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Review

Hemin/G-quadruplex DNAzyme for Designing of Electrochemical Sensors

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Guanine-rich nucleic acid sequence can fold into guanine quadruplex (G-quadruplex) that is found to complex tightly with hemin to form the hemin/G-quadruplex. Under physiological conditions, the hemin/G-quadruplex displays robust peroxidase activity and acts as horseradish peroxidase (HRP)-mimicking DNAzyme. As a kind of artificial enzyme, the hemin/G-quadruplex DNAzyme is much more stable against hydrolysis and heat treatment than natural enzymes and can be synthesized in the laboratory and modified easily. Thus, it has been considered as a promising tool for the design of sensors. Electrochemical sensor is one of the major analytical techniques for sensitive and selective detection of proteins, DNA as well as small biomolecules. In this work, we reviewed the strategies for designing of electrochemical sensors based on the signal amplification of hemin/G-quadruplex DNAzyme.

Keywords: Electrochemical sensors; hemin; G-quadruplex; DNAzyme

1. INTRODUCTION

Electrochemical sensor is one of the major analytical techniques for sensitive and selective detection of proteins, DNA as well as small biomolecules [1,2]. In this format, the crucial step is the capture and identification of analytes. Regarding selectivity, the specific binding between the target analytes and the immobilized biomolecules on electrode, such as antigen–antibody, DNA–DNA and protein–nucleic acid, has been extensively used to capture analytes. At present, the increasing demand for detection of ultralow amount of analytes is pushing the enhancement of detection sensitivity by selecting different signal amplification strategy [3]. Enzyme-amplified sensors with superior properties such as stability, reusability and availability for almost any given analytes have attracted substantial research efforts and emerged as viable alternatives to detect and quantify trace amounts of biomarkers

in biological studies, clinical diagnostics, and treatment [4-6]. Among kinds of the enzymes (horseradish peroxidase or HRP, glucose oxidase or GOx and alkaline phosphatase or ALP), HRP is the most commonly used enzyme label in connection to electrochemical monitoring of the biocatalytic reaction product. A guanine (G)-rich nucleic acid sequence in particular is predisposed to form higher order structures: e.g., G-quadruplex. Recently, it has been demonstrated that G-rich sequence could bind to hemin to form the hemin/G-quadruplex HRP-mimicking DNAzyme, which can catalyze the reduction of H_2O_2 and then generate cathodic current for measurements [7-11]. Hemin/G-quadruplex DNAzyme has thus been extensively used as a catalytic label in place of HPR for the amplified electrocatalytic detection. In this work, we reviewed the strategies for designing of electrochemical sensors based on the signal amplification of hemin/G-quadruplex DNAzyme.

2. HEMIN/G-QUADRUPLEX FOR ELECTROCHEMICAL SENSORS

The use of hemin/G-quadruplex DNAzyme as the electrocatalytic label for amplifying sensing events was first reported by Pelossof et al. in 2010 [12]. They suggested that the hemin/G-quadruplex DNAzyme (1) on electrode exhibited bioelectrocatalytic functions toward the electrocatalyzed reduction of H_2O_2 (Fig. 1A). The electrocatalytic properties of the DNAzyme were, then, used to develop electrochemical sensing platforms for analyzing the activities of enzymes and their substrates, DNA-sensors and aptasensors. As shown in Fig. 1B, the glucose oxidase (GOx) modified with a thiol-functionalized nucleic acid (**2**) was immobilized on the gold electrode covered with the thiolated nucleic acid (**3**) that includes the DNAzyme sequence and a base sequence domain complementary to (**2**). In the presence of glucose, the GOx-mediated oxidation of the substrate yielded gluconic acid and H_2O_2, and the resulting hydrogen peroxide was, then, analyzed through its electrocatalyzed reduction by the DNAzyme. The electrocatalytic cathodic currents were dependent upon the glucose concentration, facilitating the quantitative analysis of glucose. Fig. 1C outlined the analysis of DNA with the DNAzyme as an electrocatalytic label. The thiolated nucleic acid (**4**) immobilized on gold electrode showed a hairpin-stabilized structure. Domains I and II in **(4)** corresponded to the base sequence of the DNAzyme and to the sequence complementary to the target DNA (**5**), respectively. The hybridization of (**5**) to the loop domain opened the stem of the hairpin, leading to the formation of hemin/G-quadruplex DNAzyme structure in the presence of hemin. This method was also implemented to develop an electrochemical aptasensor for adenosine monophosphate AMP (**6**). The thiolated hairpin DNA (**7**) assembled on an electrode surface consists of two regions: the DNAzyme sequence (III) and the aptamer sequence (IV) for AMP (Fig. 1D). Upon interacting the modified electrode with (**6**), the AMP-aptamer complex was formed, leading to the opening of the hairpin stem and the self-assembly of the DNAzyme in the presence of hemin. The adenosine deaminase (**9**) can transform the AMP substrate into inosine monophosphate IMP (**8**) that lacks affinity for the aptamer sequence. They also found that the IMP dissociates from **(7)**, and the resulting nucleic acid refolded into the stabilized hairpin structure of **(7)**. Thus, the aptasensor electrode could be re-used. Lately, they developed a sensitive electrochemical sensing platform for Pb^{2+} detection based on the hemin/G-quadruplex nanostructure and the Pb^{2+}-dependent DNAzyme [13]. As shown in Fig. 1E, a thiolated

nucleic acid consisting of the Pb^{2+}-dependent DNAzyme sequence and the hemin/G-quadruplex DNAzyme sequence was assembled on gold electrode. In the presence of Pb^{2+}, the Pb^{2+}-dependent DNAzyme cleaved the substrate sequence, allowing for the self-assembly of the hemin/G-quadruplex.

