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Mini review

Immobilization-Free Strategies for Designing of DNA-Based Electrochemical Biosensors

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DNA has been widely utilized as a distinguish biomaterial for the development of sensitive biosensors based on its flexible structure and the specific interaction with other molecules, even cells. In recent years, with the growth of nanotechnology and biosensors, DNA-based electrochemical devices have undergone tremendous development in the field of molecular diagnosis. Importantly, immobilization-free electrochemical biosensors are increasing enormously due to their low cost, simple operation, and less time-consuming. Moreover, a wide range of signal-amplified methods have been used to improve the performances of these biosensors. This review focuses on the recent advances in immobilization-free DNA-based electrochemical biosensors for the detection of DNA, microRNA, proteins, enzymes and other molecules.

Keywords: DNA-based electrochemical biosensors; signal amplification; immobilization-free

1. INTRODUCTION

As the carrier of genetic information inside cell, deoxyribonucleic acid (DNA) play essential roles in the daily metabolic activity of living organisms and has been recognized as the important biomarker in disease diagnose. For instance, the level of human immunodeficiency virus (HIV) DNA in human serum is usually analyzed for evaluating therapy and detecting recurrence or metastasis. Thus, it is very imperative and necessary to develop more sensitive and selective methods for DNA detection. Besides, DNA, including functional nucleic acids (aptamers, nucleic acid enzymes and aptazymes) and its analogues such as peptide nucleic acid (PNA) and locked nucleic acid (LNA) have also acted as excellent biomaterials for the construction of varied biosensors for the determination of miRNA, proteins, enzymes, metal ions and so on [1, 2].

Electrochemical devices have been worldwide recognized as the potential tools for numerous applications, such as clinical diagnosis, environmental monitoring and food safety because of the advantages of excellent sensitivity, high signal-to-noise ratio, low cost and rapid detection [3-5]. To date, most of the reported DNA-based electrochemical biosensors are heterogeneous, involving an immobilization step to modify the recognition probes on the electrode surface [6-9]. Then, the target can be recognized and captured. This translates the change of probe structure and electrochemical property into a detectable electrical signal. Although the immobilization electrochemical biosensors always are high sensitivity and reusability, there are usually some disadvantages, such as tedious and time-consuming modification step, the reduced bioactivity of functional biomolecules, and the bad repeatability and reproducibility at different electrodes. Therefore, recently, immobilization-free homogeneous DNA-based electrochemical devices have intensively attracted great interest.

A typical immobilization-free homogeneous DNA-based electrochemical biosensor normally includes three essential parts, an electrode with conductive surface, a recognition probe which can specifically complex with the target, an electroactive indicator of ferrocene (Fc) or methylene blue (MB) which is labeled with the recognition probe as a tag or has priority to non-covalently interact with the recognition probe or its products. Usually, the indium tin oxide (ITO) electrode with negative charge was used as the working electrode. The change in diffusivity and electrostatic interaction between the electroactive indicator or the indicator-labeled DNA pieces and the negatively charged ITO electrode will generate significant signal change before and after the detection assay. Nonetheless, the diffusion-controlled nature will decrease the sensitivity. To overcome these drawbacks, a lot of efforts have been put into improving the sensitivity, by integrating with nuclease-assisted signal amplification strategies and DNA self-assembly signal amplification strategies.

In this review, we will focus on the recent progress in the design and applications of immobilization-free DNA-based electrochemical biosensors. First, we will mainly introduce the label-free strategy and labeled strategy for DNA detection. Especially, we discuss the elaborately designed signal-amplified methods based on nuclease-assisted cycling amplification and DNA self-assembly signal-amplified strategies. Then, the detection of various molecules or bio-related process was summarized.

2. LABEL-FREE STRATEGIES FOR DNA DETECTION

According to the reports, all of DNA bases can be electrochemically oxidized on a given pH, which endows the researchers to develop the methods for direct detection of according to the intrinsic redox activities of bases [10-12]. For example, Erdem's group developed a genomagnetic assay for label-free electrochemical detection of DNA through the guanine oxidation of the DNA target with the magnetic separation [13]. Due to the different electron transfer efficiency in single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA), the oxidation current for ssDNA is greater than that of dsDNA. Based on the intrinsic guanine signal, Ozkan-Ariksoysal's group presented an electrochemical biosensor for the detection of a sequence-specific DNA with PCR-amplified nucleic acids (Figure 1) [14]. In this research, they described two methods to monitor DNA hybridization using guanine-containing target and guanine-free probe. In the first method (method-1), the immobilized guanine-

containing target hybridized with the inosine-substituted probe, causing the decrease of the guanine oxidation peak. On the contrary (method-2), the immobilized inosine-substituted probe interacted with the guanine-containing target, resulting in the appearance of the guanine signal. Next, Jiao's group reported a label-free biosensor for the detection of DNA sequence with PCR amplification. The current change was monitored by the electrochemical oxidation of 2'-deoxyguanosine 5'-triphosphate (dGTP) [15].

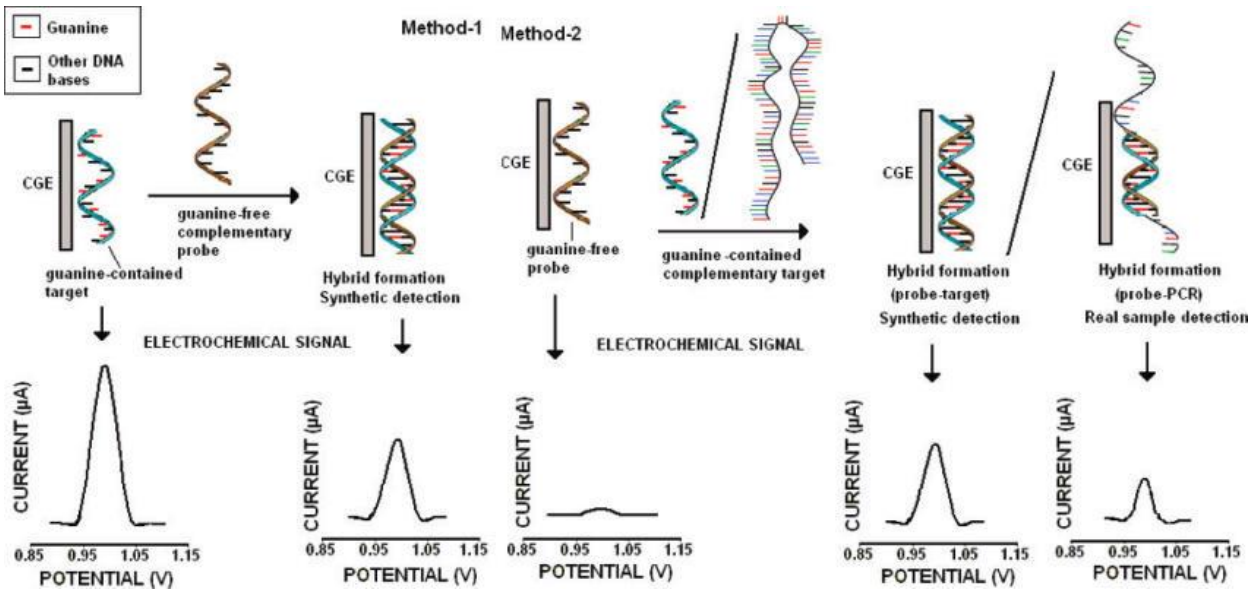

Figure 1. Detection of hybridization using guanine-containing target (method 1) and guanine-free probe (method 2) as beginning materials of the procedure. Reprinted with permission from [14]. Copyright 2008 American Chemical Society.

