

# Nucleic acid tool enzymes-aided signal amplification strategy for biochemical analysis: status and challenges

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Received: 25 September 2015 / Revised: 13 November 2015 / Accepted: 1 December 2015 / Published online: 29 December 2015  
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**Abstract** Owing to their highly efficient catalytic effects and substrate specificity, the nucleic acid tool enzymes are applied as ‘nano-tools’ for manipulating different nucleic acid substrates both in the test-tube and in living organisms. In addition to the function as molecular scissors and molecular glue in genetic engineering, the application of nucleic acid tool enzymes in biochemical analysis has also been extensively developed in the past few decades. Used as amplifying labels for biorecognition events, the nucleic acid tool enzymes are mainly applied in nucleic acids amplification sensing, as well as the amplification sensing of biorelated variations of nucleic acids. With the introduction of aptamers, which can bind different target molecules, the nucleic acid tool enzymes-aided signal amplification strategies can also be used to sense non-nucleic targets (e.g., ions, small molecules, proteins, and cells). This review describes and discusses the amplification strategies of nucleic acid tool enzymes-aided biosensors for biochemical analysis applications. Various analytes, including nucleic acids, ions, small molecules, proteins, and cells, are

reviewed briefly. This work also addresses the future trends and outlooks for signal amplification in nucleic acid tool enzymes-aided biosensors.

**Keywords** Nucleic acid tool enzymes · Signal amplification · Nucleic acid · Aptamer · Sensing

## Introduction

Nucleic acid tool enzyme is a kind of tool that can precisely cut, splice, and modify nucleic acids, and is widely used in genetic engineering. The nucleic acid tool enzymes include restriction enzymes, ligases, polymerases, nucleases, modifying enzymes, and so on. Different tool enzymes have different functions and characteristics in biology. Figure 1 schematically summarizes several prevalently used nucleic acid tool enzymes, demonstrating the unique substrate specificity and some substrate preferences. For example, polymerase replicates duplex DNA structures using nucleic acid templates as a guide [1], ligase facilitates the joining of DNA strands together by catalyzing the formation of a phosphodiester bond [2], and telomerase elongates single strands of nucleic acids along the template of its own RNA molecule [3]. The sequence-specific scission or nicking of duplex DNA substrates can hydrolyze by restriction endonucleases or nicking endonuclease, respectively [4, 5]. The duplex-specific nuclease (DSN) hydrolyzes only double strands DNA (dsDNA) or DNA in DNA/RNA hybrids, regardless of nucleotide sequence, and does not cleave single-stranded DNA (ssDNA) or RNA at all [6]. Furthermore, the RNase (RNase H) recognizes RNA/DNA heteroduplexes and specifically cleaves the RNA strands into smaller fragments [7]. Last but not least, exonuclease is a kind of sequence-independent tool enzyme that breaks phosphodiester bonds at the 3' end or the 5' end and cleaves nucleotides one at a time from the end of

Published in the topical collection featuring *Young Investigators in Analytical and Bioanalytical Science* with guest editors S. Daunert, A. Baeumner, S. Deo, J. Ruiz Encinar, and L. Zhang.

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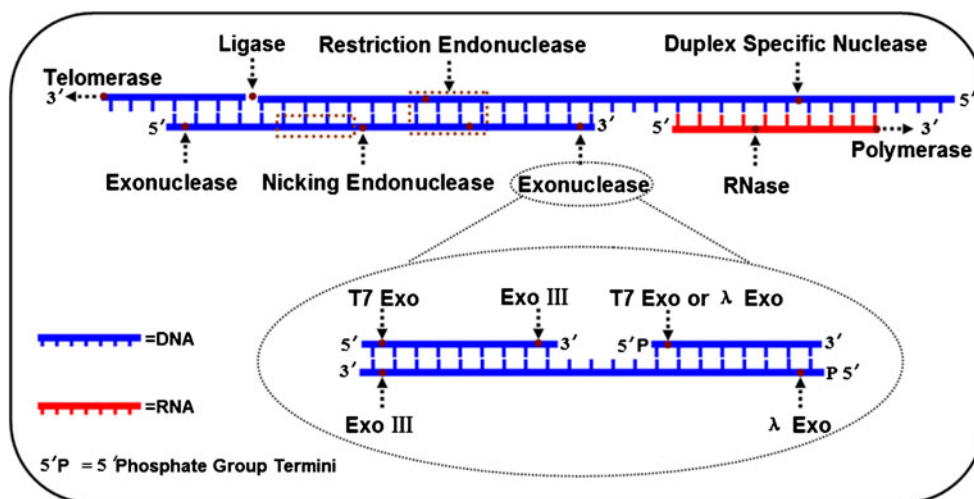
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**Fig. 1** Schematic presentation of the substrate specificity and some substrate preferences of different nucleic acid tool enzymes



polynucleotide chain [8, 9]. Although these exonucleases do not require a specific recognition sequence in their substrates, they still display some substrate preference. For example,  $\lambda$  exonuclease needs a cleavable dsDNA strand to have a phosphate group on its 5' end.

With the development of the tool enzymes, different nucleic acid tool enzymes are applied as 'nano-tools' for manipulating different nucleic acid substrates both in the test-tube and in living organisms. After the action of nucleic acid tool enzymes, the nucleic acid substrates will generate a series of structure switching, including the length of strand and the secondary and tertiary structures of substrates. Besides, they will also generate some by-products, such as repetitive ssDNA and free target, which can be used to initiate the subsequent reaction or target recycling. Thus, the diversity of nucleic acid tool enzymes can be widely applied on nucleic acids-based manipulation and nucleic acids-related signal amplification sensing. Compared with other signal amplification strategies, such as enzyme-linked immunosorbent assays [10, 11], immuno-PCR [12], mass spectrometric techniques [13], and nanomaterial-based strategies [14], the nucleic acid tool enzymes-aided signal amplification strategies have many remarkable merits: (1) the nucleic acid tool enzymes have highly efficient catalytic effects for substrates, thus the sensitivity can be guaranteed; (2) the reaction condition of enzymes involved is mild; (3) some of these enzymes display sequence-specificity or substrate preference, which can be used to design sensing mechanisms directly; (4) derived from lives cells, the nucleic acid tool enzymes display better biocompatibility in biochemistry analysis. So, the nucleic acid tool enzymes-aided signal amplification strategies have been studied in detail and developed in recent years [15–17].

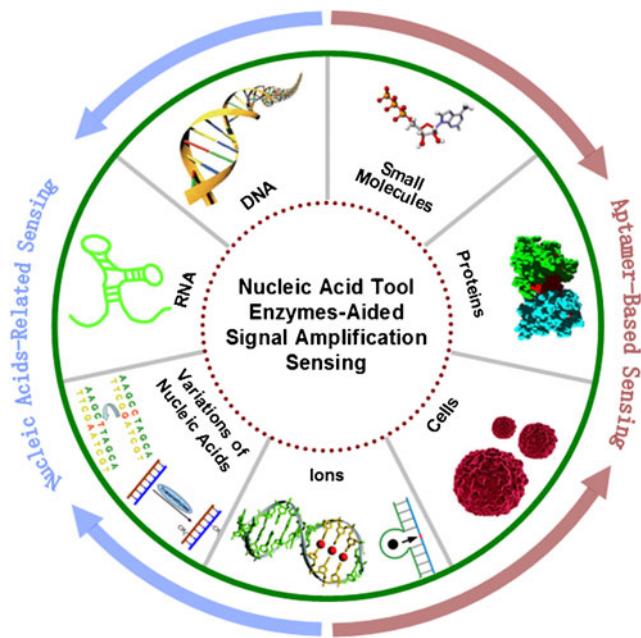
The substrates of nucleic acid tool enzymes are nucleic acids, the nucleic acid tool enzymes-aided signal amplification strategies are then mainly used for nucleic acid (DNA or RNA) amplification sensing, as well as the amplification

sensing of biorelated variations of nucleic acids such as DNA methylation and single nucleotide polymorphisms (SNPs). Moreover, some of the ions can mediate formation of non-natural base pairs in DNA duplexes, such as T-Hg<sup>2+</sup>-T or C-Ag<sup>+</sup>-C complexes [18]. Nucleic acid sequences that specifically bind Pb<sup>2+</sup>, Mg<sup>2+</sup>, or Cu<sup>2+</sup> ions were found to yield supramolecular coiled structures that cleave specific DNA sequences [19]. The nucleic acid tool enzymes therefore can be used for amplification detection of ions. Recently, aptamers, the single stranded nucleic acid molecules selected from 10<sup>12</sup> to 10<sup>15</sup> combinatorial oligonucleotide libraries by a process called systematic evolution of ligands by exponential enrichment (SELEX) [20], have attracted more and more attention because of their homothetic characteristic of antibodies. In addition to binding to complementary nucleic acids, the aptamers can bind to non-nucleic acid targets, such as metal ions, small molecules, proteins, or even whole cells, with high affinity and specificity [21]. So, the introduction of aptamers provides unprecedented opportunities for nucleic acid tool enzymes to sense non-nucleic acid targets and dramatically widens the application areas of nucleic acid tool enzymes-aided signal amplification strategies.