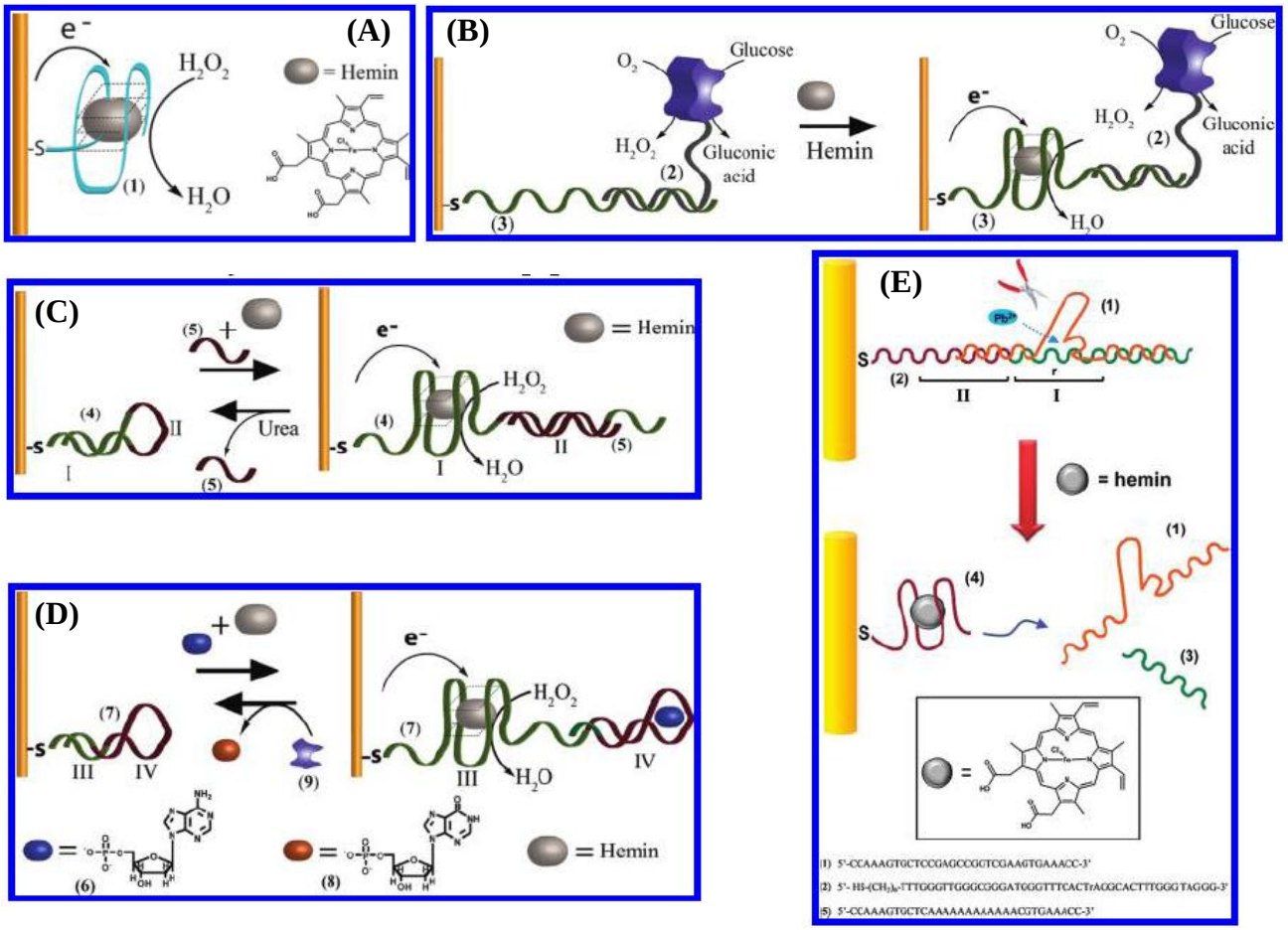

Figure 1. (A) Electrocatalyzed Reduction of Hydrogen Peroxide by the HRP-Mimicking DNAzyme-Modified Au Electrode. **(B)** Sensing of Glucose through the Bioelectrocatalyzed Reduction of H_2O_2 Generated by the GOx-Mediated Oxidation of Glucose Using a HRP-Mimicking DNAzyme Hybridized to a Nucleic Acid-Functionalized Gox. **(C)** Sensing of a Target DNA (5) by the Opening of a Thiolated Hairpin Nucleic Acid Structure (4) and the Activation of a HRP-Mimicking DNAzyme that Electrocatalyzes the Reduction of H_2O_2. **(D)** Sensing of AMP (6) by the Opening of the Thiolated Nucleic Acid Hairpin (7), Associated with the Electrode, and the Formation of an Aptamer-AMP Complex. Reprinted with permission from [12]. (Copyright 2010 American Chemical Society) **(E)** Sensing of Pb^{2+} Ions by the Pb^{2+}-Dependent DNAzyme-Catalyzed Cleavage of the Ribonuclease-Containing Nucleic Acid Sequence (2) and the Formation of a Hemin/G-Quadruplex Structure Acting as a Label. (Reprinted with permission from [13]. Copyright 2012 American Chemical Society)

The success of the initial efforts provides good motivation for further manipulation of kinds of electrochemical sensors with hemin/G-quadruplex DNAzyme as the signal tag. Lately, Shen et al. reported a hemin/G-quadruplex DNAzyme aptasensor for the amplified electrochemical detection of thrombin with a combination of hemin and guanine-rich thrombin-binding aptamer to form the HRP-

mimicking DNAzyme. The DNAzyme activity was extensively promoted by thrombin [14]. Liang et al. reported a himin/G-quadruplex-based sensor for α-naphthol detection by electrochemical impedance spectroscopy (EIS) [15]. Specifically, in the presence of K^+ and hemin, stable himin/G-quadruplex complexes formed on the electrode surface. The resulting complexes catalyzed H_2O_2-mediated oxidation of a-naphthol to insoluble 1,4-naphthoquinone on the DNA films, leading to the increase in the charge transfer resistance. Zhang et al. developed an electrochemical aptasensor for detection of recombinant human IFN-g by incorporating a specific DNAzyme sequence into a hairpin aptamer probe. The presence of IFN-g opened the hairpin structure and formed the hemin/G-quadruplex DNAzyme with subsequent addition of hemin [16]. Through the thymine–Hg^{2+}–thymine interaction mediated surface hybridization reaction, bifunctional oligonucleotide signal probe containing a mercury-specific sequence and a G-quadruplex (G4) sequence was also employed for electrocatalytic assay of Hg^{2+} [17].

Moreover, Liu et al. reported the detection of two important biomarkers, prostate specific antigen (PSA) and telomerase, by combining hemin/G-quadruplex DNAzyme with the traditional sandwich immunosensing format in one biosensing interface [18]. Specifically, PSA was captured by the antibody-coated gold electrode and then recognized by the biotinylated secondary antibody. Streptavidin was then bound to the biotinylated immunocomplex to act as a bridge for multiplex anchoring of biotinylated single-stranded oligonucleotides (telomerase primer). In the presence of telomerase, telomerization reaction was initiated to form a long single-stranded DNA with TTAGGG repeat units which were continuously added to the 3'-end of the primer in the presence of the nucleotide mixture dNTPs. Then, the produced G-rich sequences on electrode surface bound to hemin to form the hemin/G-quadruplex DNAzyme. Also, Wang et al. reported the detection of cisplatin (a cytotoxic and antineoplastic metallodrug) with the hemin/G-quadruplex DNAzyme wires containing many units of hemin/G-quadruplex DNAzyme (Fig. 2A) [19]. The combination of cisplatin with the guanine of the designed hemin/G-quadruplex DNAzyme changed the super sandwich DNA structure and decreasing the catalytic effect on H_2O_2. The specific folic acid (FA)–folate receptor (FR) interaction can prevent DNA enzymolysis by ExoI; Wang et al. also developed a signal amplification strategy for electrochemical detection of FR on DNA sequence 1 (S1) modified gold electrode [20]. As shown in Fig. 2B, in the absence of FR, the FA-linked DNA sequence (S2-FA) was hydrolyzed into mononucleotides by ExoI. In the presence of FR, FR would specifically bind to FA to form the FR-bound S2-FA (S2/FA-FR), preventing ExoI from approaching and cleaving. S1 was then hybridized with S2/FA-FR and S3 (a 48-mer oligonucleotide with ferrocene (Fc) label at the 3' end). S3 contains 3 parts: (i) 15 bases at 3' end of S3 (red part) that can hybridize with 5' end of S2, (ii) the other 15 bases (yellow part) that can hybridize with 3' end of S2, and (iii) the rest green part with 18 bases that can bind with hemin to form the DNAzyme. After hybridization, a ''DNAzyme super sandwich structure'' (S1/S2-FA-FR/S3) contained numerous Fc and hemin/DNAzyme units formed on the electrode surface. In this system, Fc acted as an electron-transfer mediator for DNAzyme [21,22]. In addition, methylene blue (MB) has also been typically used as the redox reporter or electron-transfer mediator for electrocatalysis. Wang et al. also reported an electrochemical sensing platform for adenosine triphosphate (ATP) detection using DNAzyme as the electron sink and MB as the redox reporter [23].

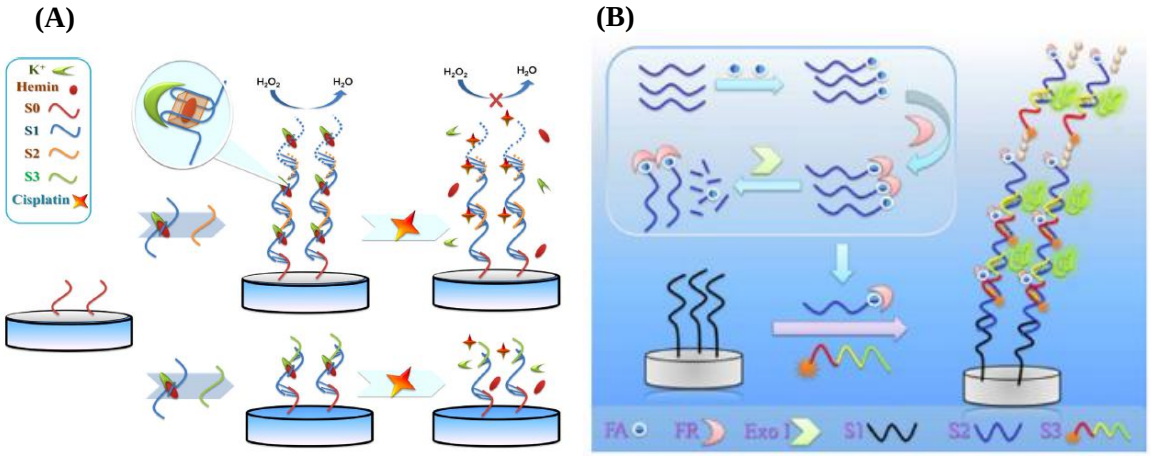

Figure 2. (A) The schematic representation of electrochemical sensor for detection of cisplatin based on hemin/G-quadruplex DNAzyme supersandwich structure. (Reprinted with permission from [19]. Copyright 2013 Elsevier) **(B)** Fabrication process of the electrochemical amplified determination of FR based on DNAzyme-supersandwich structure. (Reprinted with permission from [20]. Copyright 2013 Elsevier)

