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Short review

Electrochemical MicroRNAs Biosensors Based on Enzymatic Signal Amplification

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Recently, microRNAs (miRNAs) have been considered as reliable molecular biomarkers for early diagnosis of various diseases because their aberrant expression has been correlated with cardiovascular, diabetes, tissue injury and cancer. Electrochemical biosensors have held great promise for point-of-care diagnostics because of their high sensitivity, simplicity and rapid response. Signal amplification is the commonly used strategy for constructing electrochemical biosensors with high sensitivity. Especially, enzymes-based signal amplification has great potential in practical clinical applications. In this review, we summarized latest developments in electrochemical biosensors for miRNAs detection with enzymatic signal amplification.

Keywords: microRNAs; electrochemical biosensors; enzymatic signal amplification

1. INTRODUCTION

MicroRNAs (miRNAs), a class of small non-coding regulatory RNAs molecules comprised of 19 - 25 ribonucleotides in length), were first identified in Caenorhabditis elegans by Lee et al. and named by Ruvkun in 2001 [1, 2]. They play vital roles in various fundamental biological processes such as cellular proliferation, cell cycle progression and apoptosis. MiRNAs have been recognized in a varied range of eukaryotic organisms of animal and plant; they suppress gene expression by binding to

the target mRNAs (messenger RNA) [3, 4]. Recently, miRNAs have been believed to be reliable molecular biomarkers for early diagnosis of various diseases because their aberrant expression has been correlated with cardiovascular, diabetes, tissue injury and cancer [5, 6]. The currently used methods for miRNAs detection include Northern blotting, real-time polymerase chain reaction (RT-PCR), microarrays, and so on [4, 7]. These methods are feasible, but are usually time-consuming, less sensitivity and require fluorescent- or radio-labeling and complicated instrumentations [8, 9]. Thus, simple and highly sensitive analytical techniques for miRNAs quantification are in demand. Electrochemical biosensors have held great promise for point-of-care diagnostics because of their high sensitivity, simplicity and rapid response. To improve the detection sensitivity, signal amplification by nanomaterials and enzymes is the commonly used strategy for constructing ultrasensitive electrochemical biosensors. On behalf of the advance of nanotechnology, nanomaterials-based miRNAs biosensors have been recently summarized in many review papers [10-13]. However, although nanomaterials-based biosensors are sensitive, their practical applications are still limited due to their time-consuming and costly preparation. Instead, enzymes-based signal amplification has great potential in practical clinical applications. To the best of our knowledge, there is no review focusing on the progress in designing of enzymes-amplified electrochemical miRNAs biosensors. In this review, we first summarized kinds of enzymatic amplification strategies for developing sensitive electrochemical miRNAs biosensors.

2. ENZYMES-AMPLIFIED ELECTROCHEMICAL STRATEGIES

There are also some attempts to fabricate electrochemical biosensors for quantification of low-abundance miRNAs. These biosensors are usually made of a solid electrode**,** especially gold electrode, with an immobilized short single-stranded nucleotide probe for hybridization with the complementary sequence. This event can be then converted into a useful electrical signal by an electrochemical transducer. Two main events in fabrication of electrochemical miRNAs biosensors are the recognition layer and the transducer that reports occurrence of recognition event. The hybridization event is usually distinguished through the increased or decreased current signal of the redox activity of the nucleic acid consequent from the duplex formation or a redox indicator associating with the newly formed surface hybrid, or from variations in electrochemical parameters such as conductivity or capacitance. Enzymatic amplification strategies for electrochemical detection of miRNAs were discussed herein, including horseradish peroxidase (HRP), glucose oxidase (GOx), alkaline phosphatase (ALP), hemin/G-quadruplex DNAzyme, duplex-specific nuclease (DSN), exonuclease, endonuclease polymerase, and so on.

2.1 Horseradish peroxidase

Among kinds of enzymes-amplified detection systems, horseradish peroxidase (HRP) that can catalyze the reduction of H_2O_2 is a widely used enzyme molecule for signal output. During the

detection assay, capture of miRNAs by the oligodeoxynucleotides (ODNs) self-assembled monolayers (SAMs)-covered electrode usually induces the structure change of detection probe and/or allows for the hybridization of signal probe, which further facilitates the attachment of HPR molecules. However, note that HRP itself cannot effectively transport electron with the SAMs-covered electrode due to the intercalation of its redox sites in the insulating peptide backbone. Thus, a redox mediator such as 3, 3', 5, 5'-tetramethylbenzidine (TMB) must be used to electronically "wire" the redox center for contacting and catalyzing the electro-reduction of H_2O_2. In this method, the electro-reduced TMB is oxidized by hemin in HRP; then H_2O_2 facilitates the regeneration of hemin from its reduction form, resulting in an observed electrocatalytic peak [14, 15].

In addition, the sensitivity of SAM-based electrochemical biosensors for detection of low abundance of miRNAs is still poor because of the short length and low melting temperature as well as the reduced accessibility of miRNAs to DNA target immobilized onto the heterogeneous and locally crowded electrode surface. For this reason, Fan's group presented a nanostructured electrode surface decorated with a three-dimensional (3D) DNA tetrahedral structure [16-18]. As shown in Fig. 1A, four DNA stands containing four thiol groups at one vertex were immobilized on Au surface through the Au-S interaction to form a tetrahedral DNA nanostructure. A capture probe appended to the other vertex of the tetrahedral DNA probe leaves a free-standing at the top for hybridization with part of the target miRNAs. A biotinylated DNA strand that is complementary to the other part of miRNAs was employed for the binding of avidin-HRP or poly-HRP80. The detection limit of this method was determined to be 10 fM. Molecular beacon composing of a hairpin-like DNA stem-loop structure can improve the sensitivity and selectivity for nucleic acid detection. Recently, Fan's group also developed an electrochemical miRNAs biosensor based on the tetrahedral DNA nanostructure, in which a hairpin-like DNA strand was used as the capture probe [19]. As shown in Fig. 1B, hybridization between the capture probe DNA and the target miRNAs opened the stem-loop structure, allowing for the introduction of biotinylated signal probe on the electrode surface. With HRP enzymatic amplification, a detection limit of 1 fM was achieved. The value is lower that that (10 pM) obtained by employing a ferrocene-labeled stem-loop structure for hybridization of miRNAs and electrochemical signal output [20]. To further improve the detection sensitivity, recently, they constructed an ultrasensitive electrochemical platform for miRNAs detection based on the tetrahedral DNA nanostructure and hybridization chain reaction (HCR) amplification [21]. As shown in Fig. 1C, a DNA hairpin structure strand (miRNA-helper) was first introduced to the nanostructure surface. The HCR reaction was then carried out by miRNA-initiated hairpin polymerization, in which two biotinylated hairpin DNA strands (H1 and H2) were employed as the fuel strands for the attachment of avidin-modified HRP. The detection limit for miRNA was found to be 10 aM, which is improved by 3 orders of magnitude in contrast to the widely used supersandwich amplification.