In addition to the redox-active properties of bases, electro-active intercalators with high affinity to dsDNA can be utilized as the indicators to construct modification-free electrochemical biosensors. Based on the intercalative binding of MB with dsDNA, Park's group demonstrated an electrochemical real-time PCR system by accurately quantifying initial copy numbers of DNA templates [16]. Besides, in 2014, Zhang and co-workers first suggested that G-quadruplex sequence (EAD2) could significantly decrease the diffusion current of MB in homogeneous solution (Figure 2A) [17]. Different from the binding mechanism of dsDNA with MB by intercalation, EAD2 might mainly interact with MB through the end-stacking with π-system of the guanine quartet. They further applied the strong affinity between G-quadruplex and MB for the detection of cocaine [18]. Based on hybridization chain reaction (HCR), Li's group reported a simple, label-free and enzyme-free homogeneous "signal-off" electrochemical assay for the detection of miRNA (miRNA) (Figure 2B) [19]. The two species of metastable DNA hairpin probes as target miRNA triggers for the HCR would transform into the format of multiple G-quadruplex-incorporated long duplex DNA chains after the addition of miRNA. Thus, with MB selectively intercalated into the duplex DNA chain and the multiple G-quadruplexes, a decreased electrochemical signal was observed, linearly related to the concentration of the target miRNA. Based on the similar strategy, Li's developed a versatile and programmable DNA logic gate for the label-free homogeneous electrochemical platform (Figure 2C) [20]. Once the initiation of the

DNA machine, numerous T30695 sequences were produced, resulting in the formation of the G-quadruplexes in the presence of K^+ ions. Next, MB molecules were firmly intercalated into the formed G-quadruplexes and greatly inhibited the electrochemical diffusion current of MB.

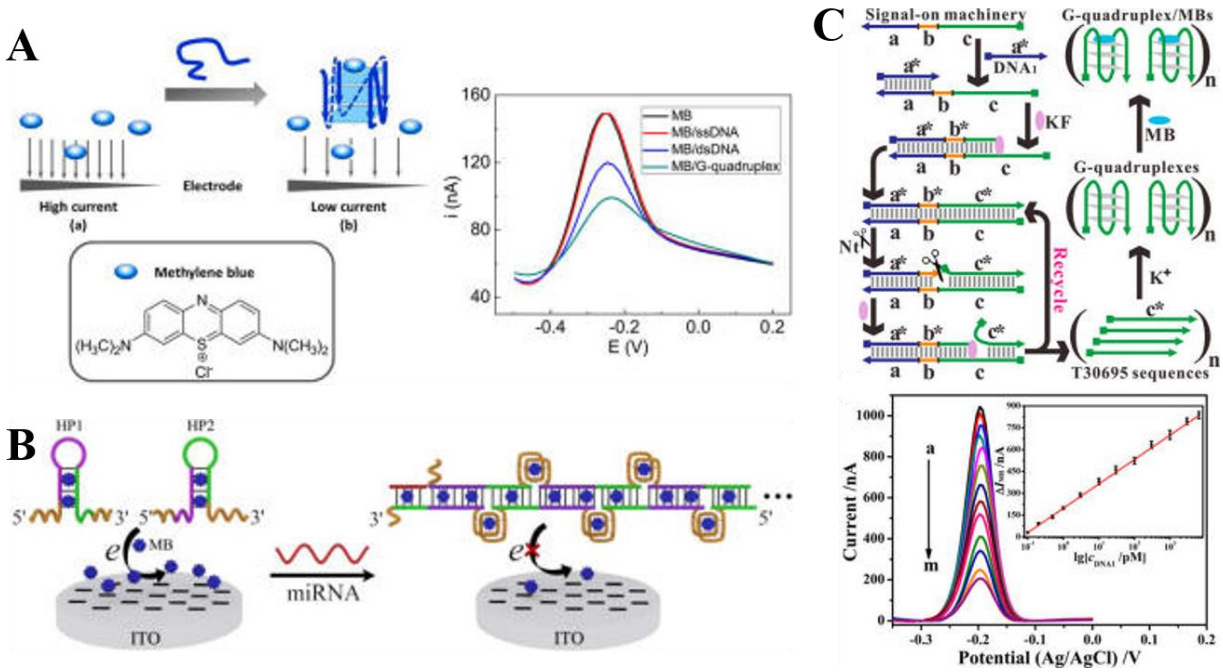

Figure 2. (A) Schematic illustration of electrochemical signal readout mode based on the binding affinity between MB and G-quadruplex. Reprinted with permission from [17]. Copyright 2014 American Chemical Society. (B) Principle of the label-free and enzyme-free homogeneous electrochemical strategy based on HCR amplification for miRNA assay. Reprinted with permission from [19]. Copyright 2015 American Chemical Society. (C) Schematic illustration of the switch-on machine for homogeneous electrochemical transducer and DPV responses of the proposed switch-on machine to different concentrations of DNA_1. Reprinted with permission from [20]. Copyright 2016 American Chemical Society.

3. LABELED DNA FOR NUCLEIC ANALYSIS

Label-free biosensors are simple and cost-effective. However, they often exhibit low sensitivity and specificity. Therefore, more and more immobilization-free electrochemical biosensors using labeled DNA probes have been constructed to improve the analytic performances.

3.1 No signal amplification

PNA consists of repeating N-(2-aminoethyl)glycine units linked by peptide bonds instead of sugar-phosphate backbones, in which the four purine and pyrimidine bases were linked to the backbone by methylene carbonyl bonds. They are one of the most useful and popular analogues of DNA. Different from the negatively charged backbone of DNA, PNA has a neutral backbone. More importantly, without the electrostatic repulsion between them, the hybridization of PNA and DNA exhibits higher affinity and specificity in contrast to the hybridization of dsDNA. Therefore, PNA has been widely used as a capture probe for sensitive detection of biomolecules [21]. Luo's group have

reported an electrochemical method for sequence-specific detection of DNA without solid-phase probe immobilization (Figure 3) [22]. This detection scheme starts with a solution phase hybridization of Fc-labeled peptide nucleic acid (Fc-PNA) and its complementary DNA (cDNA) sequence. Based on the different electrostatic interaction, the negatively charged Fc-PNA-DNA hybrid and the neutral-charge Fc-PNA have a different differential pulse voltammetric (DPV) peak current on the bare ITO electrode or the ITO electrode coated with a positively charged poly(allylamine hydrochloride) (PAH) layer. The measured electrochemical signal is proportional to the amount of cDNA present. And in terms of detection sensitivity, the PAH-modified ITO platform was found to be more sensitive (with a detection limit of 40 fM) than the bare ITO counterpart (with a detection limit of 500 fM). The decrease or increase of the peak intensity of Fc-PNA is proportional to the concentration of cDNA with a linear relationship in the range of 0.25 ~ 2 μM (for bare ITO) or 0.02 ~ 0.1 μM (for PAH ITO). This detection method holds great promise for single-base mismatch detection as well as electrochemical detection of post-PCR products. Besides, through analysis of the electrochemical melting curve, simple detection on single nucleotide polymorphism (SNP) analysis could be accomplished [23]. Luo's group has developed a novel electrochemical platform for multiplex detection of sequence-specific DNA and SNP, in which PNAs were functionalized with different electroactive indicators [24].