In view of low cost, easy functionalization and high stability against hydrolysis as well as heat treatment, new schemes based on coupling the biocatalytic amplification of DNAzyme with additional amplification processes have been developed [24-27]. Typically, duplex-specific nuclease (DSN) displays a strong preference for cleaving double-stranded DNA or DNA in DNA/RNA heteroduplexes; Zhang et al. reported a label free electrochemical biosensor based on a 2'-O-methyl modified DNAzyme and DSN assisted target recycling for amplified detection of miR-21 [25]. The sensor contains two successive steps of isothermal enzymatic amplification. A thiolated signal probe DNA (Sp) consisting of a target recognition sequence (black) and a 2'-O-methyl modified G-rich sequence (blue) was immobilized onto a gold electrode. Hybridization between target miR-21 and Sp to form a DNA–RNA heteroduplex promoted the hydrolysis of the target recognition sequence of Sp and the release of miR-21 back to the sample solution for recycling. At last, the 2'-O-methyl modified G-rich sequence part of Sp remained on the electrode surface. Upon addition of hemin, the 2'-O-methyl modified G-rich sequences bound hemin to form hemin/G-quadruplex DNAzyme. In the absence of target miR-21, Sp cannot be hydrolyzed because of the low activity of DSN against single-stranded DNA. As a result, the G-quadruplex DNAzyme could not form due to the steric-hindrance effect. Wang et al. reported a dual amplified strategy for ultrasensitive mutant DNA detection by the coupling of NEase-assisted target recycling with rolling circle amplification (RCA) [27]. After the amplification, numerous hemin/G-quadruplexcomplexes formed on the sensor surface. In this system, the dithiol-modified hairpin probes (HPs) contain four domains: the RCA primer region, the target mutant DNA recognition sequences, the NEasere cognition sequences and two complementary sequences in the duplex structure of the stem (Fig. 3A). The NEase (N.BstNBI) recognized and cleaved only one strand of a double stranded DNA four bases away from the 3'end of its recognition sequence (5'-GAGTC-3'). The target mutant p53 genes hybridized with the loop part of HPs to form double-stranded structures with full recognition sites for N.BstNBI, which preferentially bound to the recognition sites and selectively cleaved the HPs into two pieces, leaving the RCA primer sequence

regions of the HPs available for subsequent RCA reactions. At the same time, upon N.BstNBI cleavage, the target genes dissociated from the HPs and again hybridized with the un-nicked HPs to initiate the target gene recycling process, leading to permanent transformation of a large number of HPs to numerous RCA primer fragments. After incubating the resulting sensor surface with the rationally designed padlock probes and T4 ligase, the RCA reactions were initiated by adding phi29 DNA polymerase and dNTPs and massive long DNA molecules with multiple G-quadruplex units were therefore generated. These G-quadruplex units associated with hemin to form hemin/G-quadruplex complexes on the sensor surface with the help of K^+. Furthermore, Yu et al. designed an electrochemical biosensor based on an arched probe mediated isothermal exponential amplification reaction (EXPAR) [26]. The arched probe consists of two strands (Strand 1 and Strand 2), which were partially complementary at both ends (Fig. 3B). Strand 2 can prevent the hybridization of primer with Strand 1 and Strand 1 can minimize the formation of any nonspecific hemin-containing G-quadruplex from Strand 2. The recognition site of the nicking endonuclease was located in the loop region of Strand 2 and was unsuitable to bind with the enzyme. Target miRNA was complementary to the 5′ stem region and part of the loop region of Strand 1. The separation of one hybridized domain through the formation of a target-substrate complex resulted in the thermal melting of the remaining duplex. After cleavage of the arched probe, the free Strand 2 bound to hemin to form the hemin/G-quadruplex DNAzyme on the surface of the electrode. On the other hand, Strand 1 hybridized with the target was released to the solution and initiated a series of cyclic chain amplification reactions. Once the engaging primer annealed with the complementary region of Strand 1, the polymerase initiated the polymerization, which regenerated the target miRNA and synthesized a DNA duplex. As a result, the displaced miRNA was free to bind to another probe and triggered a new cycle for recycling the target and forming a DNA duplex as well. The DNA duplex generated above activated the recognition site of the nicking enzyme. After the nicking endonuclease nicked at the DNA duplex, the polymerization started and the primer part got extended plots of electrode modification at different stages.

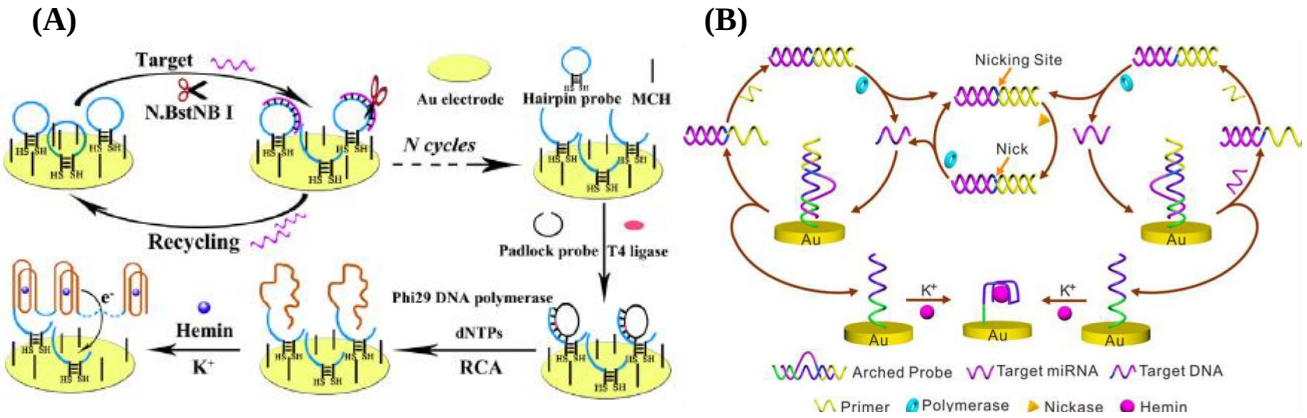

Figure 3. (A) Illustration of the dual amplified assay protocol for ultrasensitive EC detection of mutant DNA based on NEase-assisted target recycling and RCA hybrid signal amplification. (Reprinted with permission from [27]. Copyright 2014 Elsevier) **(B)** Schematic Illustration of the Arched Probe Mediated EXPAR Strategy Based on Polymerase and Nicking Endonuclease for the miRNA Assay (Reprinted with permission from [26]. Copyright 2014 American Chemical Society)

Polyaniline (PANI) is one of the most important conducting polymers due to its remarkable electrical, electrochemical and redox properties as well as good environmental stability. Li et al. demonstrated that the HRP-mimicking DNAzyme could catalyze the template-guided deposition of PANI [28]. The Pb^{2+} aptamer was first self-assembled on the electrode surface (Fig. 4A). Upon addition of Pb^{2+}, Pb^{2+} bound to its aptamer to form G-quadruplex for the formation of hemin/G-quadruplex DNAzyme with peroxidase catalytic activity. Subsequently, hemin/G-quadruplex DNAzyme catalyzed the oxidation of aniline to PANI in the presence of H_2O_2, resulting in a readily measurable ‘‘turn-on’’ electrochemical signal. Moreover, nucleic acid can guide the enzymatic deposition of nanoparticles and nanowires where the phosphate groups serve as templates; based on hybridized microRNA (miRNA)-guided deposition of PANI, Deng et al. developed a sensitive impedimetric miRNA biosensor with hemin/G-quadruplex DNAzyme. As shown in Fig. 4B, the DNAzyme catalyzed the polymerization of aniline and the hybridized miRNA strands guide the deposition of PANI, thus leading to the formation of a thin PANI film on the biosensor surface and an increase in electron transfer impedance [29].