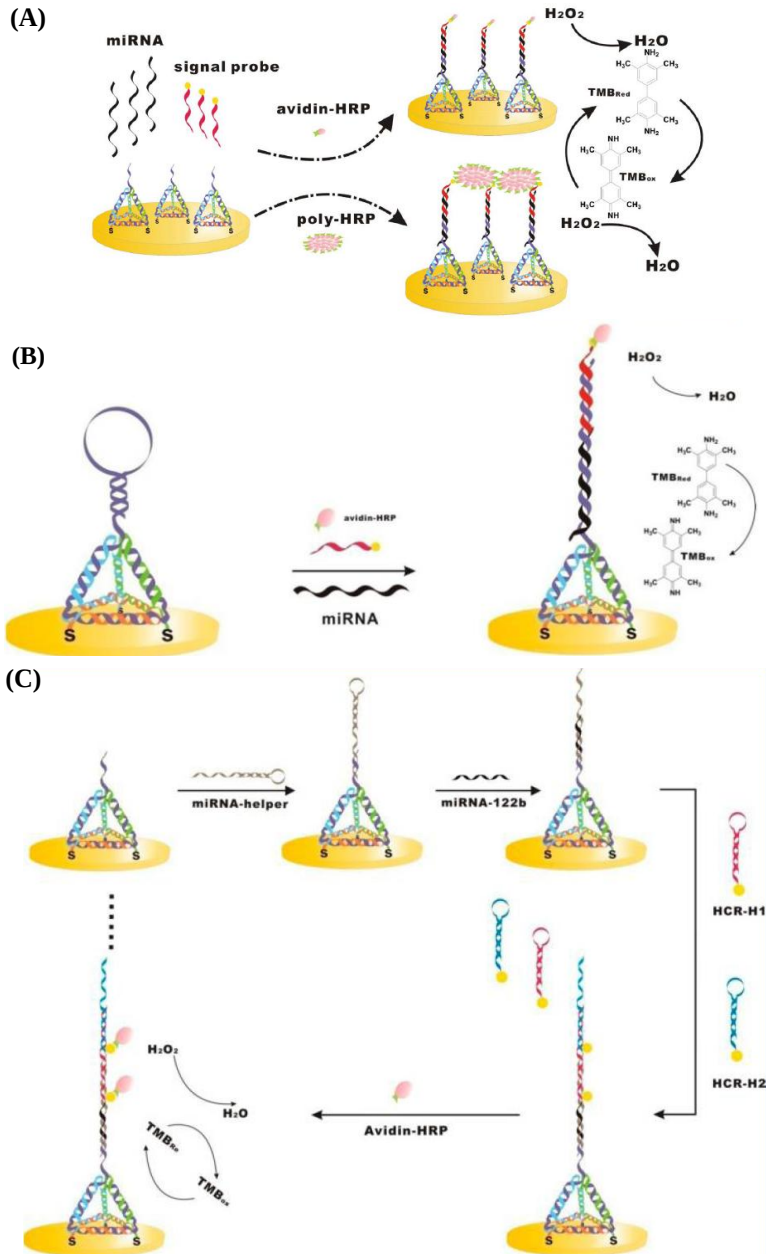

Figure 1. (A) Scheme for miRNA detection with the tetrahedron-based electrochemical miRNAs sensor (EMRS) with enzyme-based signal transduction (either avidin-HRP or high-activity poly-HRP80). Reprinted with permission from [17]. Copyright 2012 Nature. (B) The third generation of the E-DNA sensor, by controlling the surface immobilization of the stem-loop structure through the DNA tetrahedron. Reprinted with permission from [19]. Copyright 2014 ACS. (C) The detection principle is similar to what we used in DNA detection, whereas we introduce a DNA helper that can not only act as a bridge but also fold into a stem−loop structure itself to avoid nonspecific reaction. By taking advantage of the amplification via HCR amplification, we achieved ultrasensitive microRNA detection. Reprinted with permission from [21]. Copyright 2014 ACS.

Moreover, considering the advantages of strand displacement polymerization, catalytic recycling of miRNAs, and silver nanoparticle-based solid-state Ag/AgCl reaction, Miao et al. reported

a sensitive electrochemical miRNAs biosensor based on the tetrahedral DNA nanostructure with a pendant stem-loop (Fig. 2A) [22]. After the loop structure was opened by miRNAs, AgNPs-labeled signal probe was captured by the stem part. Then, Klenow fragment initiated the polymerization of signal probe. The lengthening of new strands therefore enforced the release of miRNAs, which facilitated the opening of more loops, the hybridization of more signal probes, and the occurrence of more strand displacement polymerization reactions. Based on the electro-oxidation signal of AgNPs via a solid-state Ag/AgCl reaction, the detection limit was found to be below 0.4 fM. Furthermore, they presented a highly sensitive electrochemical method for detection of miRNAs based on the tetrahedral DNA nanostructure and rolling circle amplification (RCA) amplification by phi29 DNA polymerase (Fig. 2B) [23]. AgNPs attached to the RCA products produced an enhanced electrochemical signal. The detection limit of 50 aM is lower than that of their first method.