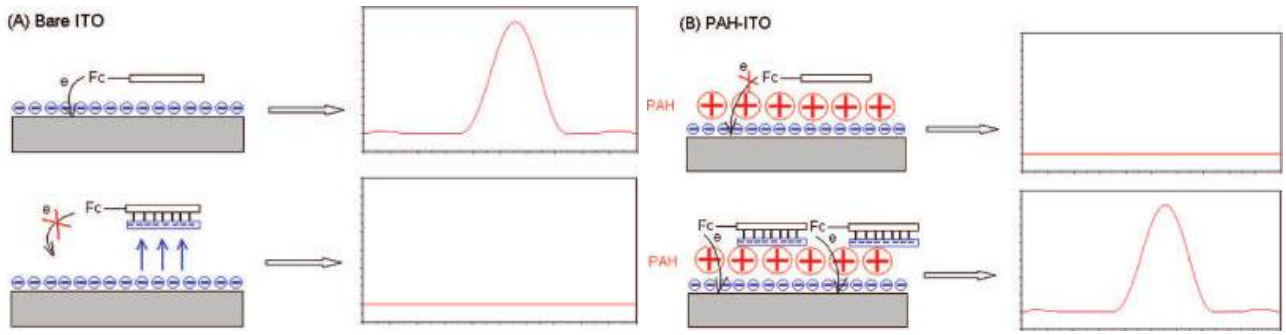

Figure 3. Working principle of immobilization-free, sequence-specific DNA detection based on negatively charged bare ITO electrode (A) or positively charged PAH-modified ITO electrode (B). Reprinted with permission from [22]. Copyright 2008 American Chemical Society.

3.2 Nuclease-induced target cycling amplification

In traditional electrochemical biosensors, the recognition probe usually hybridized with the target to form a 1:1 complex and produce a detectable electrochemical signal. Therefore, the limits of detection (LOD) of the method always are not low enough for the detection of ultralow-level of DNA. Thus, nuclease-induced target cycling strategy has been used for design of immobilization-free electrochemical biosensors.

Exonuclease III (Exo III) has a high exodeoxyribonuclease activity for duplex DNAs to stepwise remove mononucleotides in the direction from 3' to 5' terminus and limited activity on single-stranded DNA and dsDNA with the 3'-protruding end of 4 or more nucleotides. Therefore, Exo III-assisted target recycling amplification has been widely used in colorimetric assays, fluorescent sensors and electrochemical detection, especially in homogeneous electrochemical assays because of

its robustness, simplicity, and low cost. Xuan and co-works have developed a highly sensitive and selective electrochemical molecular beacon-based DNA sensor by taking the advantages of the superior exodeoxyribonuclease activity of Exo III (Figure 4A) [25]. The signal probe is a hairpin oligonucleotide containing the target DNA recognition sequence and a MB marker near the 3'-end. After the triggering of target DNA and digestion of exon III, MB-labeled electroactive mononucleotide (eNT) was released, which could be diffused to negative ITO electrode, resulting in an increase in the electrochemical signal. At the same time, the complete target DNA is freely returned to the solution and hybridized with other probes, releasing more eNT. The detection limit of the electrochemical DNA biosensor is 20 pM, which has good selectivity for the identification of single nucleotide polymorphism. Based on exon III-assisted autocatalytic target cycle strategy, Liu and co-works developed a simple, fast and ultra-sensitive DNA-based sensing platform for target DNA and protein detection (Figure 4B) [26]. Fc-labeled hairpin probe (HP1) which can recognize the target DNA is ingeniously designed to trigger the Exo III cleavage process. This resulted in the autonomous accumulation of eNT induced a distinct increase in the electrochemical signal owing to its elevated diffusivity toward ITO electrode surface. The DNA fragment acts as the secondary target analogue. This current developed autocatalytic and homogeneous strategy allowed ultrasensitive electrochemical detection of DNA and thrombin down to the 0.1 pM level. The autocatalytic biosensing system was further extended for protein detection by advising an aptamer hairpin switch with the use of thrombin as a model analyte. However, the simple dependence of the diffusion difference and the lack of the inherent interaction between the signal reporter (eNT) and the electrode surface make those methods easily influenced by environmental factors. To overcome the problems of low reliability and reproducibility, Liu's group proposed a unique and versatile immobilization-free electrochemical DNA biosensor based on the hydrophobic force between the alkyl chain of dodecanethiol monolayer on the electrode surface and the hydrophobic part of the MB-tagged mononucleotide (Figure 4C) [27]. A low detection limit of ~1 pM toward the target DNA has been achieved with an excellent selectivity under the optimized conditions. The proposed immobilization-free electrochemical biosensing strategy was also extended for the assay of Exo I and III activity.

Besides, metal ions can also be utilized as signal reporters for electrochemical DNA detection. For instance, Fu's group described a homogeneous electrochemical sensing strategy for human immunodeficiency virus (HIV) DNA detection based on Exo III-assisted target recycling amplification, in which Ag^+ facilitated the formation of HP through the cytosine-Ag^+-cytosine coordination chemistry [30]. At the same time, after the catalytic cleavage by Exo III, the released Ag^+ was readily captured by the negatively charged electrode, generating an anodic current signal. In addition, another exonuclease, T7 exonuclease (T7 Exo), is also used in Li's group to develop a novel affinity-mediated homogeneous electrochemical miRNA biosensor based on T7-Exo-assisted isothermal amplification strategy (Figure 4D).[28] In this study, the ITO surface was in situ electrochemically grafted 1-naphthalenesulfonate (NS^-) diazonium salt, which possesses the strong π–π stacking interaction between the bihexagonal cells of naphthalene and the ring structures in exposed nucleobases of ssDNA, effectively differentiating ssDNA and dsDNA. The target DNA initiated the continual and repeated cleavage of MB-labeled hairpin reporter and Fc-labeled dsDNA, leading to more Fc molecules and less MB near the surface of electrode. By measuring the distinct peak current

intensity ratios of Fc and MB tags before and after the reaction, this ratiometric homogeneous assay showed a sensitive detection limit of 25 aM. They also developed a photoelectrochemical biosensing assay for sensitive detection of miRNA (Figure 4E) [29].

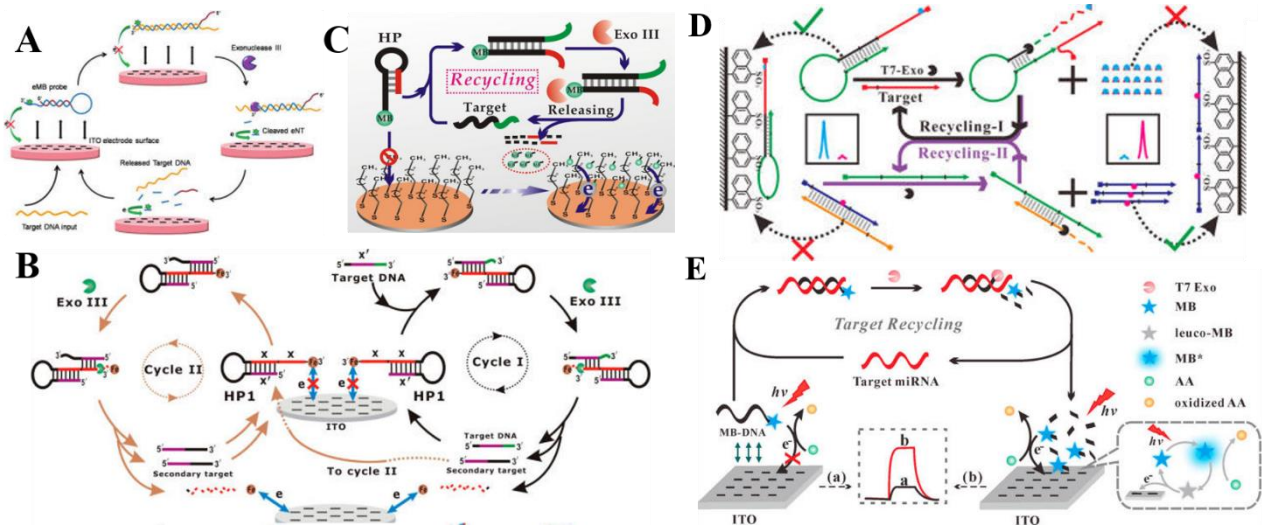

Figure 4. (A) Working principle of the exonuclease III-assisted electrochemical MB-based DNA biosensor. Reprinted with permission from [25]. Copyright 2012 American Chemical Society. (B) Schematic illustration of Exo III-assisted autocatalytic biosensing platform for the immobilization-free detection of target DNA. Reprinted with permission from [26]. Copyright 2014 American Chemical Society. (C) Schematic illustration of an electrochemical nucleic acid biosensor fabrication based on Exo III-catalyzed target recycling followed by the MB releasing and enriching effect. Reprinted with permission from [27]. Copyright 2017 American Chemical Society. (D) Schematic illustration of the design principle for the amplified ratiometric homogeneous electrochemical biosensing of miRNA on the NS−-ITO electrode. Reprinted with permission from [28]. Copyright 2017 American Chemical Society. (E) Schematic illustration of the immobilization-free diffusivity-mediated PEC strategy for miRNA assay: (a) in the absence and (b) in the presence of the target miRNA. Reprinted with permission from [29]. Copyright 2018 American Chemical Society.