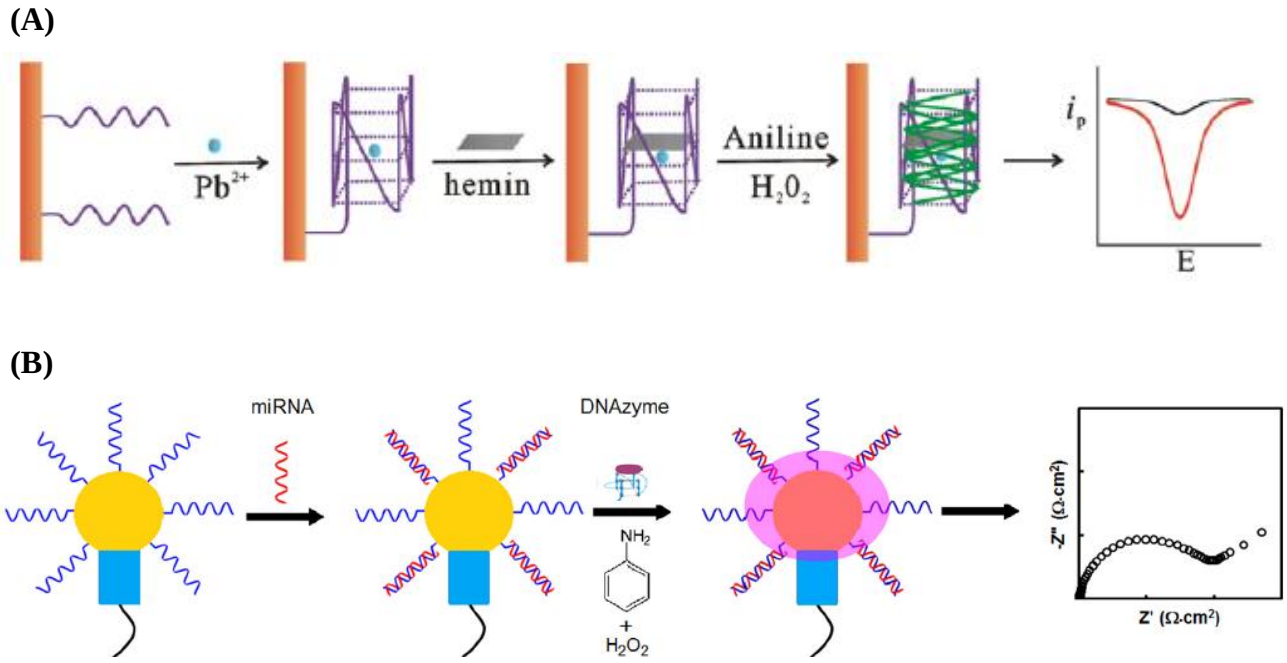

Figure 4. (A) Schematic illustration of the versatile sensing platform based on HRP-mimicking DNAzyme-catalyzed template-guided deposition of PANI. (Reprinted with permission from [28]. Copyright 2011 Elsevier) **(B)** Schematic illustration of the working principle of the label-free miRNA biosensor. (Reprinted with permission from [29]. Copyright 2014 Copyright 2011 Elsevier)

Potentiometry is a powerful electrochemical technique with high simplicity and low assay cost. Wang et al. reported the first potentiometric transducer for hemin/G-quadruplex DNAzyme-based biosensing by using potential responses of electrically neutral oligomeric phenols on quaternary ammonium salt-doped polymeric membrane electrodes [30]. In the presence of G-quadruplex/hemin DNAzyme and H_2O_2, monomeric phenols can be condensed into oligomeric phenols, inducing large

potential signals. By comparing the potential responses induced by peroxidations of 13 monomeric phenols, they found that p-methoxyphenol was the most efficient substrate for potentiometric detection of hemin/G-quadruplex DNAzyme.

3. HEMIN/G-QUADRUPLEX FUNCTIONALIZED NANOCOMPOSITES FOR ELECTROCHEMICAL SENSING

Enzyme amplification is one of the most commonly employed strategies for development of electrochemical sensors because of its high simplicity and good reproducibility. However, single amplification by enzyme label is not sufficient for detecting the ultra-low concentration of analytes. Thus, a number of research groups have been exploring various methods to improve the sensitivity by integrating redox cycling reactions and/or nanomaterials (e.g. enzymes, etallic/magnetic nanoparticles, carbon-based nanostructures and quantum dots) to further amplify the electrochemical response [31,32]. Herein, amplification strategies by nanomaterials-based hemin/G-quadruplex DNAzyme were also summarized.

As an essential aspect of bioanalysis, amplification has been successfully achieved by employing nanocontainers or nanoparticles and enzymes as amplifiers for sensitive detection of biorecognition events. Gold nanoparticles (AuNPs) have been widely used in diagnostics and detection because of their unique characteristics, such as high surface-to-volume ratio, high surface energy, ability to decrease proteins-metal particles distance, and the functioning as electron conducting pathways between prosthetic groups and the electrode surface [33-36]. With AuNPs as carriers for loading of biorecognition elements for the recognition of targets and with response molecules (redox tags or enzymes) for the signal readout, a few of hemin/G-quadruplex DNAzyme-based electrochemical sensors have been reported [37-39]. Typically, Tang's group designed a novel and in situ amplified immunoassay strategy with quadruple signal amplification by using AuNPs-functionalized DNAzyme concatamers with redox-active intercalators (Fig. 5) [37]. To construct such an in situ amplification system, streptavidin-labeled AuNP (AuNP-SA) was used for the labelling of initiator strands (S0) and detection antibody (mAb2) (denoted as mAb2−AuNP−S0), and then two auxiliary DNA strands S1 and S2 were designed for in situ propagation of DNAzyme concatamers with the hemin/G-quadruplex format. In the presence of target protein carcinoembryonic antigen CEA, the sandwiched immunocomplex was formed between the immobilized primary antibodies on the electrode and the conjugated detection antibodies on the mAb2−AuNP−S0. The carried S0 initiator strands could progress a chain reaction of hybridization events between alternating S1/S2 DNA strands to form a nicked double-helix. Upon addition of hemin, the hemin-binding aptamers could be bound to form the hemin/G-quadruplex−based DNAzymes. The formed double-helix DNA polymers could cause the intercalation of numerous electroactive methylene blue (MB) molecules. During the electrochemical measurement, the formed DNAzymes could catalyze the reduction of H_2O_2 in the solution to amplify the electrochemical signal of the intercalated MB.