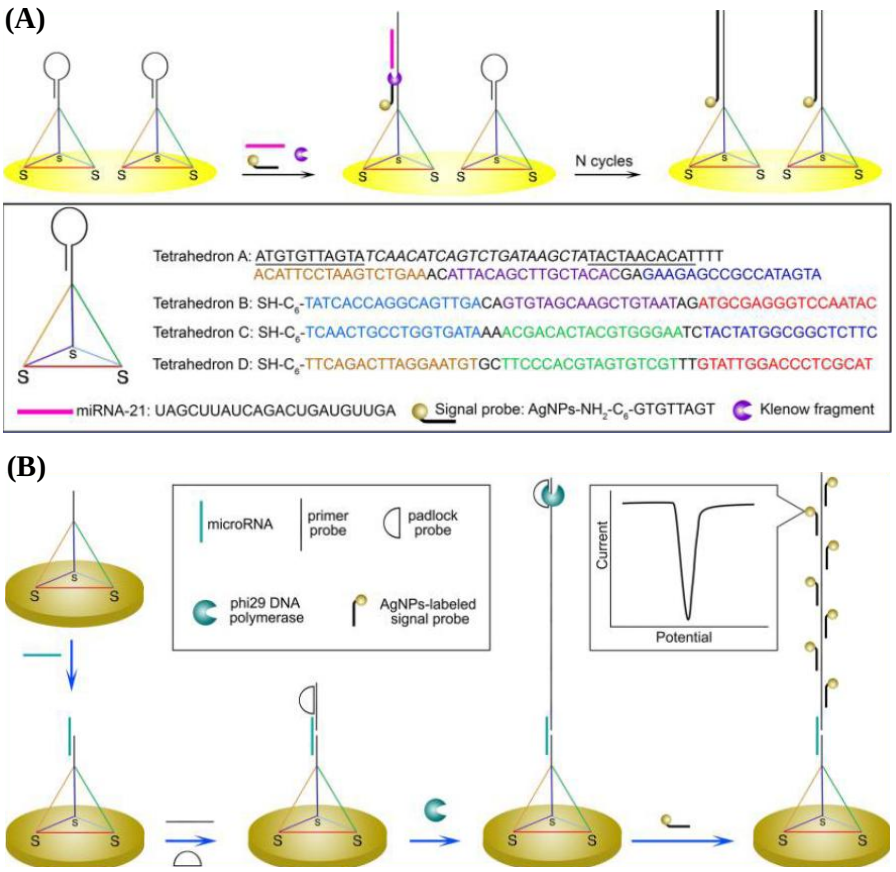

Figure 2. (A) Schematic illustration of the tetrahedral DNA nanostructure-based microRNA biosensor. Reprinted with permission from [22]. Copyright 2015 ACS. (B) Scheme of the RCA-based microRNA assay. Reprinted with permission from [23]. Copyright 2015 ACS.

With negatively charged nucleic acids as the templates, HRP can promote the deposition of polyaniline (PAn) nanowires or insulating poly(3,3′-dimethoxybenzidine) (PDB) polymer film. Thus, Gao's group presented several electrochemical miRNAs biosensors by the miRNAs-guided deposition of PAn nanowires and insulating PDB polymer film with HPR-mediated enzymatic polymerization reactions [24-26]. In these works, the electrostatic interaction between negatively charged phosphate

groups and positively charged aniline or 3,3′-dimethoxybenzidine (DB) is contributed to the formation of nanowires or polymer film. To take one example, neutrally charged morpholino capture probes were first immobilized onto an indium tin oxide (ITO)-coated glass slide (Fig. 3) [25]. The introduction of negatively charged miRNAs strands by hybridization converted the neutral surface to anionic surface, which facilitated the HPR-mediated polymerization of DB and formation of insulating PDB polymer films in the presence of H_2O_2. The signal change was then monitored by electrochemical impedance spectroscopy. Because of the high affinity of morpholino to miRNAs and the inaccessibility of DB to morpholino, the method exhibits high sensitivity with a detection limit of 2 fM.

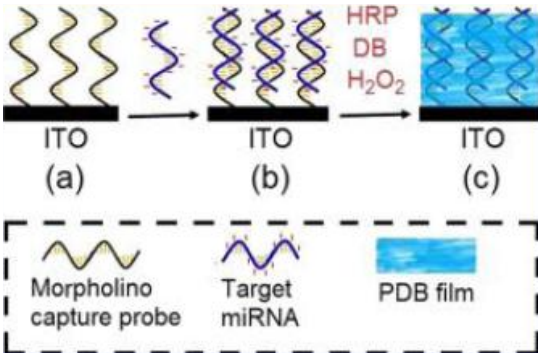

Figure 3. Schematic illustration of the working principle of the labelfree miRNA biosensor. Reprinted with permission from [25]. Copyright 2013 ACS.

To enhance the detection sensitivity, large amounts of enzymes are usually anchored on nanomaterials, such as gold nanoparticles (AuNPs) and magnetic Beads. Multienzyme report probes have been prepared by bioconjugating a large number of HRP molecules on AuNPs for signal amplification. These works have been summarized in the recent review of nanomaterials-based electrochemical miRNAs biosensors[12]. In this work, we did not introduce the electrochemical miRNAs biosensors employing enzyme-loaded nanomaterials as the signal reporters.

2.2 Glucose oxidase

Glucose oxidase (GOx) is an oxido-reductase that catalyses the oxidation of glucose to H_2O_2 and D-glucono-δ-lactone, which has also been widely used as an electrochemical reporter. Because GOx cannot effectively transfer electron from the redox centers $FADH_2$ in GOx to the electrode, the enzyme is usually electrochemically activated via covalent coupling of a redox mediator, such as $Os(bpy)_2(API)Cl$ (bpy = 2,2'-bipyridine, API = 3-aminopropylimidazole). Typically, using $Os(bpy)_2(API)Cl$-activated GOx, Gao's group reported two electrochemical miRNAs biosensors. In their first work, a DNA capture probe comprising of a miRNAs-binding segment and a detection probe-binding segment was immobilized on gold electrode [27]. If the capture probe did not hybridize with miRNAs target or hybridize with mismatched miRNAs strand, the probe would be digested by exonuclease I. However, miRNAs-hybridized probe remained intact in the presence of exonuclease I, which allowed for the attachment of activated GOx-modified peptide nucleic acid detection probe to

the bottom segment of the capture probe. The number of GOx was correlative to the level of target miRNAs. The activated GOx showed excellent catalytic activity towards electro-oxidation of glucose. As a result, a detection limit of 10 fM was achieved. In their second work, a stem-looped capture probe comprising of a miRNAs-binding segment and a detection probe-binding segment was used [28]. After hybridization with target miRNAs, the stem-loop was opened, releasing the detection probe-binding segment for the attachment of activated-GOx-tagged detection probe to the electrode surface. The detection limit of 4 fM is lower than that of their first method.