In this work, MB was used as a photoactive dye molecule to label ssDNA. Under the digestion of T7 Exo, the released eNP could easily diffuse to the surface of electrode, producing a significantly increased photocurrent signal upon visible light (λ = 627 nm) illumination.

In contrast to exonuclease, nicking endonuclease has higher and diverse specificity and has been widely used to detect single-base mismatch and single nucleotide polymorphisms due to the requirement of a specific sequence for recognition and cleavage,. For example, DNA nicking endonuclease Nt·BstNBI can specifically recognize the asymmetric sequence (5'-GAGTC-3') and cleave only one DNA strand at the 4-bases away from the 3' end of its recognition site. This has been used to construct DNA-based electrochemical biosensors based on the nicking endonuclease signal amplification (NESA) strategy. Tan's group reported a simple and ultra-selective electrochemical biosensor for target DNA related to oral cancer overexpressed 1 in saliva, firstly combining NESA with the immobilization-free electrochemical method (Figure 5A) [31]. The complementary substrate strand (eMB) of target DNA including a nicking recognition site was modified with MB at the 3' terminus. The eMB cannot diffuse easily to the negative charged ITO electrode surface because of the

abundant negative charges. The formation of dsDNA triggered by the target DNA facilitated the nicking endonuclease to recognize the site and to cleave the substrate strand of ds-DNA into two pieces. The produced 2-base ssDNA linked with MB was diffused easily to the negative charged ITO electrode surface, thus leading to the enhancement in the electrochemical response. Meanwhile, the target released intact DNA triggered the next round of hybridization, cleavage, and releasing, resulting in the signal amplification. This homogeneous DNA assay can detect target DNA as low as 0.35 pM. The proposed strategy has also been used for the quantitative determination of DNA. Furthermore, nicking endonuclease can be utilized with the aid of polymerase to repeatedly cleave and polymerize the replication product, generating numerous ssDNA for signal amplification [32]. For example, Yang group designed a programmable hairpin probe (PHP) for ultrasensitive detection of nucleic acid based on a biphasic reaction mode (Figure 5B) [33]. In this study, the ingeniously designed PHP is composed of a target-recognition region, a template sequence for enzymatic reaction and an inactivated anti-streptavidin aptamer. Under the reaction of polymerase/nicking enzymes, numerous initiator strands were released to trigger the PHP and produce a large number of activated anti-streptavidin aptamers, which can be captured by the streptavidin immobilized on the electrode, thus generating the in situ amplified electronic signal.

Besides, DNA polymerase can be solely applied to develop immobilization-free electrochemical DNA sensor based on isothermal circular strand displacement polymerization reaction [34]. For example, in 2012, Hsing's group employed a hairpin DNA with Fc-labeled PNA and DNA polymerase to analyze DNA [35]. When the target DNA hybridized with the hairpin DNA, the extension of the primer catalyzed by the DNA polymerase repeatedly liberated the neutral PNA approach the electrode surface, producing a detectable electrochemical signal.

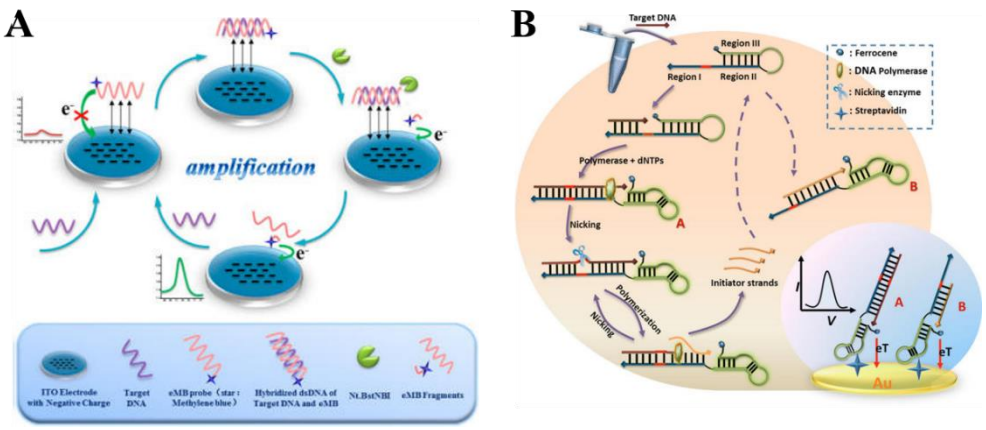

Figure 5. (A) Mechanism of the proposed homogeneous immobilization free electrochemical biosensor for DNA detection based on NESA. Reprinted with permission from [31]. Copyright 2015 American Chemical Society. (B) Immobilization-free programmable hairpin probe for highly sensitive electrochemical detection of nucleic acid with a biphasic reaction mode. Reprinted with permission from [33]. Copyright 2014 American Chemical Society.

3.3 Nuclease-induced target cycling amplification

Nuclease-induced cycling amplification strategies are sensitive but easily bring issues, such as nonspecific enzyme adsorption on bare electrode surface and strict reaction conditions. To further enhance the sensitivity and selectivity, Hsing's group reported an immobilization-free and enzyme-free electrochemical DNA assay, based on the kinetically controlled dendritic assembly of DNA and PNA (Figure 6A) [36]. In the presence of target DNA, the assembly of dendritic DNA/PNA nanostructure was triggered and numerous freely diffusible neutrally charged Fc-PNAs incorporated into DNA/PNA dendrimer. This resulted in a significant electrochemical signal reduction of Fc on a negatively charged electrode. There was a large electrostatic affinity difference between Fc-PNAs and DNA/PNA dendrimer toward the sensing electrode. The cascade-like assembly process induced an amplified signal change to quantify the number of triggering targets. This method offered a detection limit down to 100 fM, which had great potential to become a feasible approach for in situ rapid diagnosis of infectious disease. Yuan's group developed a sensitive electrochemical strategy for determining low-level of DNA (Figure 6B) [37]. They used the "open-close-open" strategy to dynamically regulate the inter-enzyme distance and regenerate the DNA tweezer for consecutive target detection. The enzyme-functionalized DNA tweezer kept at the open state with an interenzyme distance (19 ~ 24 nm). This led to a low catalytic efficiency. Oxidase (GOx) and horseradish peroxidase (HRP) were modified as model enzymes respectively with the arm of opened DNA tweezer. With the aid of target induced Mg^{2+}-dependent DNAzyme cleavage recycling amplification, the output MB-labeled DNA was used as the fuel for switching of the DNA tweezer from the open state to the close state. This led to the cascaded enzyme close enough (5-10 nm) for enhancing the catalytic efficiency. This method allowed for sensitive detection of DNA with a low detection limit (~30 fM).