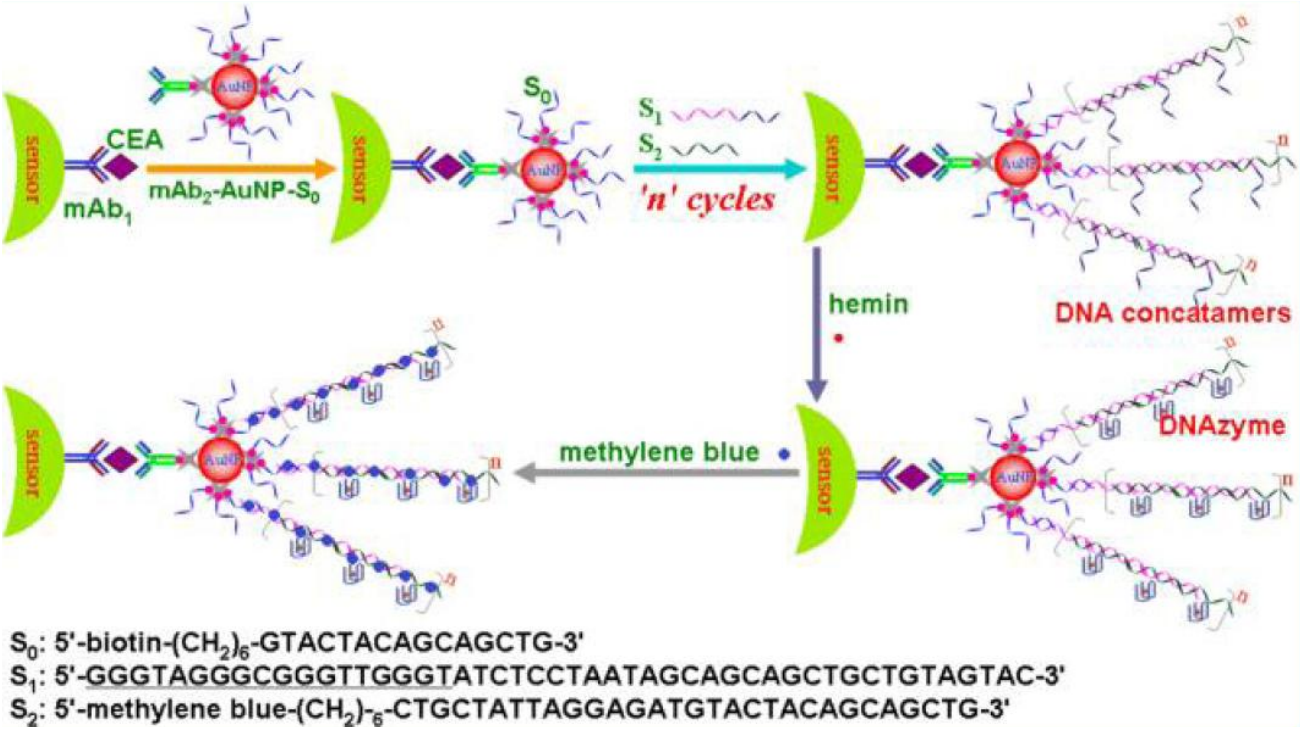

Figure 5. Schematic Representation of Nanogold-Functionalized DNAzyme Concatamers with Redox-Active Intercalators for Quadruple Signal Amplification of Electrochemical Immunoassays: Adivin−Biotin Chemistry with First Signal Amplification, AuNP-Based Second Signal Amplification, DNA Concatamer-Based Third Signal Amplification, and DNAzyme-Based Fourth Signal Amplification. (Reprinted with permission from [37]. Copyright 2013 American Chemical Society)

Besides as the signal labels, AuNPs can be used as the electrode materials to enhancing the biocompatibility, electrocatalysis and redox property of electrode. With AuNPs-modified electrode, many signal amplification approaches have been reported [40-49]. Typically, Yuan's group developed a few of hemin/G-quadruplex DNAzyme-based electrochemical sensors with AuNPs as the electrode materials and/or labels [42-46]. For example, it has been suggested that hemin/G-quadruplex could act as an NADH oxidase besides as HRP-mimicking DNAzyme [43,50]; Yuan's group constructed a new pseudobienzyme electrochemical aptasensor for thrombin detection with the hemin/G-quadruplex acting as an NADH oxidase and HRP-mimicking DNAzyme simultaneously for the first time [43]. In this method, AuNPs were loaded with thrombin aptamer in both the capture and recognition of thrombin. Moreover, hemin was intercalated into the aptamer to form hemin/G-quadruplex and the electro-active thionine was assembled on AuNPs as the electron mediator. By using autonomously assembled hemin/G-quadruplex DNAzyme nanowires for signal amplification, they reported a sensitive pseudobienzyme electrocatalytic DNA biosensor for Hg^{2+} detection on AuNPs modified glass carbon electrode [45]. As shown in Fig. 6, in presence of Hg^{2+}, the specific coordination between Hg^{2+} and T could result in the assembly of primer DNA on the electrode, which successfully triggered the HCR to form the hemin/G-quadruplex DNAzyme nanowires with substantial redox probe thionine (Thi). In the electrolyte of PBS containing NADH, the hemin/G-quadruplex nanowires firstly acted as

an NADH oxidase to assist the concomitant formation of H_2O_2 in the presence of dissolved O_2. Then, with the redox probe Thi as electron mediator, the hemin/G-quadruplex nanowires acted as an HRP-mimicking DNAzyme that quickly bioelectrocatalyzed the reduction of produced H_2O_2, which finally led to a dramatically amplified electrochemical signal. Very recently, they also developed an ultrasensitive electrochemical immunosensor for detecting the Pebrine disease related spore wall protein of Nosemabombycis (SWPN.b) based on the amplification of hemin/G-quadruplex functionalized Pt@Pd nanowires (Pt@PdNWs) [46]. The synthesized Pt@Pd NWs possessed large surface area, which could effectively improve the immobilization amount of hemin/G-quadruplex DNAzyme concatamers produced via hybridization chain reaction (HCR). In the presence of SWPN.b, the hemin/G-quadruplex labeled Pt@PdNWs bioconjugations was captured on electrode surface and thus obtained electrochemical signal.

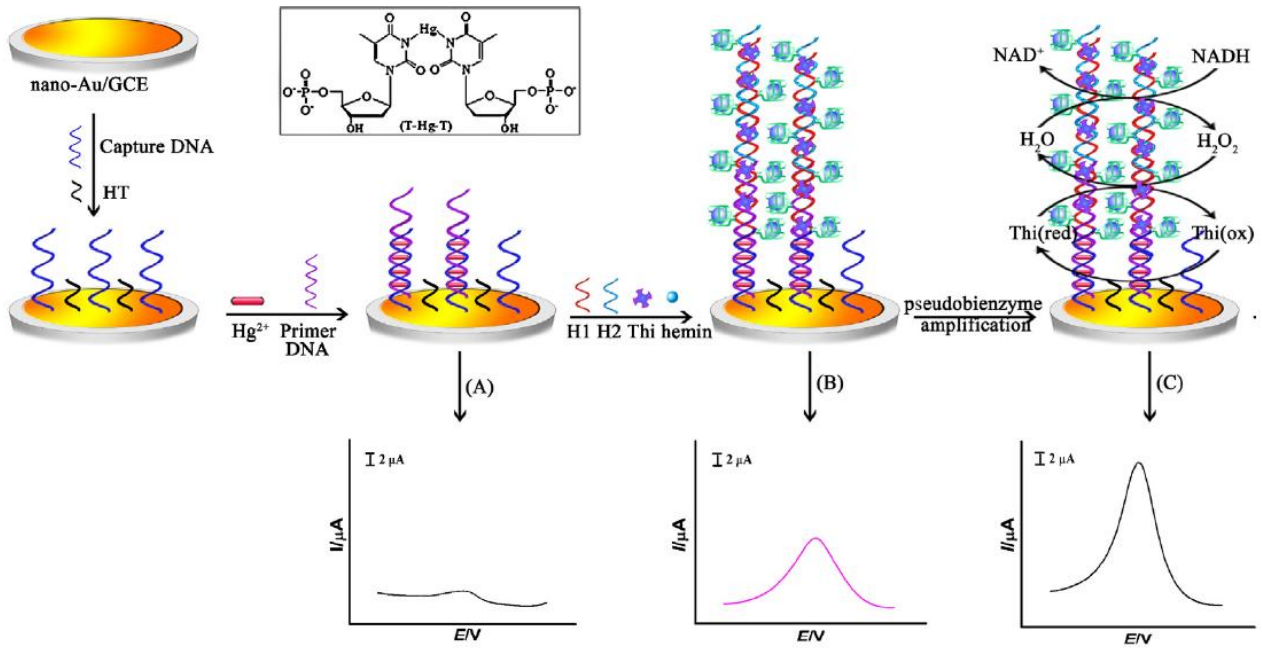

Figure 6. Schematic illustration of the sensitive pseudobienzyme electrocatalytic DNA biosensor for Hg^{2+} detection by using the autonomously assembled hemin/Gquadruplex DNAzyme nanowires for signal amplification. (Reprinted with permission from [45]. Copyright 2014 Elsevier)

Because of the extraordinary physicochemical and structural properties of carbon nanomaterials, such as grapheme and carbon nanotubes, these exciting new materials have quickly sparked tremendous interests across many fields, including biosensors, electrochemical energy storage and electronics [51]. The hemin/G-quadruplex functionalized graphene and carbon nanotubes nanocomposites have been successfully utilized as the signal labels for electrochemical biosensing typically in a "sandwich-like" format [52-56]. For example, Yuan's group reported two electrochemical thrombin biosensors on AuNPs modified electrodes using Pd nanoparticles (PdNPs)-reduced gaphene oxide (rGO) (denoted as PdNPs-rGO) [53] and dendrimer-rGO [54] functionalized with thionine- and hemin/G-quadruplex as the signal tags. As shown in Fig. 7, the thionine (TH)-

conjugated hemin/G-quadruplex-polyamidoamine (PAMAM)-rGO was captured on electrode surface. In the presence of NADH, the hemin/G-quadruplex on electrode surface firstly acted as an NADH oxidase, facilitating the oxidation of NADH to NAD^+ with the concomitant local formation of H_2O_2 in the presence of O_2. Simultaneously, the hemin/G-quadruplex acted as an HRP-mimicking DNAzyme that quickly bioelectrocatalyzed the reduction of produced H_2O_2 with dramatically improving the oxidation–reduction reaction of the TH mediator, thus leading to an increase in the redox current of TH.