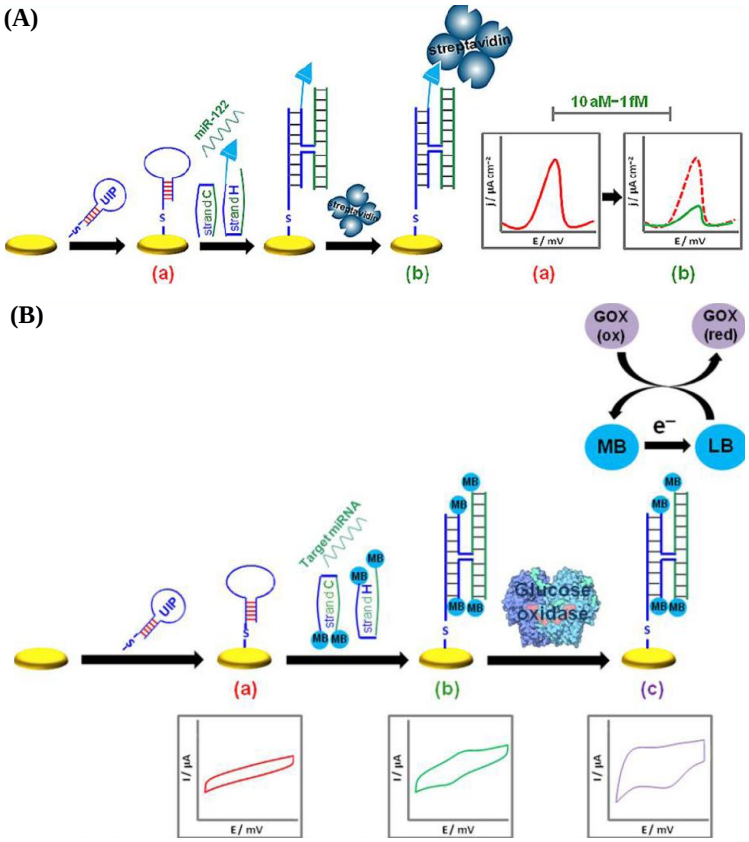

Figure 4. (A) Schematic representation of the 4J-SENS for detection of miR-122. (a) A thiol-modified UIP probe was self-assembled onto the electrode surface followed by backfilling the unoccupied spots with 2-mercaptoethanol. Afterward, the sensor was incubated with a mixture of strand C, biotin-labeled strand H, and miR-122. (b) Incubation of the sensor with streptavidin causes a decrease in the current density, measured by square wave voltammetry (SWV). Reprinted with permission from [29]. Copyright 2013 ACS. (B) Schematic representation of a protein-facilitated electrocatalytic quadroprobe sensor (SensPEQ) for detection of microRNAs. (a) Thiolmodified universal interfacial probe (UIP) was self-assembled onto the electrode surface followed surface backfilling with 2-mercaptohexanol. (b) Incubation of the sensor with a mixture of the target microRNA and the methylene blue (MB) labeled adaptor strands, namely C and H strands, results in the formation of a characteristic redox peak measured by cyclic voltammetry (CV). (c) CV in the presence of glucose oxidase showing the amplification of the MB reductive peak resulting from electrocatalysis. Reprinted with permission from [30]. Copyright 2015 ACS.

Recently, Berezovski's group developed two four-way junction based electrochemical sensors (4J-SENS) for miRNAs detection [29, 30]. In their first work, the 4J-SENS consists of a universal interfacial probe (UIP) and two DNA adaptor strands (strand C and biotinylated strand H) that cooperatively hybridize with both UIP and target (Fig. 4A) [29]. Hybridization of target miRNAs to both strand C and strand H facilitated the hybridization of surface-bound UIP to strand C and strand H, thus leading to the formation of a quadripartite complex with a four-way junction structure. Then, a large streptavidin (SA) molecule was captured by the strong biotin-streptavidin interaction, which caused an increase in the interfacial resistance of the sensing surface. In contrast, without target miRNAs, strand C and strand H did not interact with UIP since the UIP stem-loop structure is more thermodynamically stable than the associate with the adaptor strands. The detection limit of this method was found to be 2 aM. Based on the four-way junction structure, recently, they also suggested a protein-facilitated electrocatalytic quadroprobe sensor (SensPEQ) for miRNAs detection (Fig. 4B) [30]. In this work, the two DNA adaptor strands were modified with four methylene blue (MB) molecules and target miRNAs triggered the capture of the MB-tagged adaptor strands by UIP. The MB tags on the electrode surface effectively facilitated the electron transfer from $FADH_2$ in GOx to the electrode, that is, the electrochemically reduced MB (leucomethylene blue, LB) was oxidized by GOx, which led to the increase in the reduction current of MB.

2.3 Alkaline phosphatase

Differing from HRP and GOx, alkaline phosphatase (ALP) is a non-redox enzyme. It has also been commonly applied as the enzymatic label in electrochemical sensing since it can remove phosphate group from a substrate to generate an electroactive species. Using ALP as the reporter, several groups have reported the detection of miRNAs in a sandwich-like detection format [31-34]. Typically, anti-DNA:RNA antibody S9.6 exhibits high specificity and affinity for DNA:RNA hybrids but not for single-stranded DNA or double-stranded DNA and RNA [35]; Wang et al. presented a S9.6 antibody-based biosensor for miRNAs detection by ALP catalytic signal amplification [33]. In this work, the S9.6 antibody was first captured by the DNA/miRNA hybrids. Then, ALP-conjugated anti-IgG was further attached onto the electrode surface via the specific interaction of these two antibodes. As a result, ALP promoted the production of p-nitrophenol from p-nitrophenyl phosphate, leading to an electrochemical signal. However, single amplification by enzyme is les sensitive for determination of ultra-low content of miRNAs and ALP-based amplified strategy suffers from drawback due to the less stability of enzymatic products in air [36, 37]. To overcome these defects, Liu's group developed two types of ALP-based electrochemical biosensors using electrochemical-chemical (EC) and electrochemical-chemical-chemical (ECC) redox cycling, respectively [38-41]. In the EC detection system, reducing agents are added not only to prevent the auto-oxidation of enzymatic product, such as *p*-aminophenol (*p*-AP), but also regenerate *p*-AP from p-quinone imine (QI, the oxidation production of *p*-AP) in the electrochemical scanning. Based on the triple signal amplification of AuNPs, ALP and EC redox cycling as well as the differences in the structure of RNA and DNA, they reported a label-free and sensitive electrochemical method for miRNAs detection (Fig. 5A) [42]. Specifically, miRNAs

was first captured by the DNA probes on the electrode surface, which allowed for the attachment of 3-aminophenylboronicacid (APBA)/biotin-modified multifunctional AuNPs (APBA-biotin-AuNPs) through the formation of boronate ester covalent bonds. Then, SA-ALP conjugates were immobilized through the biotin-streptavidin interaction. After addition of p-aminophenyl phosphate (*p*-APP), the enzymatic conversion from *p*-APP to *p*-AP proceeded. The generated *p*-AP was cycled by tris(2-carboxyethyl)phosphine (TCEP) after its electro-oxidization, thus causing an increase in the anodic current. The detection limit of this method was found to be 3 fM. The method is sensitive, but its practical application is limited because of the complicated detection procedure and time-consuming preparation of labeled AuNPs. Furthermore, they presented a simple and ultrasensitive electrochemical biosensor for miRNAs detection based on ALP amplification and ECC redox cycling (Fig. 5B) [43]. After the hairpin structure of biotinylated DNA immobilized on gold electrode was opened by the target miRNAs, the biotin tag in DNA was forced away from the electrode surface, which allowed for the attachment of SA-ALP. After addition of L-ascorbic acid 2-phosphate (AAP), the enzymatic conversion from AAP to ascorbic acid (AA) proceeded, which triggered the ECC redox cycling. In this method, ferrocene methanol (FcM) and TCEP were used as the redox mediator and the chemical reducing reagent, respectively. The detection limit of this method was estimated to be 0.2 fM.