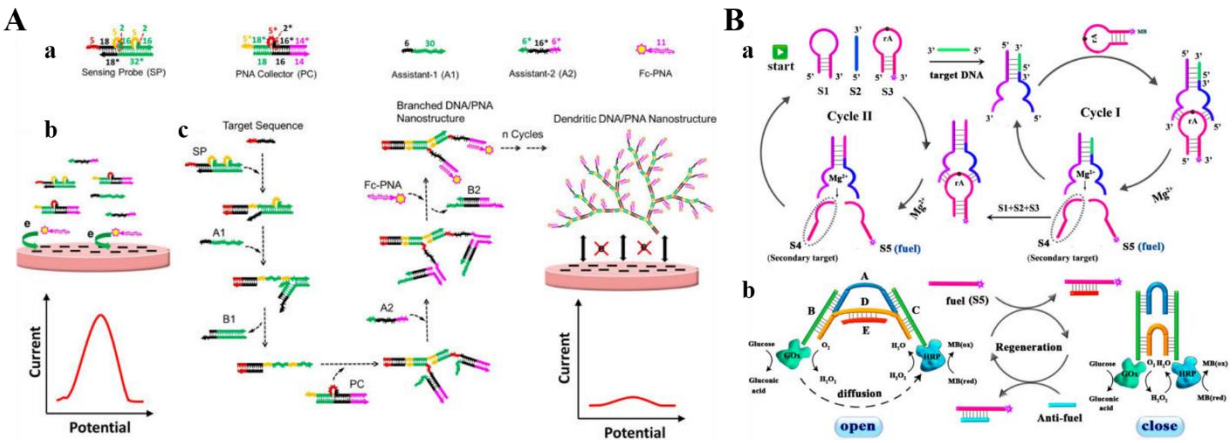

Figure 6. (A) Mechanism of the proposed homogeneous immobilization free electrochemical biosensor for DNA detection based on NESA. Reprinted with permission from [36]. Copyright 2015 American Chemical Society. (B) Illustration of the electrochemical DNA biosensor based on (a) Mg^{2+}-dependent DNAzyme cleavage recycling amplification and (b) the dynamical regulation of the enzyme cascade reaction by a regenerated DNA tweezer. Reprinted with permission from [37]. Copyright 2018 American Chemical Society.

4. ASSAYS OF OTHER BIOMOLECULES

In this section, the DNA-electrochemical biosensors for assays of DNA-related enzymes, proteins, small molecules and metal ions were discussed.

4.1 Enzymes

Methyltransferase (MTase) can catalyze the methylation of DNA by transferring a methyl group from the donor S-adenosylmethionine (SAM) to adenine or cytosine in the specific DNA palindromic sequence. It plays important functions in many biological processes, such as regulating gene transcription and genome imprinting. Thus, sensitive and selective methods for monitoring the DNA MTase activity benefit the early diagnosis of DNA methylation-related diseases. Lin's group reported a simple and sensitive immobilization-free homogeneous electrochemical method for DNA methylation detection and inhibitor screening (Figure 7) [38]. In this work, a palindromic sequence of 5'-G-AT-C-3' as the specific recognition site for Dam MTase and Dpn I was predesigned into the stem of MB-labeled hairpin. In the absence of Dam MTase, the hairpin could not be methylated and splited by Dpn I. However, in the presence of Dam MTase, the reaction of DNA methylation and the cleavage was subsequently initiated, leading to the generation of MB-labeled short oligonucleotides (eSO).

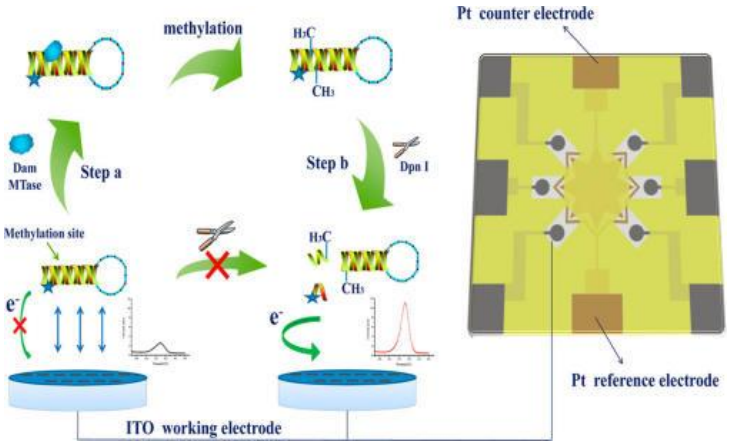

Figure 7. Mechanism of the proposed solution-phase electrochemical molecular beacon biosensor based on an ITO microelectrode chip for DNA methylation detection. Inset: ITO microelectrode chip with six ITO working electrode spots (diameter 1 mm), a Pt counter electrode, and a Pt pseudoreference electrode. Reprinted with permission from [38]. Copyright 2014 American Chemical Society.

The diffusivity and electrostatic repulsion between eSO and MB-labeled long oligonucleotides (eLO) toward the negative ITO electrode caused a different DPV signal. Furthermore, Li's group integrated the action of Dam MTase/ Dpn I with Exo III-assisted signal amplification for highly sensitive homogeneous detection of methyltransferase activity [39]. The product from the HP 1 hybridized with another HP 2 and triggered the signal amplification with the aid of Exo III.

As one of the most typical tumor markers, the rapid and sensitive detection of telomerase activity is beneficial for early cancer diagnosis, prognosis, and cancer therapy evaluation. In Liu's group, a homogeneous electrochemical biosensor based on T7 exonuclease-aided target recycling amplification was proposed for the detection of human telomerase activity (Figure 8) [40]. In the presence of telomerase, telomerase substrate (TS) primer was extended with repeats of (TTAGGG) and then hybridized with pre-designed 5′ MB-labeled HP probe. Next, the T7 exonuclease-catalyzed digestion of MB-labeled HP probe was initiated, leading to the release of a large amount of MB and the significantly amplified electrochemical signal. This strategy for highly sensitive detection of telomerase activity achieved a detection limit as low as 1 HeLa cell. Li's group also presented a sensitive, label-free electrochemical assay of telomerase activity without modification or immobilization [41]. In the absence of telomerase, the binding of MB on TS primer was weak and a large amount of MB molecules were diffused to the electrode, resulting in a strong current. When the TS primer was elongated with repeated TTAGGG in the presence of telomerase and the dNTP mixture, many MB tags bound to multiple G-quadruplexes and induced the sharp decrease in diffusion current of MB.

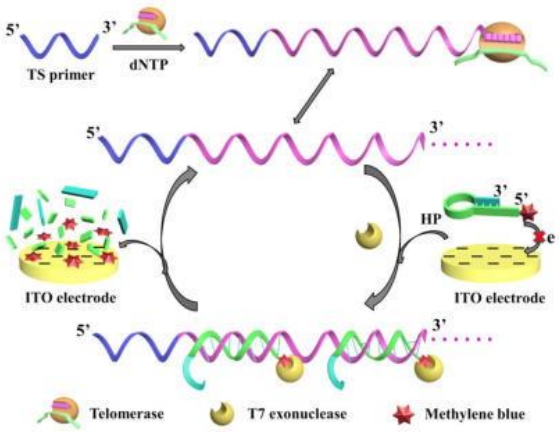

Figure 8. Principle of the homogeneous electrochemical strategy for the detection of telomerase activity based on T7 exonuclease-aided target recycling amplification. Reprinted with permission from [40]. Copyright 2015 American Chemical Society.

DNA polymerase plays an important role in the replication and repair of DNA in bacterial and eukaryotic cells. The activity of DNA polymerase has been recognized as an indicator of the cell viability of pathogens. Hsing's group developed a biosensor to monitor the activity of DNA polymerase based on the competition between the elongated DNA and the Fc-PNA to hybridize with the DNA template (Figure 9) [42]. In the presence of the DNA polymerase, the DNA primer was elongated and the Fc-PNA was released, producing a significant enhancement in the electrochemical signal of Fc. In addition, proteases can help protein catabolismby hydrolysis of peptide bonds. The activities of some proteases have been demonstrated to be relative to many diseases [43-48]. However, there are little reports on the immobilization-free electrochemical methods for protease detection. Efforts are being made in our group to develop immobilization-free electrochemical biosensors with positively or negative charged peptides as the substrates.