In this review, we focus on the recent development of nucleic acid tool enzymes-aided signal amplification strategies in nucleic acid-related sensing and aptamer-based sensing. Various analytes detection by nucleic acid tool enzymes-aided signal amplification strategies are summarized (Fig. 2), such as DNA sensing, miRNA detection, SNPs identification, DNA methylation analysis, ions detection, small molecules sensing, and proteins and cells detection.

## The detection of DNA

As one of the most exciting biopolymers in life science, DNA is not only the carrier of genetic information but also holds



**Fig. 2** Schematic illustration of nucleic acid tool enzymes-aided signal amplification sensing

great promise for future applications in nanotechnology, material science, and biomedical analysis. Rapid growth of available sequence data has made the detection of specific nucleic

acid sequences critical for gene therapy, mutation analysis, and clinical diagnosis [22–25]. Among the many methods devised for the detection of nucleic acids, amplification assays have received much attention because of the highest analytical sensitivity. As the classic target amplification method, polymerase chain reaction (PCR) can amplify trace amounts of DNA to levels that are detectable and improve the sensitivity to reach the level of single molecule detection limits [26]. However, PCR-based methods require special thermal cycling equipment and are prone to yielding false-positive results from artifact amplification, which may restrict its further application [27]. Thus, the design of alternative approaches for the sensitive detection of DNA with rapid, easy manipulation is in continuous demand. Recently, enzyme conjugates [28], DNazymes [29], nucleic acid tool enzymes [16, 30], and nanomaterials [31] have been used as amplifying labels for biorecognition, and a series of amplified methods have been developed for DNA sensing. For example, Wang et al. explored a series of electrochemical approaches for the sensitive detection of DNA by coupling of several carbon nanotubes (CNT)-derived amplification processes [32, 33]. Mao and Tian reported an improved catalytic molecular beacon for DNA/RNA detection [34]. When the target DNA was presented, the DNazymes became activated and constantly amplified the output signal through circulatory cleavage of molecular

**Table 1** Comparison of different DNA detection methods based on the nucleic acid tool enzymes

Method	Nucleic acid tool enzyme	Linear range	LOD	Year	Ref
Colorimetric	Nicking endonuclease	–	10 pM	2009	[37]
	Exo III	0.1 pM–1.0 mM	0.1 pM,	2012	[38]
	Ligase / polymerase / nicking endonuclease	–	1 pM	2012	[39]
	Ligase	50 aM–5.0 pM	20 aM	2012	[40]
	polymerase	–	1 pM	2013	[41]
Fluorescent	Nicking enzyme	–	6.3 pM	2008	[42]
	endonuclease / ligase / nicking endonuclease	–	1 fM	2011	[43]
	Nicking endonuclease	1 pM–1 nM	0.16 nM	2013	[44]
	T7 exonuclease	1 nM–50 nM	0.3 pM	2013	[45]
	$\lambda$ exonuclease	0.4 pM–4 nM	68 fM	2013	[46]
	Exo III	20 aM–200 fM	20 aM	2010	[47]
	Exo III	0.5 pM–50 pM	0.12 pM	2011	[48]
	Exo III	0.5 nM–20 nM	50 pM	2012	[49]
	Exo III	5 pm–500 pM	0.3 pM	2014	[50]
	Exo III	5 aM–70 aM.	4 aM	2015	[51]
Electro chemical	Restriction endonuclease	1 pM–10 nM	14 pM	2012	[52]
	nicking endonuclease / polymerase	10 aM–10 pM	11 aM	2012	[53]
	Exo III	10 fM–500 fM	10 fM	2013	[54]
	Exo III	0.1 pM–0.1 nM	0.1 pM	2014	[55]
	Exo III	100 fM–5 nM	92 fM	2015	[56]
	Exo III	0.01 pM–10 nM	32 fM	2015	[57]

beacon, which greatly improved the detection sensitivity. Willner et al. have reported an amplified detection method for DNA sensing by the application of rotating functional magnetic particles [35]. Compared with signal amplification by DNAzymes and nanomaterials, nucleic acid tool enzymes-aided signal amplification strategies have attracted considerable attention due to easy operation, high catalytic efficiency, and isothermal reaction condition. In this approach, each target molecule initiates multiple signaling events, and the sensitivity of DNA detection is thereby significantly increased [36]. By combining nucleic acid tool enzymes with different assay platforms, DNA can be quantified rapidly using colorimetric, fluorescent, or electrochemical assays (Table 1).

### Nucleic acid tool enzymes-mediated colorimetric DNA assays

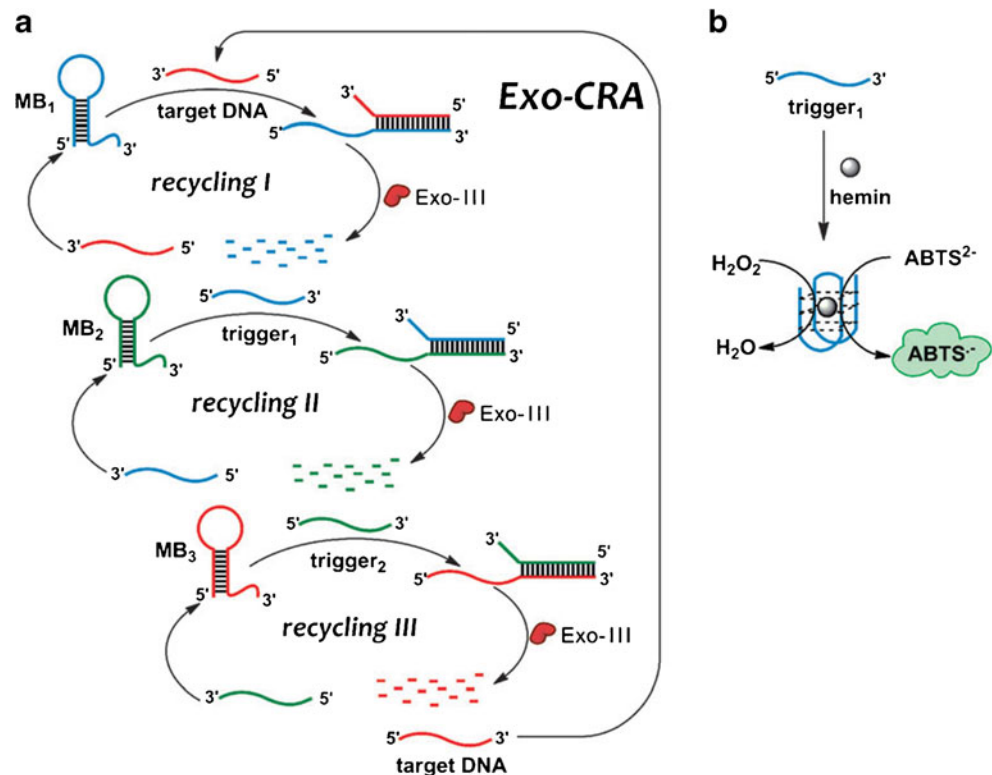
Colorimetric analysis is a simple process because the results can be monitored with the naked eye [58]. The inherent superiorities of colorimetry, such as simplicity, inexpensive, easy manipulation, and little or no required instrumentation, make it very attractive for DNA detection [59]. With the help of nucleic acid tool enzymes, the sensitivity of colorimetry gained great improvement. For example, Bi et al. reported a label-free colorimetric method for DNA detection based on target DNA triggered exonuclease-aided cascaded recycling amplification (Fig. 3) [38]. In this assay, they designed three

kinds of unlabeled stem-loop DNA molecular beacons (denoted as MB<sub>1</sub>, MB<sub>2</sub>, and MB<sub>3</sub>, respectively). In the presence of target, MB<sub>1</sub> was opened by hybridizing with target to form duplex DNA with a blunt 3' terminus. Then, the Exo III bound to the duplex region of this hybrid and partially hydrolyzed the MB<sub>1</sub> from the blunt 3' terminus, releasing the target and trigger 1.

The released target could catalyze a new cycle of probe transformation (recycling I). The trigger 1 hybridized with MB<sub>2</sub> at its 3' terminus to initiate the recycling II, which resemble recycling I. After recycling II, the trigger 2 was released and the recycling III was activated. In recycling III, the liberated single-stranded trigger was the same as that of target DNA. So the trigger, released from recycling III, in turn triggered recycling I and repeated the above cyclic process to achieve cascaded amplification. After finishing the whole network, trigger 1 could form the catalytic DNAzyme units in the presence of hemin and oxidize 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) disodium salt (ABTS<sup>2-</sup>) to produce the colored product, ABTS<sup>•-</sup>, which has a maximal absorption at 419 nm.