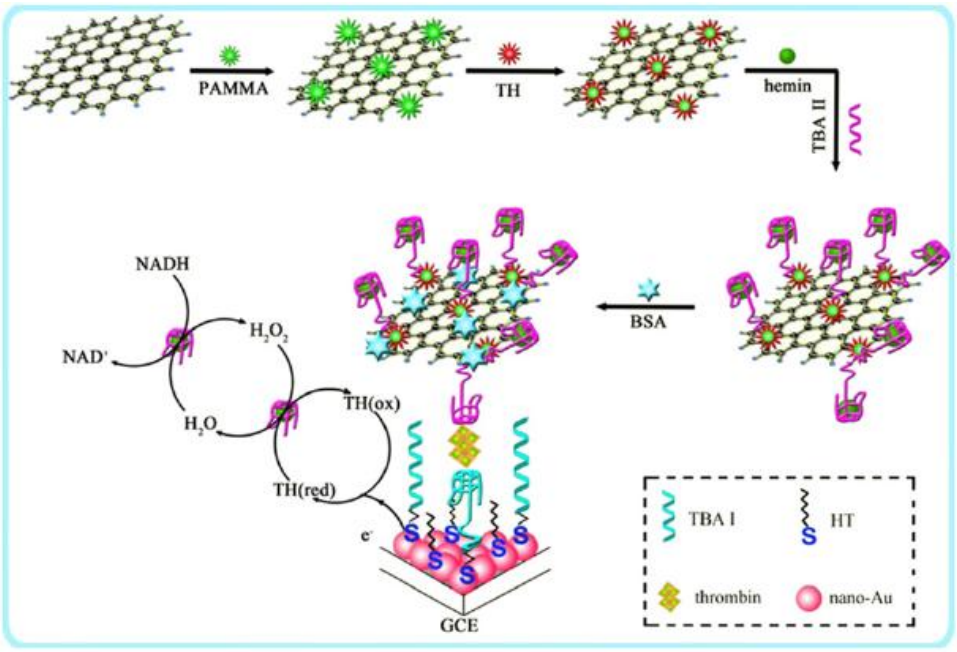

Figure 7. Schematic illustration of the pseudobienzyme aptasensor with PAMMA-rGO as nanocarrier and hemin/G-quadruplex as NADH oxidase and HRP-mimicking DNAzyme. (Reprinted with permission from [54]. Copyright 2013 Elsevier)

It has been demonstrated that the signal amplification efficiency of a bienzyme-catalyzed amplification system is obviously superior to that of a monoenzyme-catalyzed amplification system. Recently, Yuan's group, for the first time, reported several hemin/G-quadruplex triple-enzyme-catalyzed amplification systems on AuNPs modified glass carbon electrode [47-49]. For example, with the amplification of alcohol dehydrogenase (ADH)-Pt–Pd nanowires bionanocomposite and the hemin/G-quadruplex structure acting as NADH oxidase and HRP-mimicking DNAzyme simultaneously, they developed a pseudo triple-enzyme cascade electrocatalytic electrochemical aptasensor for thrombin detection (Fig. 8A) [48]. With the addition of ethanol to the electrolyte, the alcohol dehydrogenase immobilized on the Pt–Pd nanowires catalyzed ethanol to acetaldehyde accompanied by NAD^+ being converted to NADH. Then the hemin/G-quadruplex firstly served as NADH oxidase, converting the produced NADH to NAD^+ with the concomitant local formation of high concentration of H_2O_2. Subsequently, the hemin/G-quadruplex acted as HRP-mimicking DNAzyme, bioelectrocatalyzing the produced H_2O_2. At the same time, the Pt–Pd nanowires employed in the strategy not only provided a large surface area for immobilizing thrombin binding aptamer

(TBA) and alcohol dehydrogenase, but also served as HRP-mimicking DNAzyme which rapidly bioelectrocatalyzed the reduction of the produced H_2O_2. Thus, such a pseudo triple-enzyme cascade electrochemical aptasensor could greatly promote the electron transfer of hemin and resulted in the dramatic enhancement of electrochemical signal. In view of the exciting properties of gaphene such as extremely high electronic conductivity and superior mechanical strength, they also reported a hemin/G-quadruplex triple-enzyme-catalyzed amplification system with alcohol dehydrogenase- and hemin/G-quadruplex-functionalized rGO-AuNPs nanocomposites as the signal tag (Fig. 8B) [57].