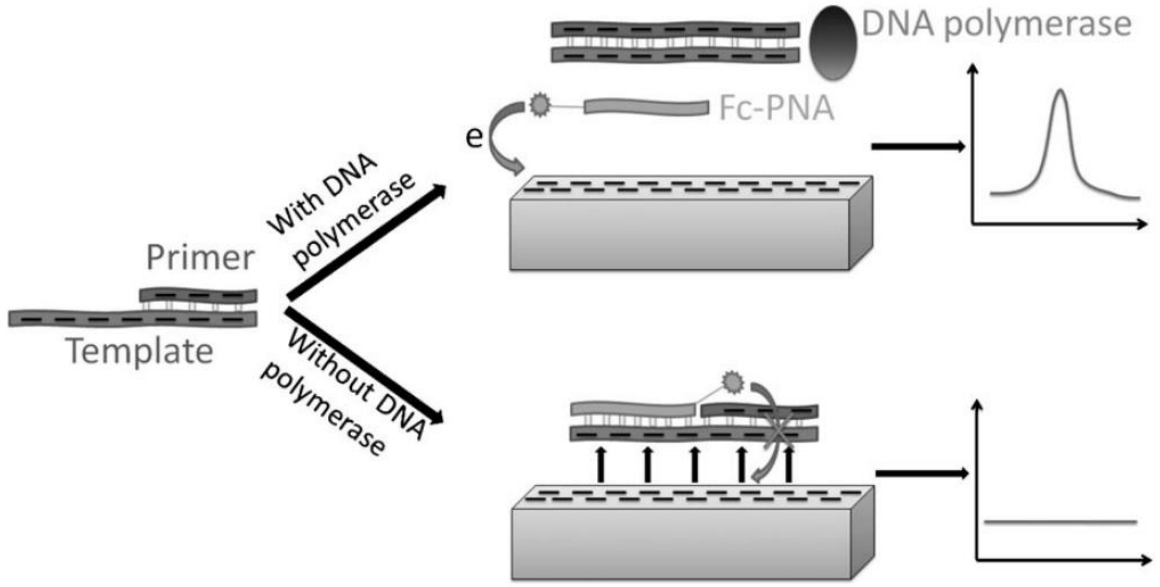

Figure 9. Schematic representation of working principle of the immobilization-free electrochemical DNA polymerase assay. Reprinted with permission from [42]. Copyright 2011 Wiley-VCH.

4.2 Proteins

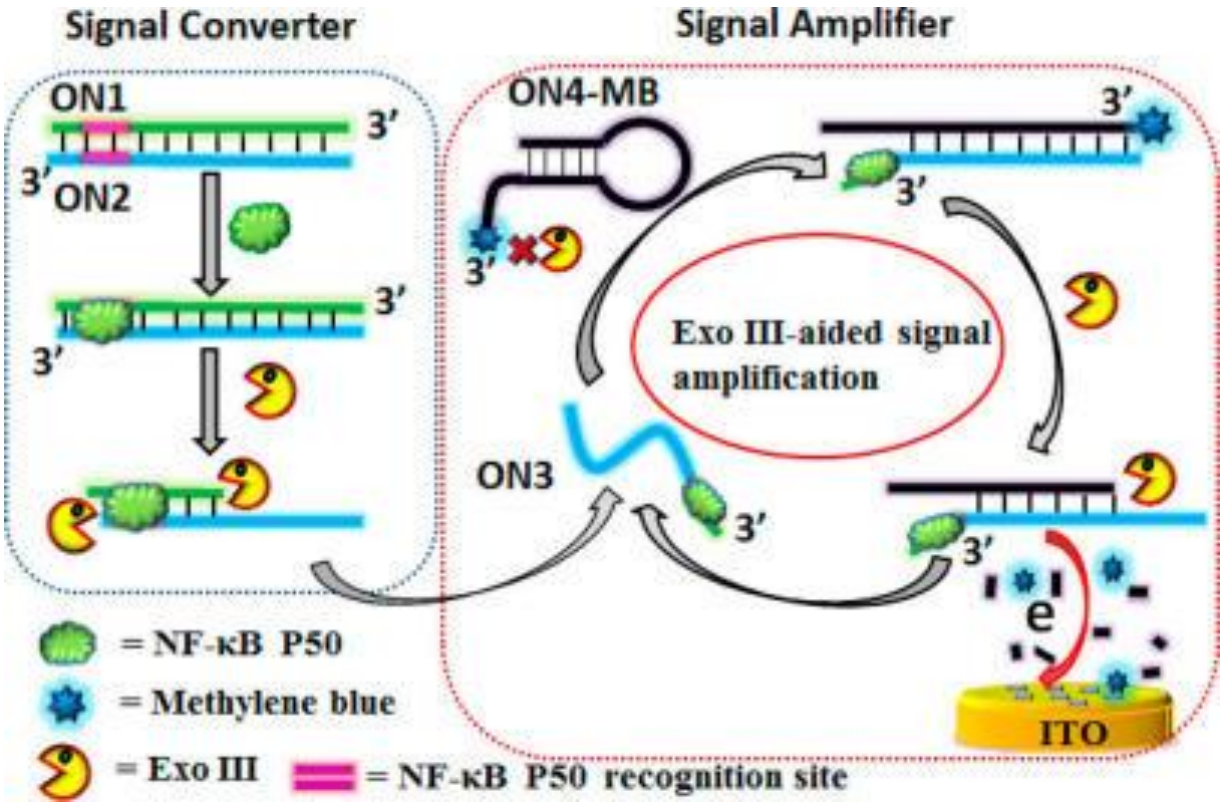

Figure 10. Mechanism of Exo III-aided homogeneous electrochemical strategy for NF-κB p50 assay. Reprinted with permission from [49]. Copyright 2017 American Chemical Society.

Transcription factors (TFs) are a family of functional proteins that bind to a specific DNA sequence with 6 ~12 base pairs [49]. Rapid and ultrasensitive monitoring of TFs is eagerly required to prevent and diagnose the TFs-related diseases. Based on the coupled isothermal cleavage reaction and Exo III-assisted cycling amplification, Lu and co-workers proposed a sensitive homogeneous electrochemical biosensor for the detection of NF-*κ*B p50 [49]. As shown in Figure 10, in the signal converter part, ssDNA ON1 and ON2 hybridized to form dsDNA which contains the binding sequence of NF-*κ*B p50 at the end. NF-*κ*B p50 protected ON2 against the digestion of the 3'-end by Exo III. Next, Exo III catalyzed the digestion of ON1, generating a large amount of ON3, which can hybridize with the MB-labeled ON4 and trigger the Exo III-catalyzed cycling amplification. Numerous MB-tagged mononucleotides were liberated, thus leading to a significant increase in the electrochemical signal.