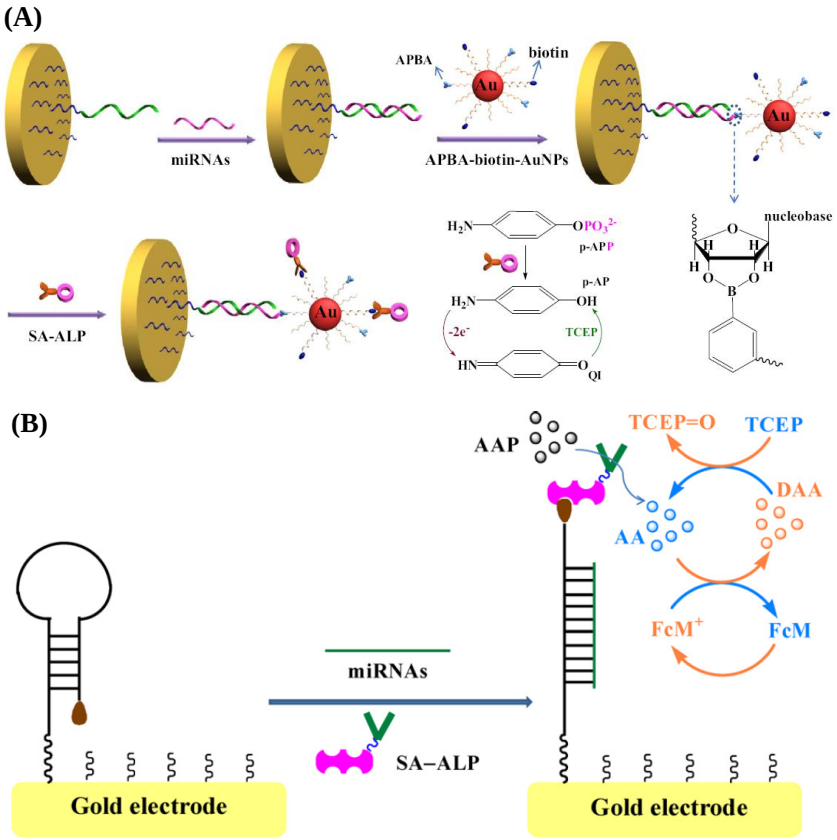

Figure 5. (A) Schematic representation of the label-free detection of miRNAs based on the triple signal amplification of APBA–biotin–AuNPs, SA–ALP and the p-AP redox-cycling reaction. Reprinted with permission from [42]. Copyright 2014 Elsevier. (B) Schematic illustration of the strategy for miRNAs detection using the hairpin DNA probe based on the signal amplification of ALP and ECC redox cycling. Reprinted with permission from [43]. Copyright 2015 Elsevier.

Moreover, Si et al. reported an ultrasensitive electrochemical method for miRNAs detection by signal amplification of gold nanoclusters (AuNCs)-incorporated ALP (ALP-AuNCs) catalytic silver deposition (Fig. 6) [44]. The incorporation of AuNCs dramatically enhanced the catalytic activity of ALP. The detection procedure included DNA/miRNA hybridization, ligase-catalyzed linking, ALP-AuNCs-catalyzed dephosphorylation, and silver deposition. Specifically, amino-silanized magnetic particles were first loaded with capture probe P1 to form Fe_3O_4-P1. Then, miRNAs were captured by Fe_3O_4-P1 and hybridized with ALP-AuNCs-P2. P1 and P2 were conjugated by the DNA ligase catalyzed linking. Finally, ALP-AuNCs hydrolyzed ascorbic acid 2-phosphate (AA-p) to generate AA and the produced AA acted as the reducing reagent for the reduction of Ag+, resulting in the deposition of Ag on the surface of AuNCs. The Ag-deposited Fe_3O_4 was immobilized on the magnetic electrode for linear sweep voltammetric measurement. The method facilitated the detection of miRNAs in blood with a detection limit of 21.5 aM.

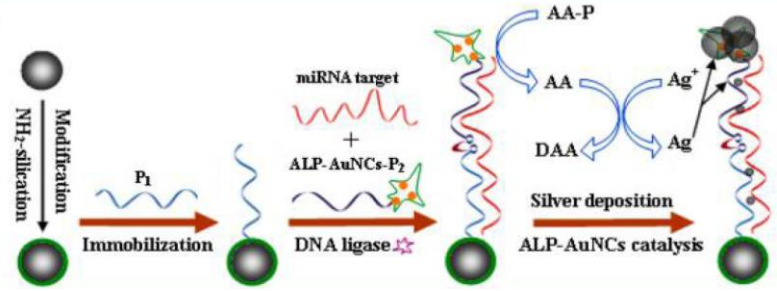

Figure 6. Schematic illustration of the principle for the sandwich-type electroanalysis of miRNAs using magnetic particles-loaded DNA capture Probe (Fe_3O_4-P1) and ALP-AuNCs-labeled DNA detection probe (ALP-AuNCs-P2), including the ligase-catalyzed linking of two DNA probes and the ALP-AuNCs-catalyzed silver deposition. Reprinted with permission from [44]. Copyright 2014 ACS.

2.4 Hemin/G-quadruplex DNAzyme

It has been suggested that guanine (G)-rich nucleic acid sequence can bind to hemin to form a hemin/G-quadruplex HRP-mimicking DNAzyme [45]. In 2010, Pelossof et al. first demonstrated that hemin/G-quadruplex DNAzyme could be used as an electrocatalytic label for amplified sensing events [46]. Recently, several groups have demonstrated the applications of hemin/G-quadruplex DNAzyme in designing electrochemical miRNAs biosensors [47, 48]. For example, Xiang et al. reported the detection of miRNAs based on endonuclease-aided target recycling and hybridization chain reaction [47]. In the work, hairpin-like capture probe containing a complementary RNA sequence of miRNAs was assembled onto AuNPs-modified electrode. After hybridization with miRNAs, the hairpin structure was opened. Then, ribonuclease A selectively cleaved the RNA segment of capture probe in duplexes, leaving the miRNAs intact for recycling. Finally, the cleaved single-strand DNA fragment remained on electrode surface and initiated hybridization chain reaction to form G-quadruplex regions. Consequently, hemin stacked into the regions to form hemin/G-quadruplex and produced amplified electrochemical signal. The biosensor showed high sensitivity with a detection limit of 0.1 pM. More

interestingly, Yu et al. developed an electrochemical biosensor based on an arched probe mediated isothermal exponential amplification reaction [48]. As shown in Fig. 7, the arched probe consists of two strands (Strand 1 and Strand 2) that are partially complementary at both ends. Strand 2 prevented the hybridization of primer with Strand 1; Strand 1 minimized the formation of hemin-binding G-quadruplex from Strand 2. The recognition site of nicking endonuclease was located in the loop region of Strand 2. Target miRNAs were 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. This biosensor shows high sensitivity toward miRNAs with a detection limits of 5.36 fM.