Compared with those organic color reagents, optical nanomaterials such as gold nanoparticles (AuNPs) have high brightness, lower cytotoxicity, and better biocompatibility, making them suitable for constructing biosensors [60, 61]. For instance, the ultrahigh extinction coefficient ( $10^8$ – $10^9$ ) of the surface plasmon resonance (SPR) absorption of AuNPs provides an alternative opportunity for improving traditional

**Fig. 3** Illustration of target DNA triggered exonuclease-aided cascaded recycling amplification. Reprinted from [38] with permission





colorimetric method [62]. In 2009, Liu et al. reported a homogeneous colorimetric DNA biosensor using nicking endonuclease-aided nanoparticle amplification, which allowed high sensitivity detection of long, single stranded oligonucleotides with single-base mismatch selectivity [37]. In this detection system, the nicking endonuclease was specifically designed to cleave only the linker DNA. In the absence of target DNA, the intact linker DNA would trigger oligonucleotide-modified gold nanoparticles aggregation and a concomitant color change. If the target was present, the linker strand would be cleaved by nicking endonuclease and particle aggregation would not occur.

### Nucleic acid tool enzymes-mediated fluorescent DNA detection

Although colorimetric assays are amenable to point-of-care (POC) testing, the low sensitivity of colorimetry precludes it from being a strong competitor among various analysis strategies. Fluorescent analysis strategies are typically based on the measurement of the fluorescence intensity induced upon specific binding between fluorescence-labeled probes and target molecules. Compared with colorimetric assays, fluorescence assays enable extraordinary sensitivity and multiplex detection capability as well as automation. The nucleic acid tool enzymes-aided signal amplification has also been adopted in fluorescence assays for improving the sensitivity of DNA detection. Two well-known nucleic acid tool enzymes-aided target recycling for DNA detection assays, the nicking enzyme assay [42] and exonuclease III assay [47], are illustrated in Fig. 4a and b, respectively. In the nicking enzyme assay, a nicking enzyme recognition sequence was embedded into the loop portion of the molecular beacon (MB). When the target DNA was presented, it hybridized with MB and formed a full recognition site for the nicking enzyme. The nicking enzyme nicked the beacon strand and separated fluorophore and quencher, generating a high fluorescence signal. The released target strand could then hybridize with another MB to initiate the next cycle of cleavage, resulting in an amplified fluorescence signal. The exonuclease III assay mainly utilizes the cleavage function of Exo III on blunt or recessed 3' hydroxyl termini of duplex DNAs. In this assay, an MB was modified with a CAL Fluor Red 610 (FR610) fluorophore at its 5' end and a Black Hole Quencher (BHQ) at an internal position. When the target DNA was absent, the MB self-hybridized to form a stem-loop structure with a 3' protruding terminus, which could resist Exo III hydrolysis. This closed stem-loop structure held the fluorophore in close proximity to the quencher, resulting in low fluorescence. However, in the presence of target DNA, the stem-loop structure was opened and formed a double-stranded structure with a blunt 3' terminus.

Then, Exo III catalyzed the hydrolysis of beacon strand from blunt 3' terminus, releasing the fluorophore and the

target DNA. The released target DNA then hybridized with another MB probe to initiate a new round of hydrolysis.

Even though the Exo III is assumed to be dsDNA-specific exonuclease in most literature, recent research shows that Exo III has the activity of hydrolyzing ssDNA [63]. The hydrolysis of ssDNA target is one of the main causes for the ceasing of target recycling amplification. In order to solve this problem and obtain higher amplification, Yang et al. developed a dual-signal amplification method for DNA detection based on the hydrolyzing activity on ssDNA of Exo III. In this method, an X sequence was specially designed that could resist the Exo III digestion. The X sequence could cyclically trigger the molecular beacons digestion for a long time, resulting in four orders of magnitude in the detection limit [50].

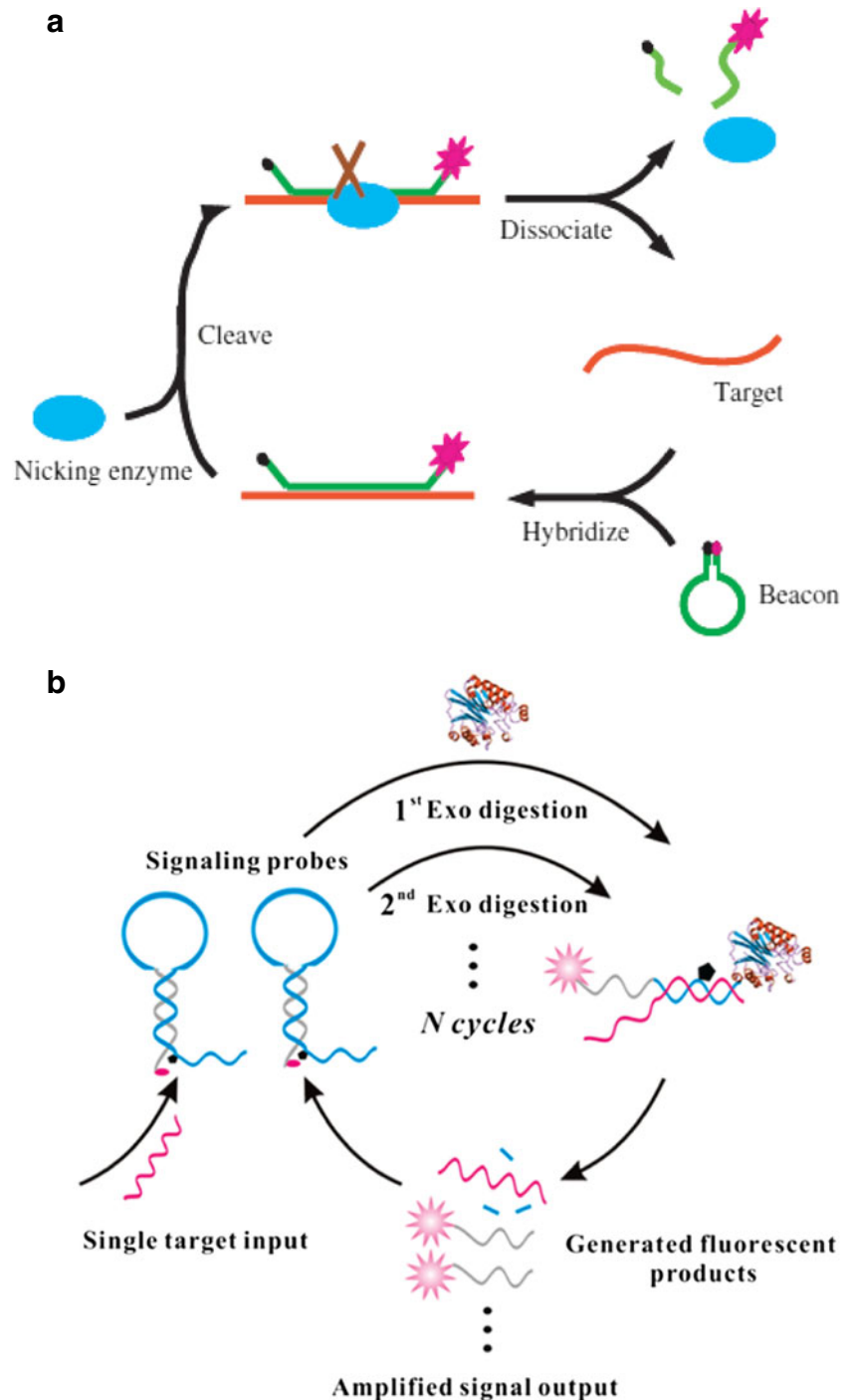
### Nucleic acid tool enzymes-mediated electrochemical DNA sensing

Electrochemical biosensor-based DNA detection is popular because of its simplicity, rapid response, high sensitivity, and low fabrication cost [64].

The reaction is monitored electrochemically, based on the measurement of current (amperometry), potential (potentiometry), or changes in the conductive properties of the medium between the electrodes (conductometry) [65]. Many electrochemical DNA sensors have also been developed based on nucleic acid tool enzymes-aided amplification [52–55]. For example, Ye et al. proposed a rapid, single step, ultrasensitive electrochemical DNA sensor using the Exo III to achieve the aim of signal amplification. As depicted in Fig. 5, a signaling probe with a thiol group at 5' terminus self-assembled onto the gold electrode. Then, the fabricated gold electrode was exposed in RuHex buffer to adsorb RuHex molecules, in which RuHex/DNA/electrode system gave an initial redox signal arising from electron transfer between RuHex molecules and the gold electrode. Upon addition of the target DNA and Exo III, the signaling probe hybridized with target DNA to form a double-stranded structure with a 3' blunt end. The Exo III specifically recognized the duplex and catalyzed the hydrolysis of signaling probe from blunt 3' terminus, releasing the target DNA. The released target DNA could hybridize with another signaling probe to initiate a new round of digest process. After manifold cycles, most of the signaling probes were hydrolyzed and only a small quantity of RuHex was adsorbed onto the gold electrode, resulting in a decrease redox signal. By employing Exo III-aided target recycling strategy, the sensitivity of the proposed sensor has been greatly improved, to the detection limit of 20 fM [66].