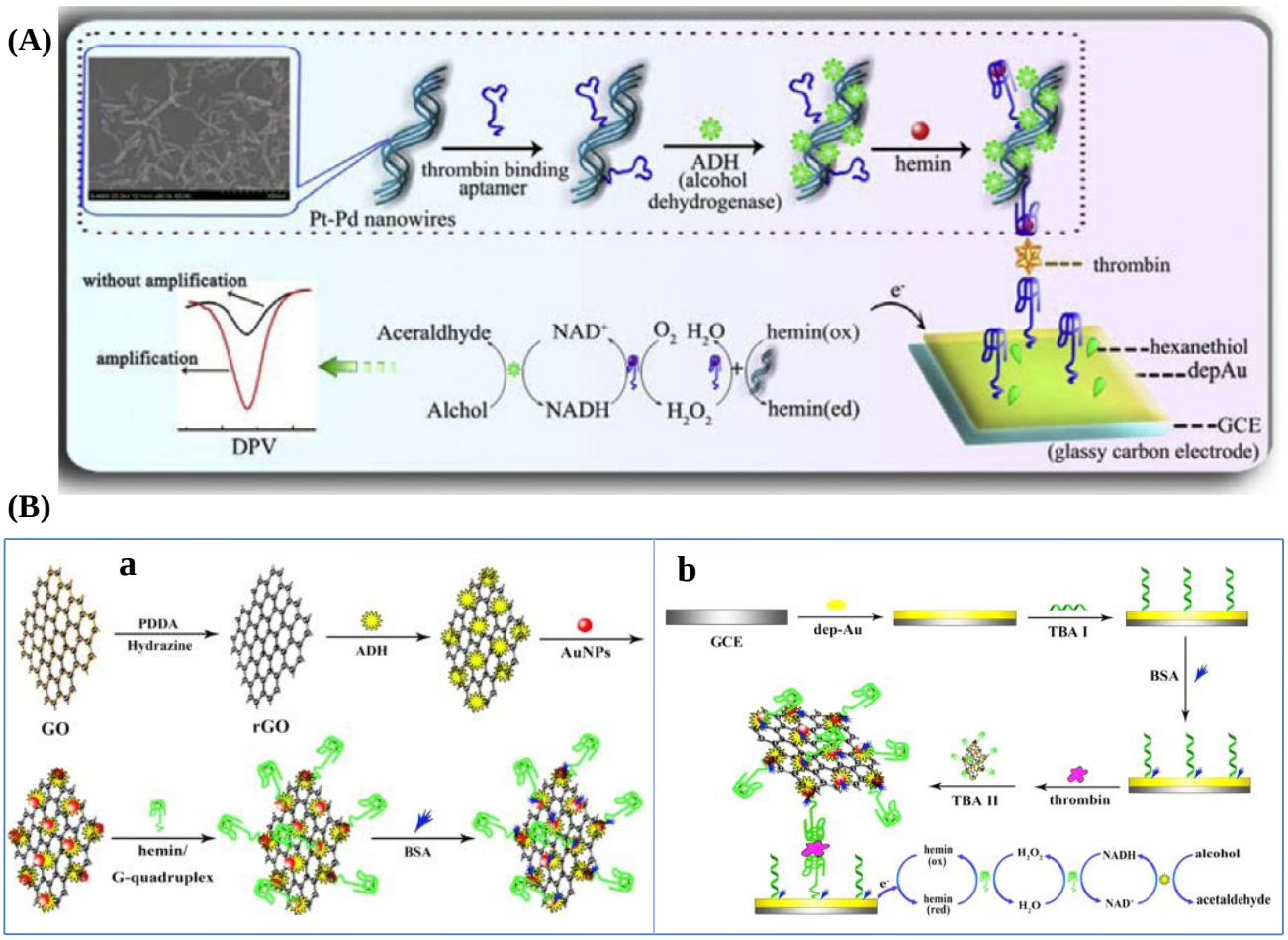

Figure 8. (A) The schematic diagrams of fabricated pseudo triple-enzyme electrochemical aptasensor based on the electrochemical signal amplification of Pt–Pd nanowires and hemin/G-quadruplex. (Reprinted with permission from [48]. Copyright 2014 Elsevier) **(B)** The preparation process of the second thrombin aptamer (a) and the sandwich-type electrochemical aptasensor (b). (Reprinted with permission from [57]. Copyright 2014 Copyright 2014 Elsevier)

On the basis of exonuclease-assisted target recycling and hybridization chain reaction (HCR) amplification strategy, Yuan's group further developed an ultrasensitive mediator-free triple-enzyme cascade electrocatalytic aptasensor for thrombin detection [49]. As shown in Fig. 9, the double strands constructed by the hybridization of thrombin binding aptamer (S1) with its complementary strand (S2) were firstly assembled on the electrode. Upon addition of target to the system, the S1 recognized

thrombin and left off electrode to make space for assembly of hybrid-primer probe (H0). Then, the H0 triggered the HCR to form the multi-functional hemin/G-quadruplex DNAzyme nanowires. In the mediator-free triple-enzyme cascade electrocatalytic amplification system, the hemin/G-quadruplex DNAzyme nanowires simultaneously played three roles: the redox probe, NADH oxidase and HRP-mimicking DNAzyme, respectively. Additionally, the exonuclease-assisted target recycling amplification was used for the continuous removal of S1, improving the assembly amount of hemin/G-quadruplex DNAzyme nanowire.

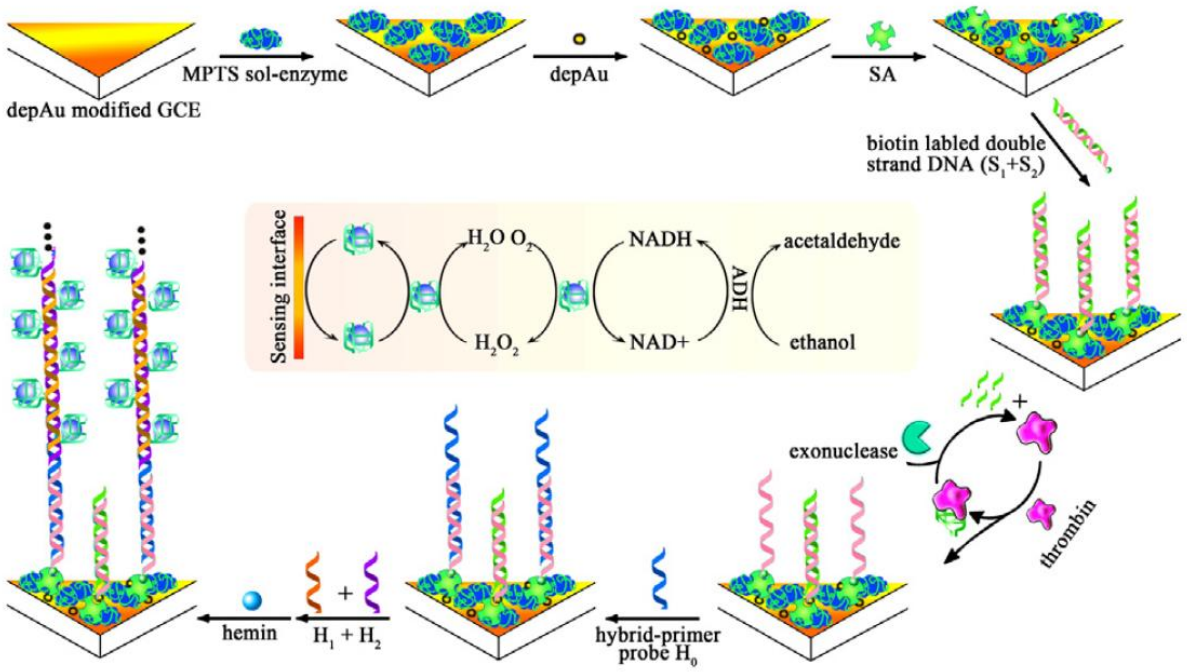

Figure 9. Schematic illustration of the multi-functional hemin/G-quadruplex DNAzyme nanowires-based mediator-free triple-enzyme cascade electrocatalytic electrochemical aptasensor with exonuclease-assisted target recycling and hybridization chain reaction amplification. (Reprinted with permission from [49]. Copyright 2014 Elsevier)

Based on the hollow PtCo nanochains (HPtCoNCs) functionalized by bi-enzyme-HRP mimicking DNAzyme and glucose oxidase as well as ferrocene-labeled secondary thrombin aptamer (Fc-TBA 2), Yuan's group constructed a highly sensitive electrochemical thrombin aptasensor [58]. The HRP mimicking DNAzyme contains a special G-quadruplex structure with an intercalated hemin. With the surface area enlarged by HPtCoNCs, the amount of immobilized Fc-TBA 2, hemin and glucose oxidase was enhanced. Under the enzyme catalysis of glucose oxidase, D-glucose was rapidly oxidized into gluconic acid accompanying with the generation of H_2O_2, which was further electrocatalyzed by Pt nanoparticles and HRP mimicking DNAzyme to improve the electrochemical signal of Fc. Furthermore, they fabricated an ultrasensitive sandwich-type electrochemical aptasensor for thrombin based on a triplex signal amplification strategy of hemin/G-quadruplex, blocking reagent-HRP and iron telluride nanorods (FeTe NRs) [59]. In this work, the FeTe NRs decorated by AuNPs (AuNPs@FeTe NRs) were not only used as carriers of secondary thrombin aptamer, electron mediator thionine and HRP, but also catalyzed the electrochemical reaction of thionine in the presence of H_2O_2.

Using hemin/G-quadruplex as the signal label and Fe_3O_4–Au nanocomposites with glucose oxidase and hemin/G-quadruplex as the signal enhancers, Yuan's group also developed a novel electrochemical thrombin aptasensor [60]. Due to the glucose oxidase-mimicking enzyme activity, AuNPs on the surface of the Fe_3O_4–Au nanocomposites effectively catalyzed the oxidization of glucose in the presence of dissolved O_2, accompanied by the production of H_2O_2. Both the Fe_3O_4 cores of Fe_3O_4–Au nanocomposites and hemin/G-quadruplex with H_2O_2-mimicking enzyme activity could catalyze the reduction of the generated H_2O_2, which promoted the electron transfer of hemin and amplified the electrochemical signal. At the same time, Sun et al. reported a sensitive electrochemical aptasensor for thrombin by using porous platinum nanotubes (PtNTs) labeled with hemin/G-quadruplex and glucose dehydrogenase (GDH) as labels (Fig. 10) [61]. Coupling with GDH and hemin/G-quadruplex as both NADH oxidase and HRP-mimicking DNAzyme, the cascade signal amplification was achieved by the following ways: in the presence of glucose and NAD^+ in the working buffer, GDH electrocatalyzed the oxidation of glucose with the production of NADH. Then, hemin/G-quadruplex as NADH oxidase catalyzed the oxidation of NADH to in situ generate H_2O_2. Based on the corporate electrocatalysis of PtNTs and hemin/G-quadruplex toward H_2O_2, the electrochemical signal was significantly amplified.