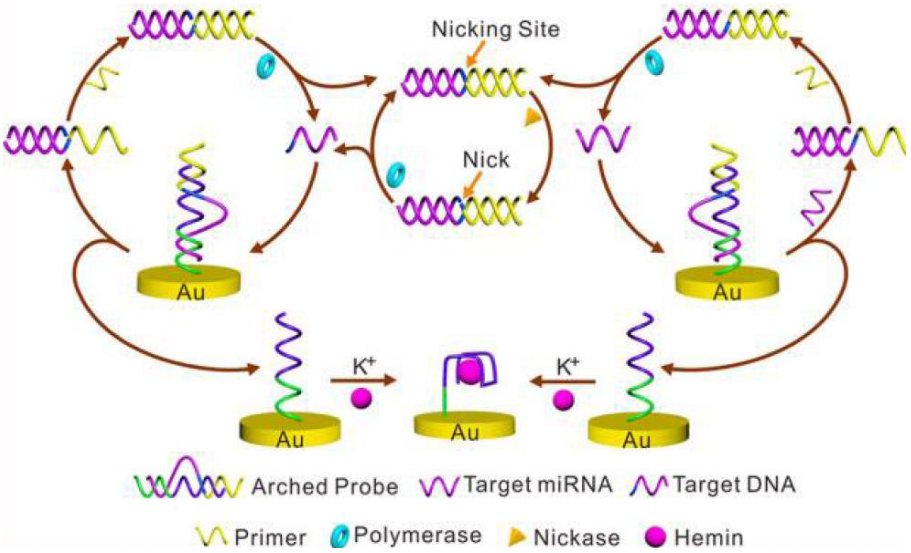

Figure 7. Schematic illustration of the arched probe mediated EXPAR strategy based on polymerase and nicking endonuclease for the miRNA assay. Reprinted with permission from [48]. Copyright 2014 ACS.

Hemin/G-quadruplex DNAzyme can catalyze the oxidation of aniline to polyaniline (PANI) in the presence of H_2O_2 [49]. Consequently, a readily measurable ‘‘turn-on’’ electrochemical signal could be observed. As mentioned above, nucleic acid guides the enzymatic deposition of nanoparticles and nanowires where phosphate group serves as the template. For this reason, Gao’s group constructed an impedimetric miRNAs biosensor with hemin/G-quadruplex DNAzyme as the catalyzer [50]. In this process, the DNAzyme promoted the formation of a thin PANI film on the miRNAs hybridized surface. Based on the increase in the electron transfer impedance, a detection limit of 0.5 fM was achieved. In addition, Wan et al. also presented the ultrasensitive impedimetric assays of miRNAs based on hemin/G-quadruplex-initiated formation of an insulating film [51]. In this work, the DNAzyme-capped AuNPs were anchored onto the electrode surface through a sandwich-like

hybridization reaction after the hairpin-like DNA probes pre-immobilized on the gold electrode were opened by miRNAs. The DNAzyme catalyzed the oxidation of 4-chloro-1-naphthol (CN) to an insoluble product. The insulating layer formed by the insoluble product on the electrode surface introduced a barrier for the electron transfer of redox probes, which caused an increase in the electron-transfer resistance. As a result, a detection limit of 15 aM was achieved.

2.5 Duplex-specific nuclease

Duplex-specific nuclease (DSN) is enzyme that shows a strong preference for cleaving double-stranded DNA or DNA in DNA/RNA heteroduplexes. This enzyme is practically inactive toward single stranded DNA, or single- or double-stranded RNA and shows a good capability to discriminate between perfectly and nonperfectly matched short duplexes. Ren et al. reported a DSN-based label-free electrochemical miRNAs biosensor (Fig. 8) [52]. Specifically, thiolated DNA caption probes were assembled onto the gold electrode through the formation of Au-S bound. After hybridizing with the target miRNAs, the DNA probes in the DNA/miRNA heteroduplexes were cut by DSN, releasing miRNAs back to the solution for recycled hybridization, thus forming an isothermal amplification cycle and causing the cleavage of more DNA probes. Based on miRNAs-induced change of electrochemical impedance, miRNA at the concentration down to femtomolar could be determined. Lately, Yang et al. reported a multiplexed electrochemical platform for simultaneous detection of miRNAs from different cancer cells based on redox labels with distinct voltammogram and DSN-assisted isothermal amplification cycle (Fig. 9) [53]. Thiolated redox-labeled hairpin probes were immobilized onto a gold electrode to produce two strong electrochemical signals. Hybridization of miRNAs with the pre-immobilized probes resulted in the formation of DNA/miRNA heteroduplexes. Cleavage of redox-labeled DNA probes in the duplexes DSN led to the release of miRNAs for recycling and great decrease in the peak currents. This method allowed for multiplexed detection of miRNA-141 and miRNA-21 down to 4.2 and 3.0 fM, respectively. Liu's group also developed a DSN-based electrochemical sensing platform for miRNAs detection based on signal amplification of ALP plus redox cycling reaction [54]. In the work, biotinylated DNA capture probes were assembled on a gold electrode for the attachment of SA-ALP. The captured ALP catalyzed the production of electroactive p-AP from p-APP. The formation of DNA/miRNA heteroduplexes led to the cleavage of probe DNA by DSN, making biotin tag stripping from the electrode surface and SA-ALP incapable of attachment onto the electrode. The results indicated that the oxidation current of p-AP decreased with the increasing miRNAs concentration. The detection limit of the "turn-off" electrochemical biosensor was 0.2 fM, which is lower that that obtained by the above two DSN-based electrochemical methods. Moreover, based on signal amplification of hemin/G-quadruplex DNAzyme and DSN-assisted target recycling, Zhang et al. presented a label-free electrochemical miRNAs biosensor, in which miRNAs at the concentration as low as 8 aM could be detected [55].