In order to avoid the enzyme recognition sites located on the target DNA/capture DNA duplexes, the targets should contain special sequences. Our group introduced a 'Y' junction structure, formed by a capture probe, an assistant probe, and a target DNA, for detecting different target DNAs. In this

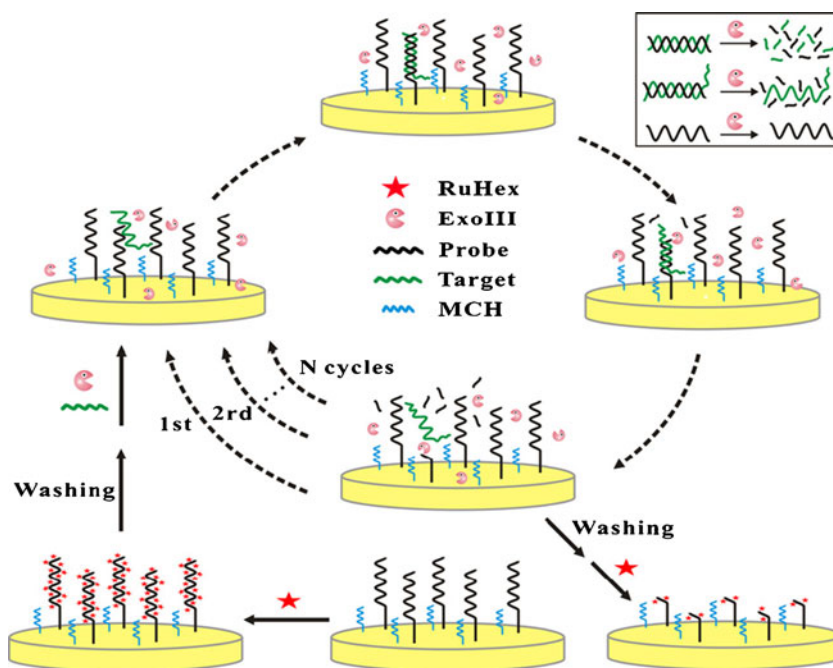
**Fig. 4** Working principle of nicking enzyme (a) and exonuclease III (b) aided signal amplification. Reprinted from [42] and [47] with permission



assay, a methylene blue labeled capture probes self-assembled onto the gold electrode through Au-S linkages. Upon addition of target DNA and assistant probe to this system, the target DNA was complementary to the capture probes at one-half of the segment and complementary to the assistant probe at the other half-segment. Meanwhile, the rest of assistant probe also hybridized with the remaining segment of capture probe. The specific cleavage site for restriction endonuclease was located on the assistant probe/capture probe duplexes, and isolated

from target DNA. Thus, the restriction endonuclease recognized the specific cleavage site in assistant probe/capture probe duplexes and digested the capture probe, releasing the methylene blue labeled piece. At the same time, the target DNA could also be released and initiate a new round of digest process. By changing the sequences of assistant and capture strands, the proposed sensor was suitable for detecting different target sequences with high sensitivity [52]. More recently, in order to avoid the limitation of "signal-off" strategy, the

**Fig. 5** Schematic representation of the label-free electrochemical DNA sensor based on Exo III-aided target recycling. Reprinted from [66] with permission



ratiometric electrochemical assay, combining “signal-on” and “signal-off” strategies, has also gained widespread development and application because of its good analytical performance [57, 67, 68].

### The detection of miRNA

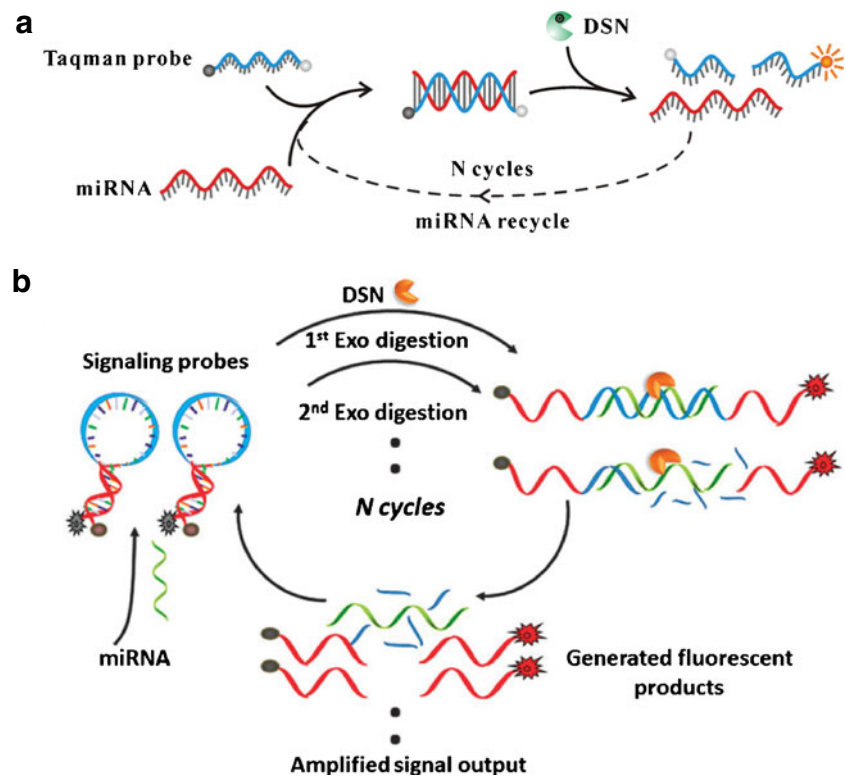
miRNAs are a group of short, endogenous, noncoding RNAs involved in a variety of biological processes. Recent studies have indicated that abnormal miRNA expression is highly correlated with various diseases [69]. Nowadays, miRNAs are regarded as biomarker candidates in clinical diagnosis and therapy [70–72]. However, the unique characteristics of miRNAs, such as their sequence homology, small size, low abundance in total RNA samples, and susceptibility to degradation, make them difficult to be detected [73]. The Northern blotting technology and microarrays are the traditional approaches for miRNA detection. But it is unsatisfactory because of their low sensitivity and specificity [74, 75]. Recently, various methods have been developed for miRNAs detection, such as nanoparticle-based amplification strategy [76] and nucleic acid tool enzymes-aided amplification strategy [77, 78]. Among these approaches, multiple self-assembly steps or complicated operational requirements are needed by the former. The nucleic acid tool enzymes-aided amplification strategies have attracted considerable attention because of easy operation and isothermal reaction. However, some of these nucleic acid tool enzymes, such as nicking endonucleases and restriction endonucleases, require a specific nucleotide sequence for recognition and cleavage. Limited by the

intrinsic properties of the nucleases used, most of the previous DNA amplification detection strategies are not suitable for the detection of miRNA. Fortunately, some sequence-independent nucleic acid tool enzymes, such as Exo III, S1 nuclease, and (DSN), can catalyze the cleavage of phosphodiester bonds in either DNA or RNA and have been widely used for miRNA detection [79–82].

For example, DSN has a substrate preference for hydrolyzing dsDNA and DNA in DNA-RNA hybrid duplexes, and does not cleave ss-DNA or RNA. Based on this property of DSN, Ye et al. developed a single-step, sensitive method for miRNA detection [83]. As shown in Fig. 6a, a Taqman probe, modified with a fluorophore and a quencher, acted as signal output. In the presence of target miRNA, the Taqman probe hybridized to a target miRNA to form DNA/RNA heteroduplex, which could be recognized and digested by DSN. After digestion by DSN, the fluorophore and quencher were separated, resulting in significant fluorescence signal. Meanwhile, the target miRNA was released and started a new digest process. This method allowed the detection of miRNA in the femtomolar range within 30 min, nearly five orders of magnitude lower than the conventional method.

