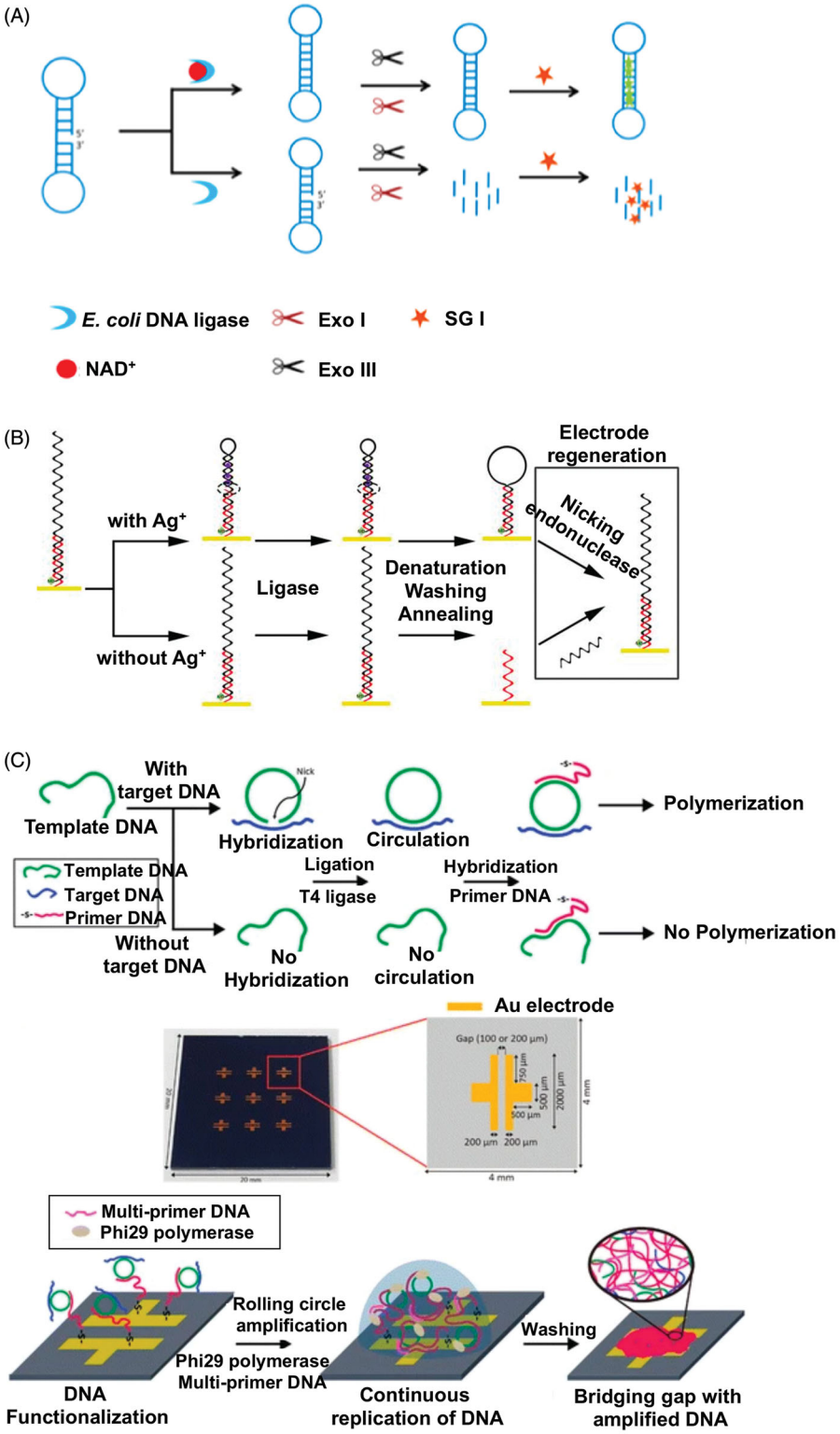

Figure 5. Biomolecule-detecting strategies of DNA ligase-assisted biosensors. (A) Schematic illustration of fluorescence detection of NAD^+. Reproduced with permission from reference [19]; (B) Schematic illustration of an electrochemical Ag^+ sensor. Reproduced with permission from reference [58]; (C) Schematic illustration of an electrical pathogen DNA biosensor. Reproduced with permission from reference [59].

been investigated [67]. For high specificity and sensitivity, a recent study has also used the DNA walker system to initiate cascade amplification [25]. In this system, mismatched DNA fragments were detected by combining a DNA walking machine with an electrode with a porous structure and large surface area, resulting in a

0.6 fM LOD with a high signal-to-noise ratio. Furthermore, some studies have showed that even single-nucleotide mismatches could be detected at low target concentrations by the use of a DNA polymerase-assisted SDA reaction (100 amol LOD for miRNA, [68]; 0.14 fM LOD for miRNA, [69]).

One challenge when developing POC systems is decreasing the assay time (Table 2). The assay time required by DNA-modifying enzyme-based biosensors depends on how efficiently the enzyme catalyzes the reaction on the substrate. This can also help determine whether the DNA and enzymes should be added to the reaction mixture consecutively or all at once. One-tube enzymatic reactions are simple, but can have low signal-to-noise ratios. Subsequent exogenous component addition provides high signal-to-noise ratio, but requires a more complicated, multi-step process with a long assay time that may prevent practical point-of-care use [52]. Some recent studies have used the stacking primer technique, with increased strand replacement rates up to 10^6 fold [70].

Simultaneous detection of different molecule types in one assay is also a challenge for detecting and identifying low-concentration biomolecules (Table 2). For example, each cell contains about 1000 miRNA molecules. To meet high target demands, enzyme-based amplifications were combined with various methods, such as magnetic separation technology [37], voltammetry using various electroactive labels [71], and various fluorescence labels [72].

Diverse ideas and efforts are continuously being developed, which will lead to the production and commercialization of sensitive, accurate, inexpensive, and multiplexed biosensor systems. These systems will facilitate the detection of biomolecules that could have several applications, such as in POC disease monitoring, environmental monitoring, food control, and biomedical research.

Disclosure statement

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