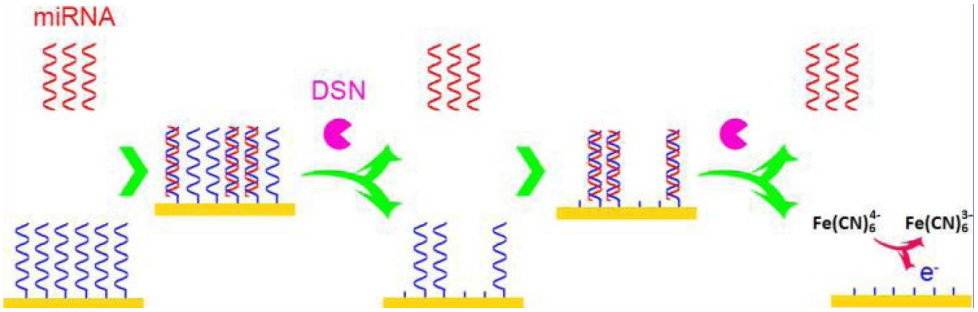

Figure 8. Schematic illustration of the working principle of the label-free electrochemical biosensor. Reprinted with permission from [52]. Copyright 2013 ACS.

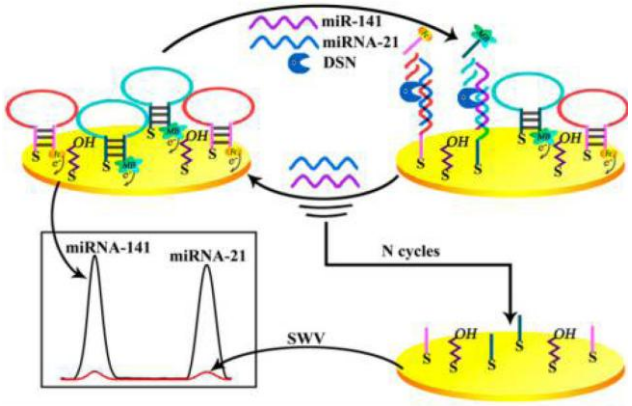

Figure 9. Schematic illustration of multiplexed and amplified electrochemical detection of miRNA-141 and miRNA-21 by coupling different redox labels with DSN assisted target recycling signal amplification. Reprinted with permission from [53]. Copyright 2014 ACS.

2.5 Other enzymatic amplification

T7 exonuclease is a sequence independent nuclease which catalyzes the removal of 5′ mononucleotides from the 5′ termini of double stranded but not single-stranded DNA. Moreover, it works on DNA or RNA in the RNA/DNA duplexes from 5′ to 3′, while it is not active on single stranded and double stranded RNA. In view of the special activity of T7 exonuclease, Wang et al. reported a label-free miRNAs biosensor based on the cyclic enzymatic amplification (Fig. 10) [56]. After hybridization of miRNAs with the DNA probes pre-immobilized onto the AuNPs-modified gold electrode, DNA in the miRNA/DNA duplexes was digested by T7 exonuclease, which resulted in the release of miRNAs from the electrode surface back to the solution. The released miRNAs further hybridized with DNA remained on the electrode surface. Because of the isothermal amplification cycle, a low detection limit of 0.17 fM was obtained by measuring miRNAs-induced change of electrochemical impedance.

Endonuclease is an enzyme that cleaves the phosphodiester bond within a polynucleotide chain. Recently, Miao et al. reported a sensitive electrochemical platform for miRNAs analysis using two DNA probes and one endonuclease [57]. Thiolated MB-labeled capture probe was first immobilized on

AuNPs-modified glassy carbon electrodes. Hybridization of capture probe (CP) to target miRNAs and auxiliary probe (AP) caused the formation of a star trigon structure on the electrode surface. Subsequently, endonuclease recognized and cleaved CP on the CP/AP duplex, resulting in the release of miRNAs and AP from the electrode surface back to the solution. The two regenerated strands further caused the formation of another star trigon with other CP molecules remained on the electrode surface. After multi-cycles, the electrochemical signal of MB decreased greatly. The proposed method showed a high sensitivity with a detection limit as low as 30 aM.

Polymerase is an enzyme that is responsible for the synthesis of long-chain nucleic acids. Peng et al. proposed a polymerase-based electrochemical strategy for miRNAs detection based on signal amplification of streptavidin-conjugated AuNPs (SA-AuNPs) and biotinylated ALP (biotin-ALP) [58]. Thiolated DNA probes were immobilized on a gold electrode for the capture of target miRNAs. The probes also acted as the primers and were extended along the template in the presence of DNA polymerase and dNTPs. A biotin tags were introduced into the duplexes by addition of biotin-11-dUTP in the solution. Then, biotin-ALP molecules were anchored on the electrode surface with SA-AuNPs as linkers, which enhanced the electrochemical signal. The detection limit of this method was determined to be 99.2 fM.

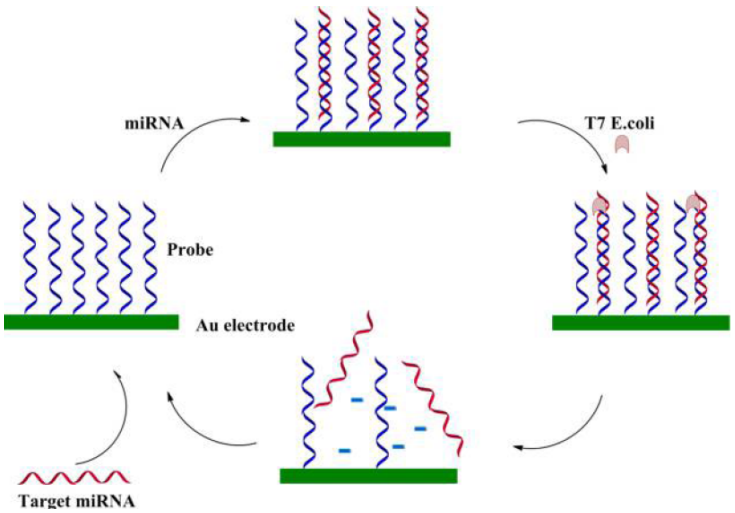

Figure 10. Biosensor Fabrication and miRNA Detection Principle Based on T7 Exonuclease Assisted Signal Amplification. Reprinted with permission from [56]. Copyright 2014 ACS.

4. CONCLUSION

MiRNAs play significant roles in developmental and physiological processes and have been considered as reliable molecular biomarkers for early diagnosis of various diseases. Thus, simple and highly sensitive analytical techniques for miRNAs quantification in complex samples are in demand. Recent studies demonstrated that electrochemical biosensors provide a number of advantages over traditional techniques. At present, signal amplification by enzymes is still the most popular strategy to improve the detection sensitivity, but there is no review paper to address the recent progress in designing of enzymes-amplified electrochemical miRNAs biosensors. In this review, enzymatic

amplification strategies for electrochemical detection of miRNAs were discussed, including HRP, GOx, ALP, hemin/G-quadruplex DNAzyme, DSN, exonuclease, endonuclease, polymerase, and so on. Moreover, we note that multiplexed detection of different samples with high-throughput assays in multichannel electrochemical cells would provide more reliable information for early diagnosis. This work should be valuable for the development of new types of biosensors and likely lead to many applications in clinical studies.