Moreover, multiplexed miRNAs detection could be easily realized by designing different fluorophore-labeled Taqman probes because DSN enzyme has no requirement for specific recognition sequence. Similarly, Yang et al. [84] designed a sensitive method for miRNA detection based on a specific molecular beacon (MB) and DSN-aided target recycling (Fig. 6b). Since DSN can also cleave dsDNA, the stem of the MB is prone to be recognized and digested by DSN. To overcome this problem, a specific MB, modified with 2-OME-

**Fig. 6** **a** Schematic representation of miRNA direct detection based on duplex-specific nuclease signal amplification. Reprinted from [83] with permission. **b** Working principle of the 2-OMe-RNA-modified MB and DSN-aided target recycling for miRNA detection. Reprinted from [84] with permission



RNA on its stem, was designed to resist DSN hydrolysis. In the absence of miRNA, the MB showed low fluorescence intensity since the fluorophore and the quencher were in close proximity. In the presence of miRNA, the MB would bind to the target miRNA and produce a fluorescence signal because of the spatial separation of the fluorophore from the quencher. The formed MB–miRNA duplex would be recognized and digested by DSN, releasing the target miRNA. Subsequently, the released miRNA could hybridize with another MB, which leads to a cyclic reaction and fluorescent signal amplification. In order to further improve the sensitivity, by combining T7 exonuclease-aided cyclic amplification and rolling circle amplification strategy, they developed a dual amplification method for ultrasensitive detection of miRNA [85]. This dual amplification method could achieve a detection limit as low as 12 fM. More recently, based on polydopamine nanospheres-protected ssDNA probes and DNase I-aided signal amplification strategy, they developed a simple fluorescent method for sensitive miRNA detection [86].

## The analysis of biorelated variations of nucleic acids

### The analysis of single nucleotide polymorphisms

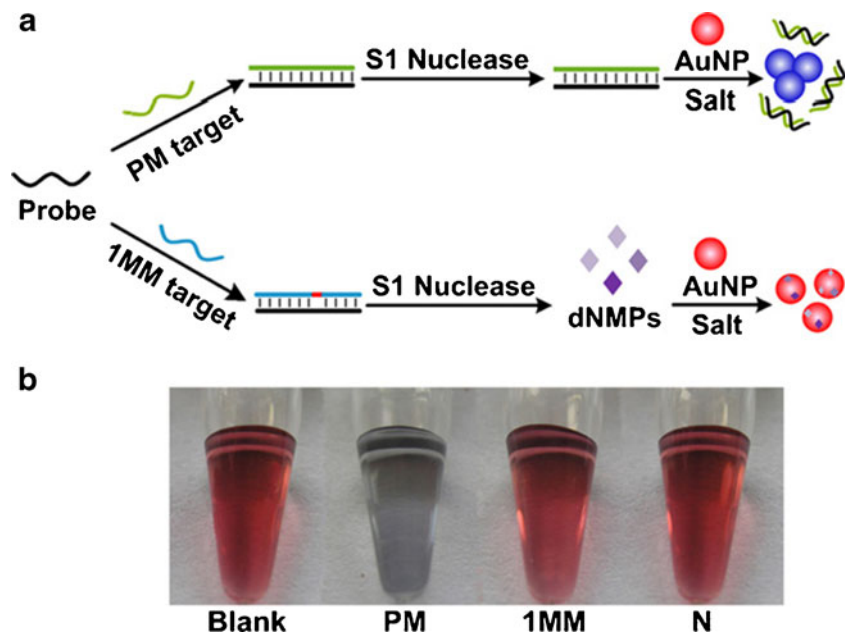
Because gene mutations play an important role in carcinogenesis, the identification of DNA variations is another important aspect of cancer therapy. Single nucleotide polymorphisms

(SNPs) are the most common type of genetic variation, and they have been regarded as the key diagnostic markers for cancer progression, genetic disease, and pharmacogenomics [87–89]. SNPs identification is therefore of vital importance for early diagnosis, clinical prognostics, and disease treatment. Usually, the detection of single-base mismatch is the basis of all hybridization-based detection approaches for SNPs analysis. Up to now, many nucleic acid tool enzymes-based methodologies have been proposed for single base mismatch detection [90–93]. For example, Zhao et al. reported a novel colorimetric method for single-base mismatch detection based on S1 nuclease and gold nanoparticles (Fig. 7) [94].

S1 nuclease has a substrate preference for cleaving ssDNA and base-pair mismatches in dsDNA, and does not cleave perfectly matched dsDNA [95]. In previous work they have found that nucleoside monophosphates (dNMPs) could better stabilize unmodified gold nanoparticles (AuNPs) than ssDNA and dsDNA with the same base-composition and concentration. As shown in Fig. 7, the 16-bp perfectly matched duplex DNA could not be cleaved by S1 nuclease. The intact dsDNA was not able to stabilize AuNPs, leading the AuNP aggregation upon adding salt solution and resulting in purple-blue solution. In contrast, in the presence of single-base mismatched duplex DNA, the color of AuNPs slightly changed into red-purple because of the incomplete cleavage of the single-base mismatched dsDNA by S1 nuclease. Single-base mutations present in target DNA with varied sites were all effectively detected by this method.



**Fig. 7** Schematic illustration of the label-free optical detection of single-base mismatches. Reprinted from [94] with permission



In order to improve the detection sensitivity of AuNPs-based SNPs assay, Xiao et al. [96] presented a remarkably simple platform that enables sensitive naked-eye detection of SNPs based on the Exo III aided target recycle at room temperature. In this assay, one set of DNA capture probe (CP) was modified into the AuNPs with Au-S linkages. If the target sequence is perfectly matched to the CP sequence on the AuNPs, the intact dsDNA could digest by Exo III, leading to AuNP aggregation and yielding a blue color solution. However, in the case of a single-base mismatch, the sensor failed to aggregate and remained red. This method could achieve a detection limit as low as 2 nM by naked eye, which was 100 times lower than that unmodified AuNPs.

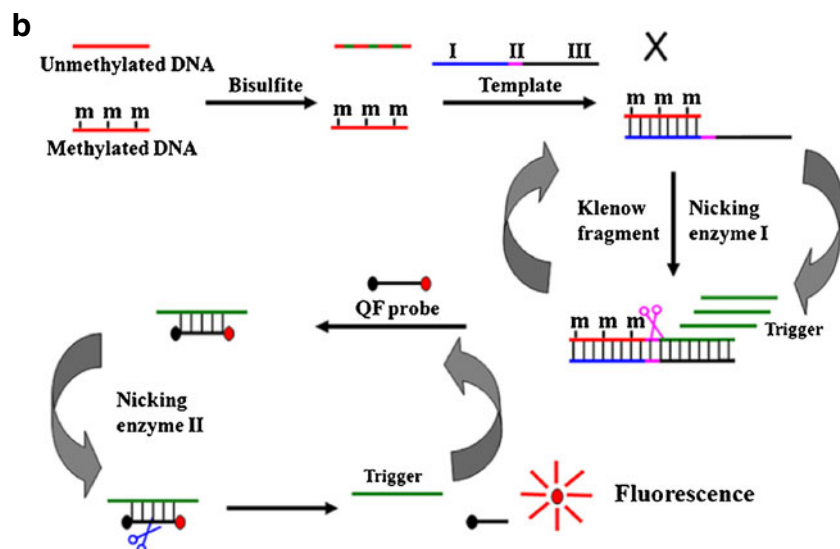
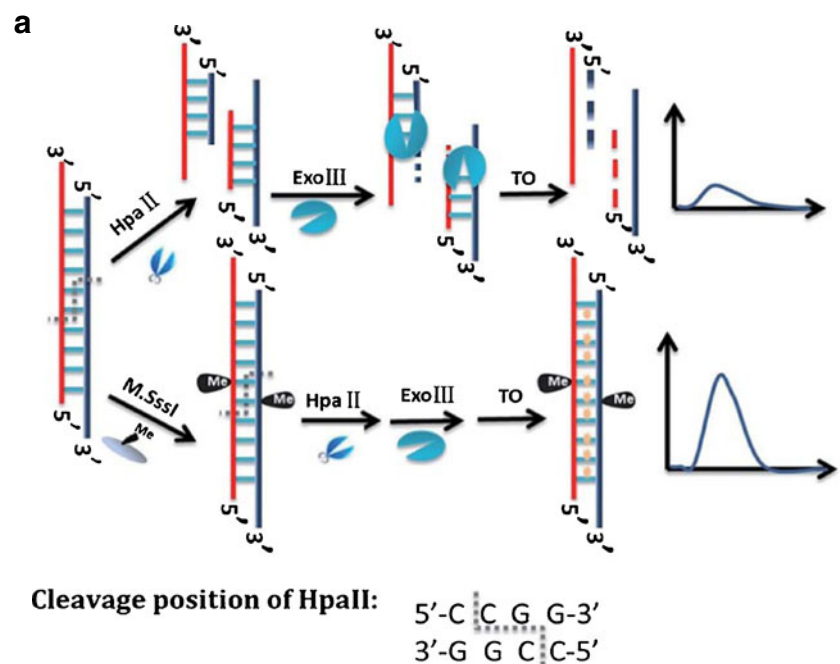
### The detection of DNA methylation and DNA methyltransferases