4.3 Small molecules

As a naturally produced mycotoxin, ochratoxin A (OTA) can cause a tremendous threat to human health and has been widely used as a model molecule to construct electrochemical method for mycotoxins, in which the corresponding OTA aptamer was utilized as the recognition probe. Among these methods, a few exonuclease-based assays have been designed for signal amplification. For example, Lin's group employed RecJf exonuclease to instruct an immobilization-free electrochemical aptasensor (Figure 11A) [50]. RecJf exonuclease is a ssDNA-specific exonuclease that can catalyze the cleavage of deoxy-nucleotide monophosphates from DNA in the 5'→3' direction. In this work, the OTA aptamer first hybridized with the complementary MB-labeled probe DNA. In the presence of OTA, the OTA-aptamer dsDNA was separated into the MB-labeled ssDNA and the OTA-aptamer complex, which are both digested by RecJf exonuclease for the release of a MB-labeled electroactive mononucleotide and the target OTA to participate in the next reaction cycling. Based on the differential electrostatic repulsion between long and short DNA on a negatively charged ITO electrode surface, this method showed a detection limit of 0.004 ng/mL. Wang and co-workers reported the homogeneous electrochemical detection of OTA using DNase I, in which thionine-labeled OTA aptamer was absorbed on the surface of graphene oxide and diffused close to the electrode, resulting in a weak electronic signal [51]. The addition of OTA triggered the DNase I-catalyzed release of free thionine and resulted in an enhancement in the electrochemical signal. Exo III-assisted recycling amplification was also employed by Wang and co-workers to determine OTA (Figure 11B) [52]. The metastable hairpin probe (HP) consisted of three regions, including the aptamer for the OTA recognition, poly(dA) sequence as extended DNA and the target DNA. The probe is complementary to a part of aptamer and MB-labeled DNA. Once OTA was introduced, the structure of HP changed from hairpin format to the open state. The change led to the hybridization between T-DNA and MB-DNA. Next, the repeated Exo III cleavage was initiated for the amplification of electrochemical signal. Besides, Lin's group developed a homogeneous and label-free electrochemiluminescence (ECL) biosensor. The method is based on the difference of electrostatic repulsion between free $Ru(phen)_3^{2+}$ and dsDNA-$Ru(phen)_3^{2+}$ to the negatively charged ITO electrode [53].

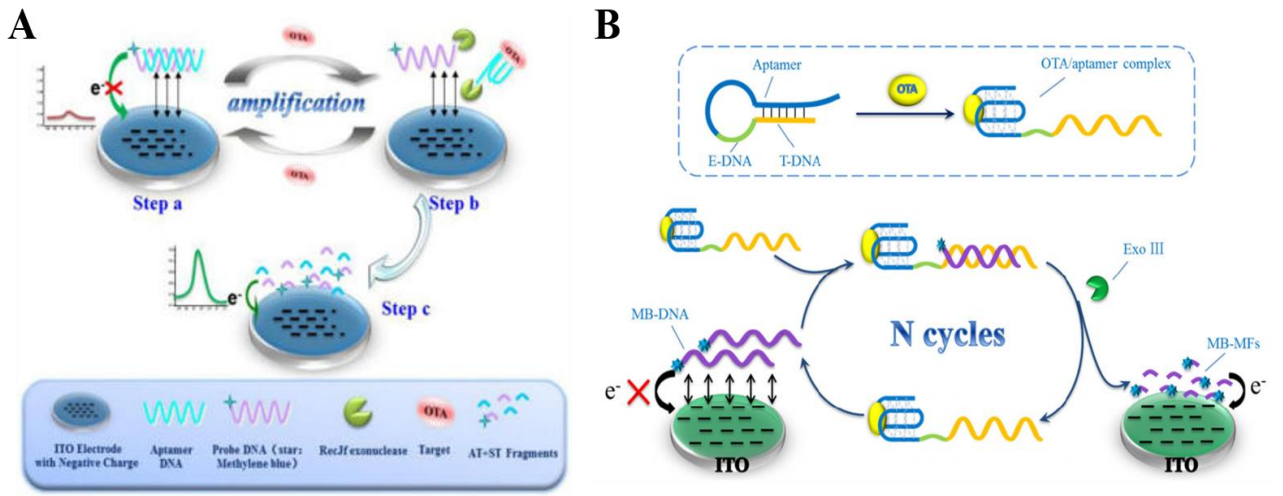

Figure 11. (A) Scheme of the immobilization-free electrochemical aptasensor for OTA based on DNA triplex structure and Exo III-assisted recycling amplification. Reprinted with permission from [50]. Copyright 2015 American Chemical Society. (B) Schematic illustration of the proposed homogeneous electrochemical method for OTA. Reprinted with permission from [52]. Copyright 2017 American Chemical Society.

Melamine (MA) is a nitrogen-rich toxic contaminant in milk industry and has been widely used as a chemical substance used in chemical industries. Due to the intrinsic structure, MA can be specifically inserted in the hydrophobic basic site and bind to the opposite through hydrogen bonding interaction between thymine and melamine, facilitating the formation of a T-MA-T triplex structure. By integrating DNA triplex with Exo III-based recycling amplification, Lin's group presented homogeneous electrochemical biosensor for MA (Figure 12A) [54]. When MA was added, T1, T2, and 8A formed a stable T-MA-T DNA triplex structure and further hybridized with MB-labeled ssDNA (dMB) to generate a T-shaped DNA structure. Next, the dMB was hydrolyzed with the aid of Exo III to produce short MB-labeled mononucleotide fragments (MB-MFs). The released T-MA-T complex could repetitively trigger the cycle of hybridization, cleavage, and release. Because of the higher diffusivity to the negatively charged ITO surface than the dMB probe, numerous produced MB-MFs caused an increased electrochemical signal. Li's group also developed a analytical platform for homogeneous detection of MA based on the similar strategy [55]. Differently, MB-labeled ssDNA can form a T-MA-T DNA triplex structure with the hairpin DNA and can be hydrolyzed by Exo III when DNA triplex formed.

Organophosphate pesticides (OPs) and carbamate pesticides have been worldwide used for protecting crops from pests to obtain higher yields in agricultural products and environmental samples. Therefore, it is highly necessary to develop effective and sensitive strategies to detect trace level pesticide residues in ensuring food and environment safety. On the basis of acetylcholinesterase (AChE)-mediated DNA conformational switch and rolling circle amplification (RCA), Li's group designed a label-free homogeneous "signal on" method for pesticide detection (Figure 13) [56]. AChE catalyzed acetylthiocholine (ACh) chloride to produce thiocholine (TCh), which further triggered the

DNA conformational change by competitively removing Hg^{2+} from T-Hg^{2+}-T base pairs in the unmodified hairpin.

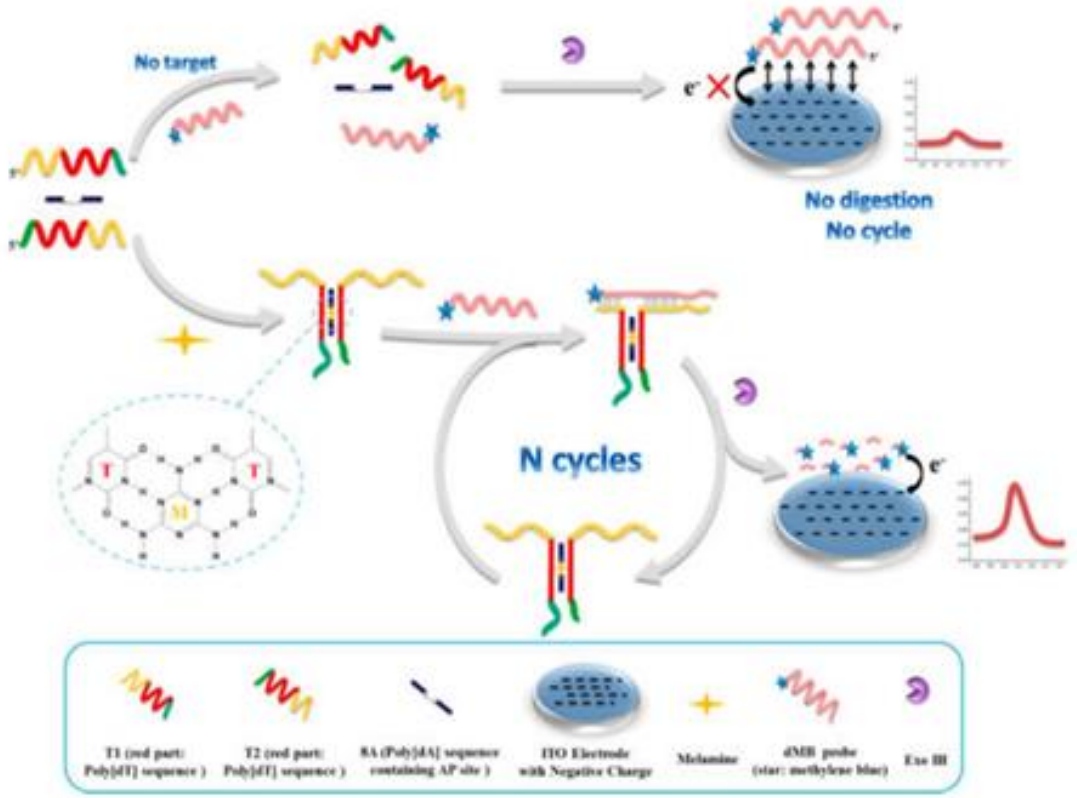

Figure 12. Schematic illustration of the proposed homogeneous electrochemical biosensor for melamine detection based on DNA triplex structure and Exo III-assisted recycling amplification. Reprinted with permission from [54]. Copyright 2016 American Chemical Society.