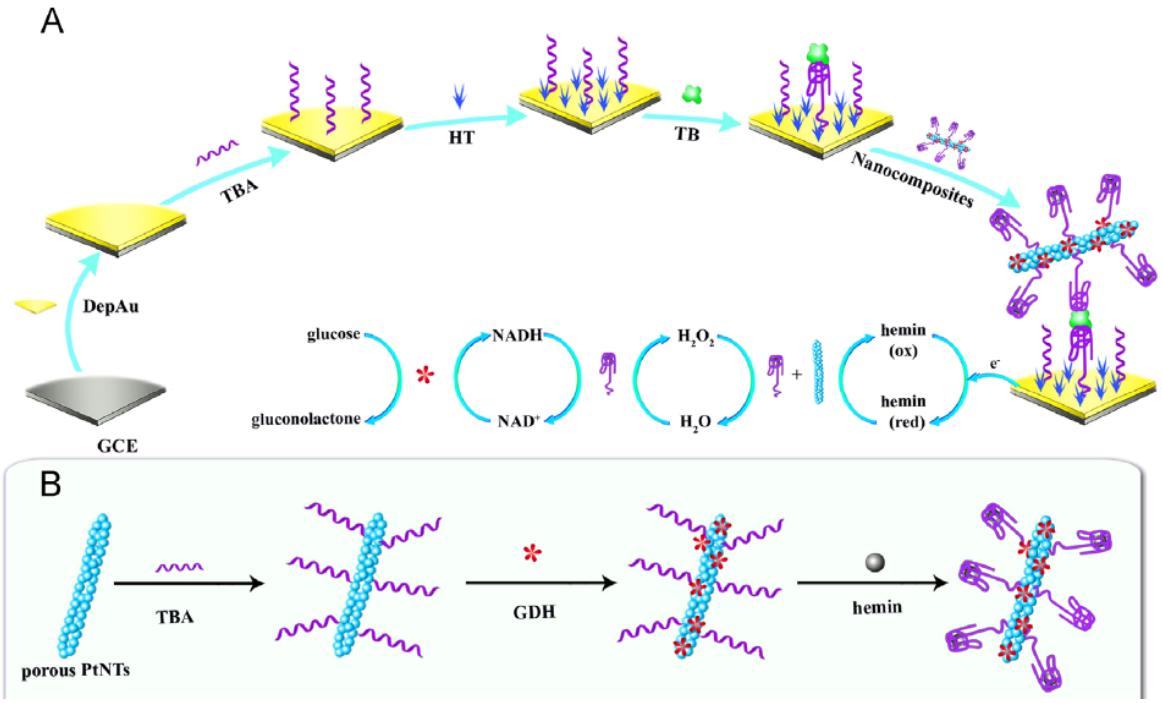

Figure 10. (A) Schematic diagram for the fabrication of the cascade amplified aptasensor based on hemin/G-quadruplex–GDH–PtNTs for sensitive TB detection. (B) Preparation of porous PtNTs labeled with hemin/G-quadruplex and GDH. (Reprinted with permission from [61]. Copyright 2014 Elsevier)

Among kinds of signal amplification approaches, polymerase chain reaction (PCR), rolling circle amplification (RCA), loop-mediated isothermal amplification (LAMP), nicking endonuclease

signal amplification, strand displacement amplification (SDA), hybridization chain reaction (HCR) and the isothermal exponential amplification reaction (EXPAR), the HCR and EXPAR attracted researchers' considerable attention to design sensitive assays for the analysis of targets. Recently, a new signal amplified strategy was also constructed in Yuan's group based on EXPAR and HCR generating the HRP-mimicking hemin/G-quadruplex nanowires as signal output component for the sensitive detection of thrombin (Fig. 11) [44]. The hemin/G-quadruplex structures simultaneously served as electron transfer medium and electrocatalyst to amplify the signal in the presence of H_2O_2. Specifically, the HCR was achieved and the hemin/G-quadruplex complexes were formed on the surface of an electrode to give a detectable signal after the EXPAR reaction process had occurred. The proposed strategy combined the amplification power of the EXPAR, HCR, and the inherent high sensitivity of the electrochemical detection.

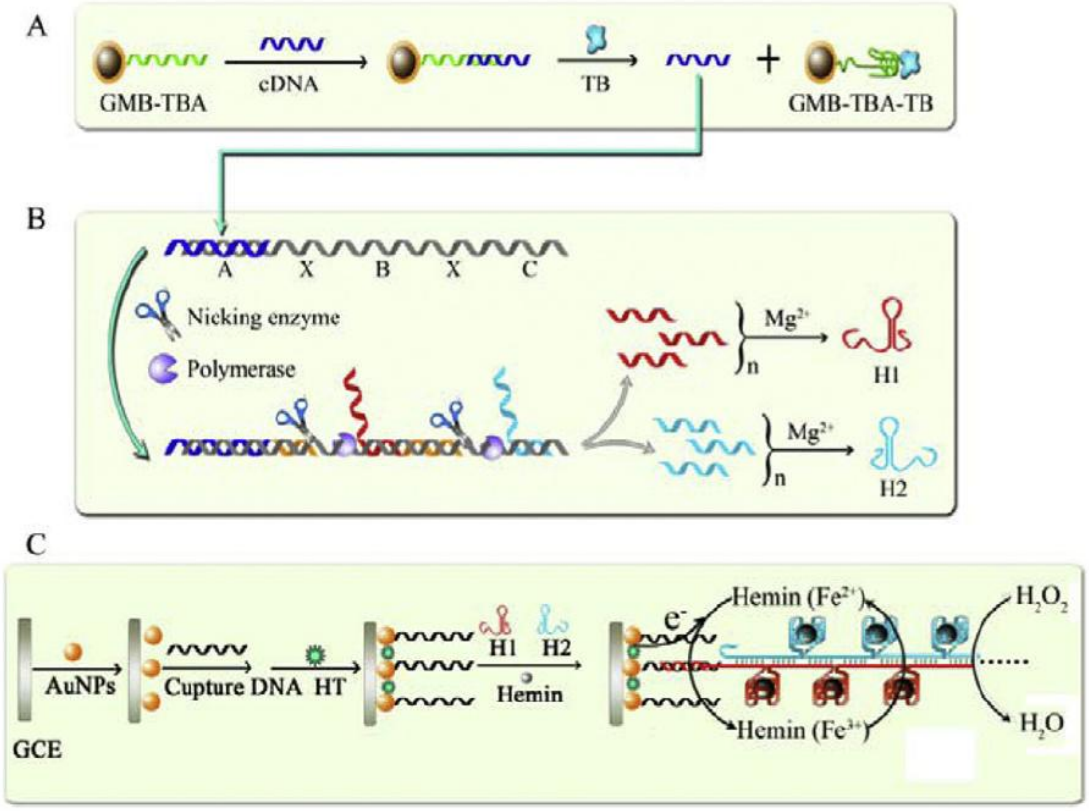

Figure 11. Schematic illustration of TB detection based on isothermal exponential amplification and hybridization chain reaction generating the hemin/G-quadruplex HRP-mimicking DNAzymes nanowires. (Reprinted with permission from [44]. Copyright 2014 Elsevier)

4. CONCLUSION

Hemin/G-quadruplex DNAzyme is a promising tool in the design of biosensors and chemical sensors because of its outstanding advantages over natural enzymes, such as thermal stability, high catalytic efficiency, easy preparation and modification, and lower cost than proteins. Over the past five years, it has been a constant source of inspiration for chemists in their efforts to develop hemin/G-

quadruplex–based electrochemical sensors in view of the advantages of electrochemical techniques. In this review, we have presented an overview of recent advances in the development of hemin/G-quadruplex–based electrochemical sensors. In particular, combining the advantages of DNAzyme and nanomaterials, electrochemical sensors with high sensitivity and selectivity were developed to operate in complex environments and detect targets at low concentrations. Although the applications of hemin/G-quadruplex DNAzyme are still at the basic research level, the rapid progress in the field and the multidisciplinary applications of these biocatalytic materials hold great promises for additional developments.

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