DNA methylation is an important component of epigenetic regulation and plays an essential role in regulating gene expression in organisms [97]. DNA methylation is catalyzed by different DNA methyltransferases (MTases) [98, 99]. As abnormal DNA methylation can be regarded as biomarkers for early cancers and DNA MTases is regarded as a potential target for anticancer drugs, the detection of DNA methylation and assay of MTase activities is of significance for both fundamental biochemical research and drug discovery [100–102]. So far, the methods for detection of DNA methylation mainly include immuno-based recognition [103], methylation-sensitivity restriction enzyme digestion [104], bisulfite treatment [105, 106], and various polymerase chain reaction techniques [107]. For the immuno-based methods, the preparation of antibody is labor-intensive and expensive,

which restrict its further application. In the restriction enzyme-based assay, a dsDNA with specific recognition site for restriction enzyme was usually designed. The restriction enzymes recognize the methylation/demethylation position, and selectively scissor the specific DNA sequence. When the specific position is demethylated/methylated, the cleavage will be inhibited. As in Fig. 8a [108], first, target DNA hybridized to the probe and formed dsDNA with two 3' protruding termini, which could be resistant to hydrolysis by Exo III. Hpa II endonuclease was used to recognize the 5'-CCGG-3' sequences and cleave them, resulting in two split short dsDNA with 3' recessed termini. Then, Exo III could bind to the split dsDNA and hydrolyze it from recessed 3' hydroxyl termini to generate ssDNA. The fluorescence intensity of the added thiazole orange (TO) was weak because of weak interaction between the TO and ssDNA. If dsDNA was methylated by M.SssI MTase, the function of Hpa II was inhibited and Exo III could not work. The added TO intercalated into its double strand and showed strong fluorescence intensity.

The methods based on restriction enzymes can accurately determine the methylation sites, but are confined to detect limited DNA methylation due to the selectivity of restriction enzymes. In the bisulfite-based assay, the cytosine (C) in substrate is deaminated and converted to uracil (U) through the treatment of bisulfite. If C was methylated (mC), mC remains almost unchanged. Therefore, this method could identify the methylated cytosine at any site in DNA and is therefore considered to be a gold standard assay. As in Fig. 8b [109], after the sodium bisulfite treatment, the cytosine in both methylated and unmethylated DNA was changed to uracil, but 5-methylcytosine (mC) remained unchanged. The methylated DNA specifically bound to the domain I of template and

**Fig. 8** **a** Schematic illustration of the label-free fluorescent method for the detection of site-specific DNA methylation and MTase activity based on restriction enzyme and Exo III. Reprinted from [108] with permission. **b** Schematic illustration of DNA methylation assay through the treatment of bisulfite. Reprinted from [109] with permission



worked as a primer to initiate an extension reaction in the presence of dNTPs and polymerase. With the assistance of nicking enzyme I, the extension reaction could generate a large number of trigger, which could hybridize to the QF probes and initiate the nicking enzymeII-aided signal amplification.

The unmethylated DNA with six-base mismatch was unable to hybridize with the template and could not initiate the subsequent signal amplification. More recently, by combining the advantages of LCR amplification and AuNPs-based colorimetric strategy, Li et al. developed a low-cost method for detection of DNA methylation with high sensitivity [110].

In many cases, the DNA methylation states are linked to the activity of DNA MTase [111, 112]. Alterations of MTase

activity may lead to abnormal DNA methylation. So, the DNA MTase has also been regarded as a potential therapeutic target in many types of cancer [113]. Strategies to detect DNA MTase are therefore also important in the area of clinical diagnostics. Recently, isothermal amplification techniques, such as strand displacement amplification (SDA) [114], rolling circle amplification (RCA) [115, 116], nicking enzyme signal amplification (NESAs) [117, 118], and methylation-sensitivity restriction enzyme digestion [119–121], are frequently applied for DNA MTase assay because of the wide variety of choices. In 2013, Zhang et al. [116] developed a highly sensitive chemiluminescence method for the MTase assay based on hairpin probe (HP) and rolling circle amplification (RCA). In the presence of DNA MTase, the HP was

methylated and cleaved by the methylation-sensitive restriction endonuclease. The cleaved HP could function as a primer to initiate RCA, which generated a large number of horseradish peroxidase-mimicking DNAzyme chains. The DNAzyme chains could fold into a biocatalytic G-quadruplex/hemin structure and catalyze the oxidation of luminol to generate distinct chemiluminescence signal in the presence of  $\text{H}_2\text{O}_2$  and luminol. In the absence of Dam MTase, the methylation and cleavage reaction of HP would not occur. The intact HP could not function as a primer to initiate subsequent RCA, and no chemiluminescence signal was obtained. More recently, they developed a sensitive fluorescence method for DNA MTase detection based on DSN and T7 RNA polymerase aided signal amplification [122].

### The amplification detection of ions

As is known, DNA forms a double-helix structure mainly through the formation of Watson-Crick base pairs, such as adenine-thymine (A-T) and guanine-cytosine (G-C) base pairs [123]. Recently, the Ono and Urata groups in a series of articles have reported that silver specifically stabilizes the cytosine-cytosine (C-C) mismatches (C-Ag-C) and mercury ions specifically stabilize the thymine-thymine (T-T) mismatches (T-Hg-T) in oligodeoxynucleotide duplexes [19, 124, 125]. In these non-natural base pairs, the hydrogen bonds present in natural DNA are replaced by coordinative bonds. These features were widely applied to develop T/C-rich DNA for the detection of  $\text{Hg}^{2+}$  or  $\text{Ag}^+$  through the ion-induced conformational switch [126, 127]. Both the objective of metal ion mediated non-natural base pairs and nucleic acid tool enzymes' substrates are nucleic acids, and the nucleic acid tool enzymes-aided amplification strategies were also applied in the detection of  $\text{Hg}^{2+}$  and  $\text{Ag}^+$  ions [128–132]. For example, Hsing and Xuan [128] reported an ultrasensitive electrochemical method for  $\text{Hg}^{2+}$  detection based on  $\text{Hg}^{2+}$  trigger digestion of the designed e-T-rich probe by Exo III (Fig. 9a). In this assay, an e-T-rich probe was modified with methylene blue at an internal T base close to the 3' end. In the presence of  $\text{Hg}^{2+}$ , the linear e-T-rich probe could fold into a hairpin structure with a blunt 3' terminus and activate the hydrolytic ability of the Exo III enzyme, releasing a thymidine monophosphate with the methylene blue tag (eNT). The eNT possesses a higher diffusivity toward the negatively charged ITO electrode than that of the e-T-rich probe due to its less negative charge and smaller size.

Thus, methylene blue could close to the ITO electrode and generate a distinct increase in the electrochemical signal. The released  $\text{Hg}^{2+}$  can bind with other e-T-rich probe to initiate a new cycle of cleavage. However, in the absence of  $\text{Hg}^{2+}$ , the linear e-T-rich probe could hardly get into contact with the ITO electrode and therefore a negligible electrochemical

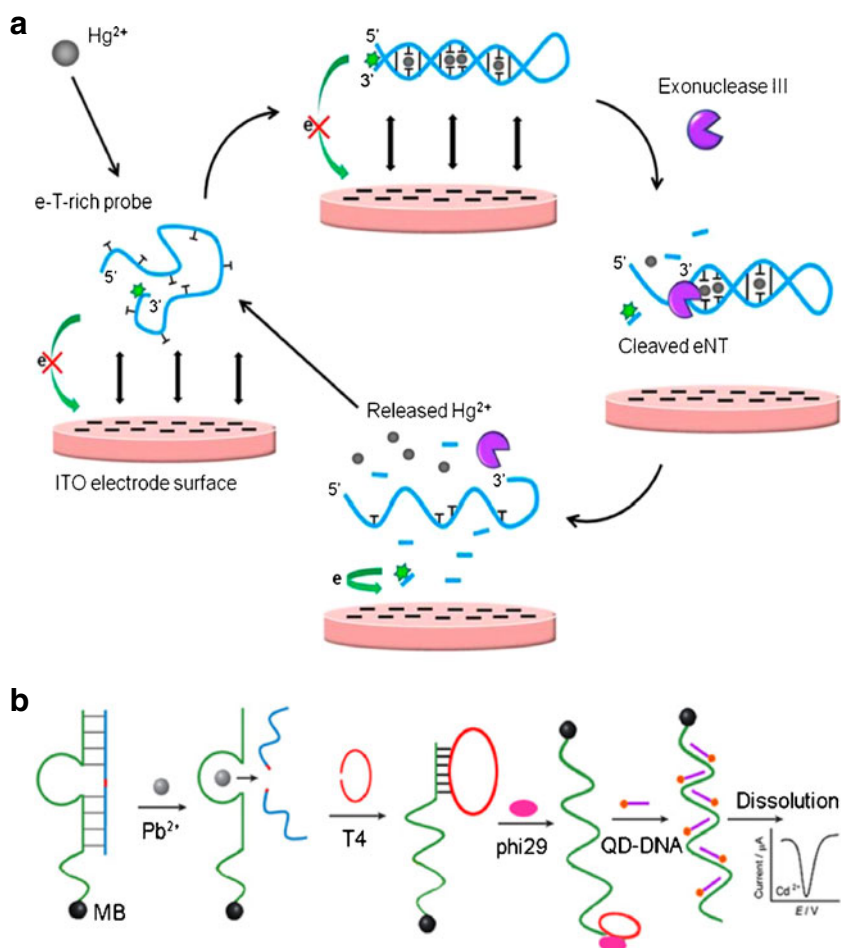
response was observed. Through the Exo III aided signal amplification, this immobilization-free electrochemical  $\text{Hg}^{2+}$  sensor platform has a detection limit of 0.2 nM and a total assay time within minutes. Similarly, Zhang et al. presented a new strategy for the detection of  $\text{Ag}^+$  based on ion-triggered conformation change and Exo III-aided target cycle.