Once ssHP hybridized with the padlock into a complex, RCA reaction was initiated with the catalysis of phi29 DNA polymerase, resulting in a long ssDNA with the repeated same sequence. The product folded into a series of G-quadruplex structures in the presence of K^+. Then, a large number of electroactive MB molecules were captured by G-quadruplex structures and the diffusion to the surface of ITO electrode was hampered, causing a great decrease in the electrochemical response. However, in the presence of OPs, the activity of AChE was inhibited and the positively charged MB molecules could easily move to the surface of ITO electrode, thus generating a strong diffusion current. With the same strategy, this group presented an Exo I-aided homogeneous electrochemical biosensor for OPs (diazinon) detection [57]. Moreover, Guo's group employed the profenofos aptamer and T7 Exo to develop homogeneous electrochemical aptasensor for sensitive detection of profenofos residues [58].

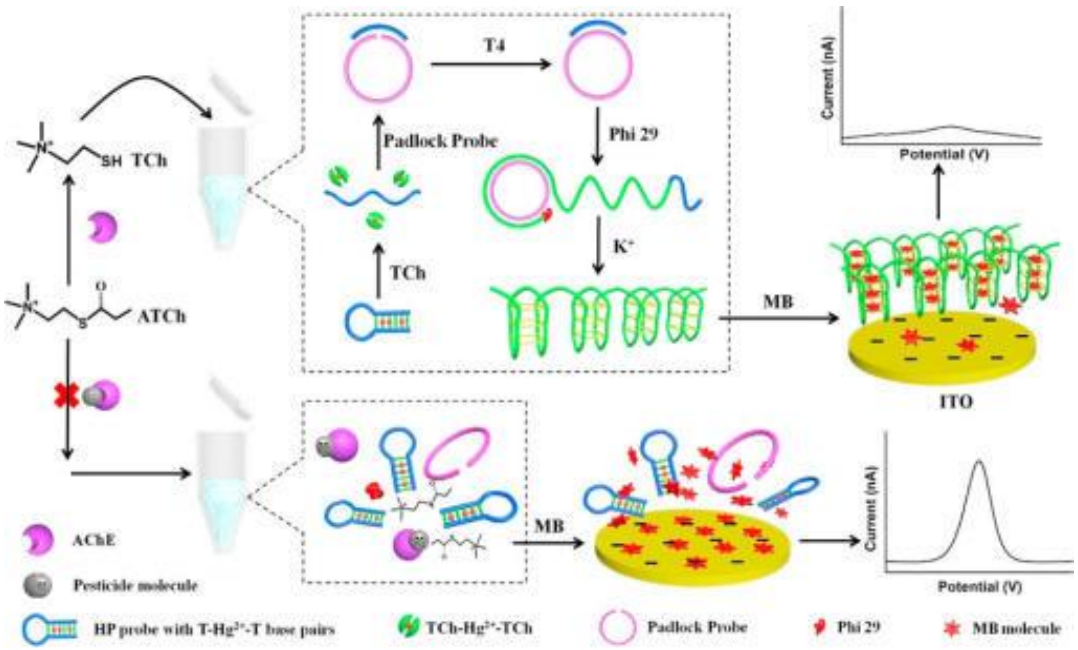

Figure 13. Principle of this label-free homogeneous electroanalytical method for pesticide assay based on acetylcholinesterase-mediated DNA conformational switch integrated with rolling circle amplification. Reprinted with permission from [56]. Copyright 2017 American Chemical Society.

Sensitive detection of drug molecules is important to screen drugs and monitor the metabolism. Li's group presented a homogeneous electrochemical bleomycin (BLM) assay based on the BLM-induced cleavage of methylene blue (MB)-labelled ssDNA [59]. Zhang's group constructed a homogeneous label-free electrochemical aptasensor for cocaine detection based on a designed miniaturized electrochemical device (Figure 14) [18]. The proposed electrochemical biosensor consisted of a disposable micropipet tip. Homogeneous DNAzyme catalytic activity was measured at a microliter level with a repeatable carbon fiber ultramicroelectrode. When cocaine was added, the hairpin was opened and the DNAzyme sequence was released, which possessed catalytic activity with hemin. This "mix-and measure" aptasensor realized the quantification of cocaine ranging from 1 to 500 μM with high specificity.

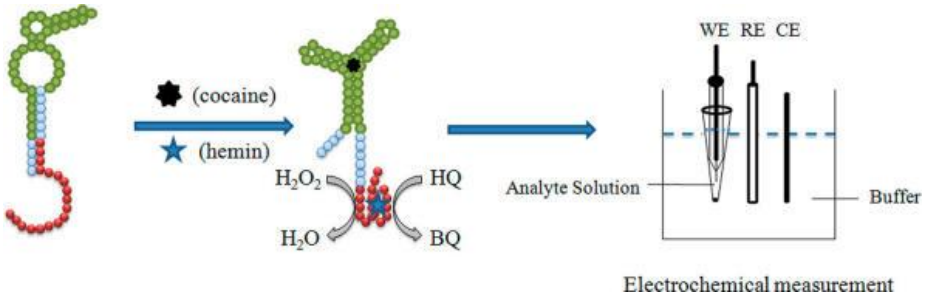

Figure 14. Principle of homogeneous label-free electrochemical aptasensor based on aptamer-DNAzyme hairpin for cocaine detection. Reprinted with permission from [18]. Copyright 2013 American Chemical Society.

4.4 Metal ions

Heavy metal ions in waste water threat the environment and health of animals all over the world. Qiu's group constructed an immobilization-free electrochemical method for Pb^{2+} detection with Pb^{2+}-dependent DNAzyme, in which the DNAzyme substrate was conjugated with MB at the 3' terminal [60]. The product of the cleavage of the DNAzyme/substrate complex, MB-labeled short-oligonucleotide, can diffuse easily to the surface of the negatively charged ITO electrode than the DNAzyme/substrate complex with a larger negative charge. However, when Hg^{2+} bind with thymine to form a stable T-Hg^{2+}-T structure and trigger the mismatch of the T-T base pair between two ssDNA probes, the dsDNA was produced. Based on the specific interaction, Chen's group presented a homogeneous electrochemical sensor for Hg^{2+} detection with the help of Exo III-assisted recycling amplification [61].

4. CONCLUSION

DNA-based electrochemical biosensors exhibit many applications in the fields of disease diagnosis, environmental monitoring, food control and biomedical research. In summary, we mainly reviewed the DNA-based immobilization-free electrochemical biosensors for the detection of DNA, miRNA, proteins, enzymes, metal ions and small molecules. To explore the applications of immobilization-free biosensors, more elaborately-designed functional nucleic acids and nuclease with high specificity should be integrated with DNA self-assembly technology to establish more sensitive biosensors in the future.

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