In addition, since it was first found by two independent research groups in the late 20th century, the SELEX technique was widely used to select aptamers or DNAzymes. The DNAzymes are specific single strand DNA sequences that can be activated in the presence of added ions. For example, nucleic acid sequences that specifically bind  $\text{Pb}^{2+}$ ,  $\text{Mg}^{2+}$ , or  $\text{Cu}^{2+}$  ions were found to yield supramolecular coiled structures that cleave specific DNA sequences [18]. Based on this, researchers developed some new biosensors for the detection of these ions in recent years [133, 134] that overcome the limitations of the traditional protocols. Recently, by combining the powerful amplification of RCA and the highly specificity cleavage efficiency of ion-dependent DNAzyme, Zhang et al. [135] developed a novel, highly sensitive, and selective electrochemical sensing system for lead ions detection (Fig. 9b). In the absence of  $\text{Pb}^{2+}$ , the DNAzyme was inactivated, and neither cleaving nor RCA reaction could be initiated. When the  $\text{Pb}^{2+}$  was present, the DNAzyme was activated and the substrate strand was cleaved into two DNA fragments. The free catalytic strand could be hybridized with the padlock probe. After addition of ligase, polymerase, and dNTPs, the RCA process was triggered, producing a long ssDNA with repeating sequence units. Subsequently, the signal probes (CdS QD-ssDNA) were hybridized with the long ssDNA and formed a long DNA duplex. After magnetic separation, the unhybridized CdS QD-ssDNA was removed; the captured CdS QDs were then dissolved by nitric acid. The released cadmium cations could be quantified by square wave voltammograms. In order to further simplify the detection process, Guo et al. employed a DNA circular template instead of the padlock probe, which makes the RCA process easier and more accurate for application in the detection [136]. Similarly, by combining the self-cleaving DNAzyme and the nucleic acid tool enzymes-aided reaction cycles, Cao et al. report a label-free fluorescent assay for the amplified detection of copper ions [137].

### Aptamer-based signal amplification sensing

Aptamers are single-stranded nucleic acid molecules isolated from random-sequence nucleic acids libraries by SELEX [138, 139]. They generally have high binding affinity and selectivity for specific targets, ranging from small molecules to macromolecules, and even cells [21].

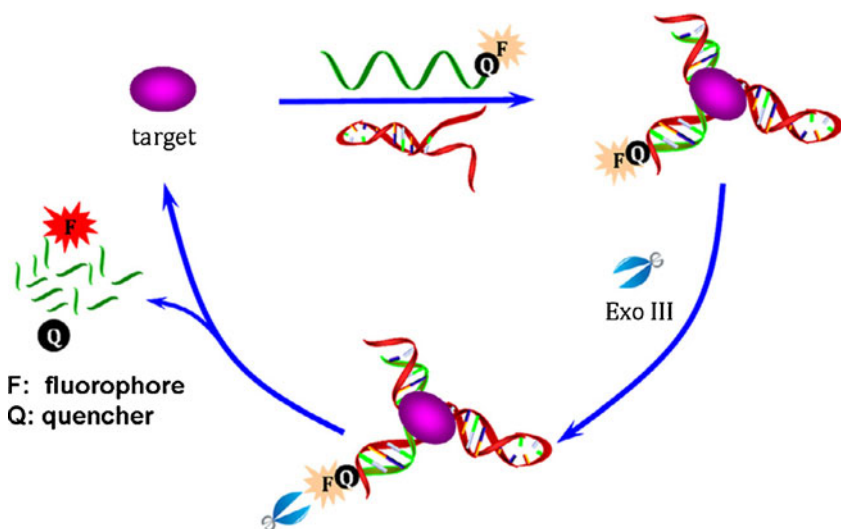
**Fig. 9** **a** Illustration of the ion-triggered Exo III-aided digestion of e-T-rich probe for immobilization-free electrochemical  $\text{Hg}^{2+}$  sensing. Reprinted from [128] with permission. **b** Principle of the RCA-based electrochemical sensing system for  $\text{Pb}^{2+}$  detection. Reprinted from [135] with permission



Being oligonucleotides, aptamers share the common properties of nucleic acids, including programmability through Watson-Crick base pairing, predictable secondary or tertiary structures, and high chemical stability. The binding of aptamers to their specific targets depends on their secondary and tertiary structures [140]. A variety of structure

switching mechanisms can be developed to facilitate the transduction of target recognition events to either target- or signal-based amplifications [141–146]. Therefore, the introduction of aptamers provides unprecedented opportunities to detect non-nucleic acid targets, such as small molecules, proteins, and cell, which dramatically widen

**Fig. 10** The mechanism of Exo III-aided cyclic amplification method for the detection of cocaine using linear molecular beacons as signal beacons. Reprinted from [48] with permission





the application areas of nucleic acid tool enzymes-aided signal amplification strategies.

### The amplification detection of small molecules

Evaluation of small molecules associated with different disease states can also serve as useful information for identification of disease and cancer pharmacogenomics [147].

Many small molecules, such as thrombin, adenosine, cocaine, and vascular endothelial growth factor, have been utilized as target for aptamers identification [148, 149]. Based on target molecules triggered aptamers structure switch and nucleic acid tool enzymes aided signal amplification, many novel signal amplification strategies have also been developed for these molecules [150–152]. For example, by integrating structure-switching aptamers with Exo III, Yang et al. developed a rapid, selective, and ultrahighly sensitive approach for nucleic acid and cocaine detection (Fig. 10) [48]. In the cocaine sensing assay, the aptamer of cocaine was been split into two fragments, fragment 1 and 2, to eliminate false negative signals. Fragment 2 was designed to be a linear molecular beacon with a fluorophore and quencher attached to 3' terminal and penultimate nucleotides, respectively. In the absence of cocaine, the two fragments remained irrelevant with no fluorescent signal owing to the fluorophore and the quencher in close proximity. However, in the presence of cocaine, the two aptamer fragments interacted with the target and assembled into an aptamer–target complex. The linear molecular beacon formed a duplex-like structure with a blunt 3' terminus, which could serve as the substrate of Exo III. Exo III

hydrolyzed this duplex-like structure from the blunt 3' terminus, liberating the fluorophore and releasing the target. This method could be used to detect cocaine down to 0.67  $\mu\text{M}$ .

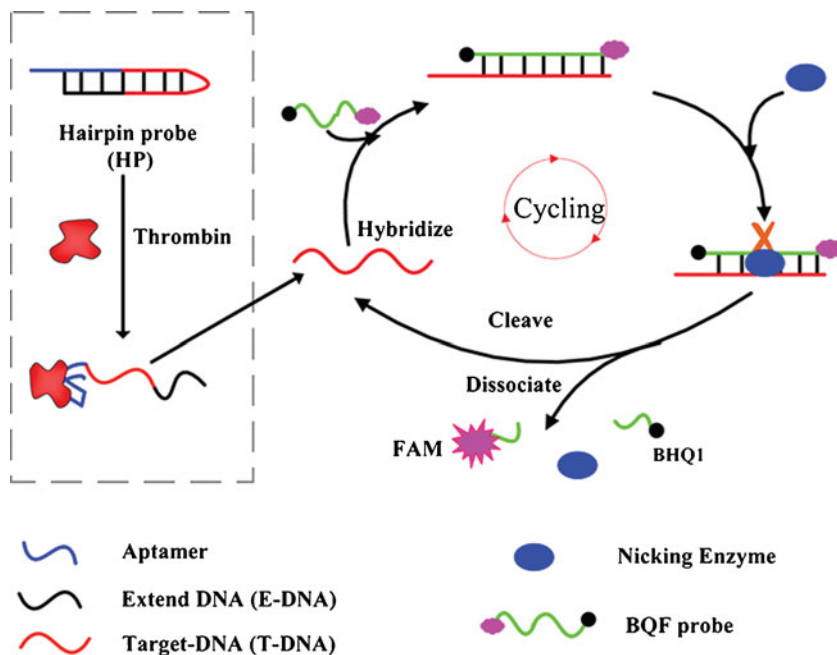
### The amplification detection of proteins

Similar to the detection of small molecules, nucleic acid tool enzymes-aided signal amplification strategies are also used in protein sensing [153–155]. For example, Xing et al. developed a novel, simple, and homogeneous approach for protein detection based on nicking enzyme aided fluorescence signal amplification [153]. They extended an aptamer to form a hairpin probe (Fig. 11).

The binding of the aptamer to thrombin opened the hairpin, which enabled hybridization with a signaling sequence that is dual labeled with a fluorophore and a quencher. This initiated cyclic cleavage of the signaling sequence, which generated an amplified fluorescence signal. A detection limit of 100 pM was obtained. More recently, this method was further used in detecting membrane protein on single living cells in microfluidic droplets [156].

Willner et al. combined the split aptamer subunits with Exo III-aided target recycling for the analysis of proteins [157, 158]. In their system, one of the aptamer subunits was modified with a fluorophore at the 5' terminus and a quencher at the 3' terminus, respectively. In the absence of target protein, the fluorophore was in close proximity to the quencher, resulting in a low fluorescence signal. When the target protein was present, it induced the aptamer subunits to self-assemble into a structure with recessed 3' terminus, resulting in cyclic enzymatic amplification and fluorescence enhancement. Similarly,

**Fig. 11** Aptamer and NEase-aided fluorescence signal-amplification detection of protein. Reprinted from [153] with permission



by converting the protein detection to nucleic acid detection, our group developed a simpler fluorescence method for protein detection based on enzyme-aided strand displacement amplification [159]. This method was dependent on the simultaneous and proximal recognition of target-protein by pairs of proximity probes.

### The amplification detection of cells

Many high-affinity aptamers that specifically bind to different target cancer cells have also been created using cell-SELEX in recent years [146]. The aptamers selected are also used for highly selective detection of cancer cells because of their good selectivity [160–162]. So, many novel aptasensors for cancer cells have been developed by nucleic acid tool enzymes-aided signal amplification [163–167].

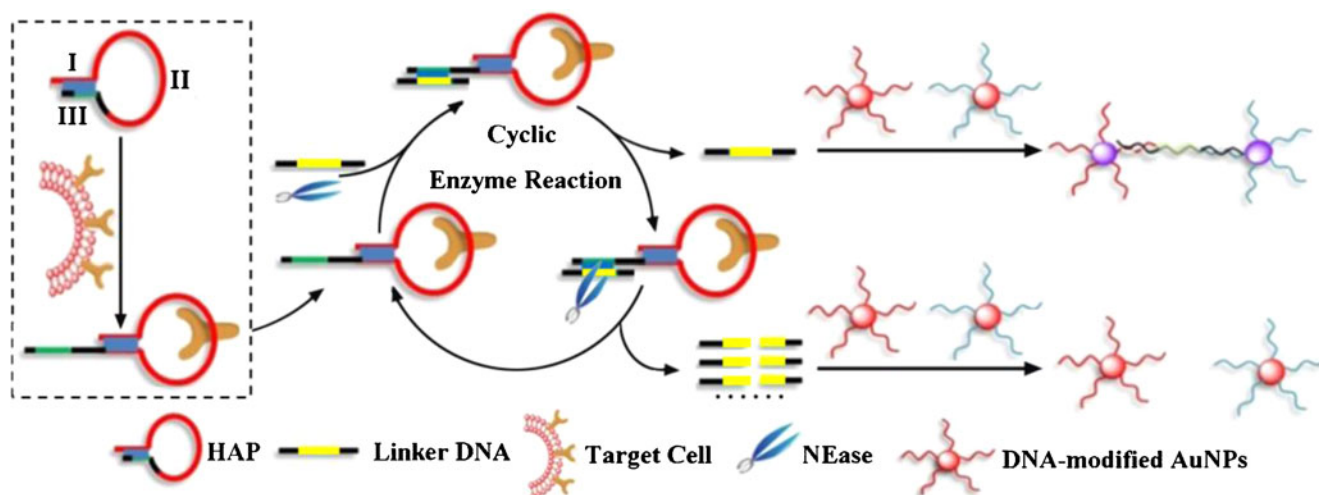
For example, Kong et al. [163] developed a simple, highly sensitive colorimetric method for visually detecting rare cancer cells based on cell-triggered cyclic enzymatic signal amplification (Fig. 12). In the absence of target cells, the hairpin aptamer probes (HAPs) and linker DNAs stably coexist in solution. Then, the linker DNA could assemble DNA-AuNPs, producing a purple solution. In contrast, when the HAP was incubated with target cancer cells, the specific binding of HAPs to the target cells triggers a conformational switch that results a nicking endonuclease-strand scission cycle. In the subsequent step, the cleaved linker DNA could no longer assemble two DNA-AuNPs and the solution was red. This method could detect target cells with high sensitivity and high selectivity. The linear response for target CCRF-CEM cells in a concentration range from  $10^2$  to  $10^4$  cells was obtained with a detection limit of 40 cells, which is approximately 20 times lower than the normal AuNP-based methods without amplification.

Moreover, Ding et al. [164] constructed a cascade signal amplification strategy for detection of cancer cells by combining the RCA technique with oligonucleotide functionalized nanoparticles, and anodic stripping voltammetric detection. Based on DNA-RCA-directed enzyme-catalyzed polymerization, Zheng et al. demonstrated an ultrasensitive electrochemical sensor for cancer cells and successfully applied it for the electrochemical cytosensing of MCF-7 cells [167].

### Conclusions and perspective

The need for ultrasensitive bioassays and the trend towards fast assays has made the nucleic acid tool enzyme one of the most useful tools for manipulating nucleic acids and for signal amplification. A wide variety of nucleic acid tool enzymes with different catalytic properties have been introduced into biosensing for signal amplification because of their highly efficient catalytic effects. Because the substrates of nucleic acid tool enzymes are nucleic acids, the nucleic acid tool enzymes-aided signal amplification strategies are mainly used to detect nucleic acid, as well as biorelated variations of nucleic acids. On the other hand, based on different configurations of aptamers bound to targets, nucleic acid tool enzymes-aided signal amplification strategies have also been used for amplified detection of non-nucleic acid targets such as ions, small molecules, proteins, and cells.

In addition, some biosensors incorporating multiple tool enzymes are developed for cascade signal amplification and achieving higher sensitivity. For example, exonucleases and endonuclease can be used to regenerate primers for RCA reactions [116]; endonuclease can produce blunt 3' termini for exonuclease digestion [168]; nicking endonuclease can be used to



**Fig. 12** Schematic illustration of a highly sensitive colorimetric method for the detection of rare cancer cells based on cell-triggered cyclic enzymatic signal amplification. Reprinted from [163] with permission

generate primers for polymerization cycle [169]; RCA reactions can generate prolonged ssDNA for nicking endonuclease-based amplification [39]. In order to further improve sensor performance, the combination of enzymatic and other non-enzymatic amplification approaches is becoming popular. For instance, Li et al. report a dual-amplification strategy for highly sensitive detection of DNA based on the dumbbell-shaped DNA probe with a magnetic bead at 5' terminus and a fluorescein-encapsulated liposome at 3' terminus [51].

All these nucleic acid tool enzymes-aided signal amplification strategies provide an effective tool for quantitative analysis trace amounts of analytes and supply valuable information for biochemical research, clinical diagnostics, and environmental monitoring. There are still many challenges in the development of this field. (1) With amplification, the detection time of some assays may be prolonged, which is unpopular in fast detection. So, more advanced, efficient, and stable nucleic acid tool enzymes urgently need to be explored for potential applications. (2) Their performance in practical samples is unsatisfactory (for example, cell detection), and commercialization of these nucleic acid tool enzymes-aided biosensors is difficult. Therefore, the reaction conditions need to be strictly controlled and the pretreatment of complex samples should be progressed. (3) Up to now, the number of aptamers with specific targets is limited. Only a few non-nucleic acid targets could be detected using such strategies. In order to extend the potential utility of nucleic acid tool enzymes for non-nucleic acid targets, more converting mechanisms are needed to transfer the non-nucleic acid targets detection to nucleic acid detection. With the persistent development of nucleic acid tool enzymes, we envision that the utilization of nucleic acid tool enzymes will bring higher sensitivity, reagent-less biosensing, and point-of-care diagnosis.

**Acknowledgments** This work was supported by the Key Project of Natural Science Foundation of China (grant no. 21175039, 21322509, 21305035, 21190044, and 21221003), Research Fund for the Doctoral Program of Higher Education of China (grant no. 20110161110016), and the project supported by Hunan Provincial Innovation Foundation for Postgraduate (grant no. CX2015B072).

#### Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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