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References

1. R. C. Lee, R. L. Feinbaum, V. Ambros. *Cell* 75 (1993) 843.
2. G. Ruvkun. *Science* 294 (2001) 797.
3. M. Mori, R. Triboulet, M. Mohseni, K. Schlegelmilch, K. Shrestha, F. D. Camargo, R. I. Gregory. *Cell* 156 (2014) 893.
4. H. Dong, J. Lei, L. Ding, Y. Wen, H. Ju, X. Zhang. *Chem. Rev.* 113 (2013) 6207.
5. N. Xia, L. Zhang, G. Wang, Q. Feng, L. Liu. *Biosens. Bioelectron.* 47 (2013) 461.
6. S. S. Hebert, B. De Strooper. *Science* 317 (2007) 1179.
7. B. N. Johnson, R. Mutharasan. *Analyst* 139 (2014) 1576.
8. R. Tavallaie, S. R. M. De Almeida, J. J. Gooding. *WIREs Nanomed. Nanobiotechnol.* 7 (2015) 580.
9. C. D. Chen, M. La, B. B. Zhou. *Int. J. Electrochem. Sci.* 9 (2014) 7228-7238
10. F. Degliangeli, P. P. Pompa, R. Fiammengo. *Chem. Eur. J.* 20 (2014) 9476.
11. A. K. L. Teo, C. L. Lim, Z. Gao. *Electrochim. Acta* 126 (2014) 19.
12. N. Xia, L. Zhang. *Materials* 7 (2014) 5366.
13. M. Labib, M. V. Berezovski. *Biosens. Bioelectron.* 68 (2015) 83.
14. F. Wei, P. Patel, W. Liao, K. Chaudhry, L. Zhang, M. Arellano-Garcia, S. Hu, D. Elashoff, H. Zhou, S. Shukla, F. Shah, C.-M. Ho, D. T. Wong. *Clin. Cancer Res.* 15 (2009) 4446.
15. F. Wei, J. Wang, W. Liao, B. G. Zimmermann, D. T. Wong, C.-M. Ho. *Nucleic Acids Res.* 36 (2008) e65.
16. N. Lu, H. Pei, Z. Ge, C. R. Simmons, H. Yan, C. Fan. *J. Am. Chem. Soc.* 134 (2012) 13148.
17. Y. Wen, H. Pei, Y. Shen, J. Xi, M. Lin, N. Lu, X. Shen, J. Li, C. Fan. *Sci. Rep.* 2 (2012) 867.
18. Y. Wen, G. Liu, H. Pei, L. Li, Q. Xu, W. Liang, Y. Li, L. Xu, S. Ren, C. Fan. *Methods* 64 (2013) 276.
19. M. Lin, Y. Wen, L. Li, H. Pei, G. Liu, H. Song, X. Zuo, C. Fan, Q. Huang. *Anal. Chem.* 86 (2014) 2285.
20. S. Liu, W. Su, Z. Li, X. Ding. *Biosens. Bioelectron.* 71 (2015) 57.
21. Z. Ge, M. Lin, P. Wang, H. Pei, J. Yan, J. Shi, Q. Huang, D. He, C. Fan, X. Zuo. *Anal. Chem.* 86 (2014) 2124.
22. P. Miao, B. Wang, X. Chen, X. Li, Y. Tang. *ACS Appl. Mater. Interfaces* 7 (2015) 6238.
23. P. Miao, B. Wang, F. Meng, J. Yin, Y. Tang. *Bioconjugate Chem.* 26 (2015) 602.
24. Y. Fan, X. T. Chen, A. D. Trigg, C. H. Tung, J. M. Kong, Z. Q. Gao. *J. Am. Chem. Soc.* 129 (2007) 5437.

25. Z. Gao, H. Deng, W. Shen, Y. Ren. *Anal. Chem.* 85 (2013) 1624.
26. W. Shen, H. Deng, Y. Ren, Z. Gao. *Biosens. Bioelectron.* 44 (2013) 171.
27. Z. Gao, Y. Peng. *Biosens. Bioelectron.* 26 (2011) 3768.
28. Z. Gao. *Analyst* 137 (2012) 1674.
29. M. Labib, S. M. Ghobadloo, N. Khan, D. M. Kolpashchikov, M. V. Berezovski. *Anal. Chem.* 85 (2013) 9422.
30. M. Labib, N. Khan, M. V. Berezovski. *Anal. Chem.* 87 (2015) 1395.
31. T. Kilic, S. N. Topkaya, D. O. Ariksoysal, M. Ozsoz, P. Ballar, Y. Erac, O. Gozen. *Biosens. Bioelectron.* 38 (2012) 195.
32. Y. Zhang, Y. Yan, W. Chen, W. Cheng, S. Li, X. Ding, D. Li, H. Wang, H. Ju, S. Ding. *Biosens. Bioelectron.* 68 (2015) 343.
33. M. Wang, B. Li, Q. Zhou, H. Yin, Y. Zhou, S. Ai. *Electrochim. Acta* 165 (2015) 130.
34. S. Y. Wu, H. Chen, Z. Zuo, M. N. Wang, R. Luo, H. B. Xu. *Int. J. Electrochem. Sci.* 10 (2015) 3848.
35. A. J. Qavi, J. T. Kindt, M. A. Gleeson, R. C. Bailey. *Anal. Chem.* 83 (2011) 5949.
36. N. Xia, F. Ma, F. Zhao, Q. He, J. Du, S. Li, J. Chen, L. Liu. *Electrochim. Acta* 109 (2013) 348.
37. M. La, Y. X. Feng, C. Y. Yang, C. D. Chen. *Int. J. Electrochem. Sci.* 9 (2014) 6985.
38. L. Liu, Q. He, F. Zhao, N. Xia, H. Liu, S. Li, R. Liu, H. Zhang. *Biosens. Bioelectron.* 51 (2014) 208.
39. L. Liu, Y. Gao, H. Liu, J. Du, N. Xia. *Electrochim. Acta* 139 (2014) 323.
40. N. Xia, L. Liu, R. Wu, H. Liu, S.-J. Li, Y. Hao. *J. Electroanal. Chem.* 731 (2014) 78.
41. L. Liu, N. Xia, M. Jiang, N. Huang, S. Guo, S. Li, S. Zhang. *J. Electroanal. Chem.* 754 (2015) 40.
42. L. Liu, N. Xia, H. Liu, X. Kang, X. Liu, C. Xue, X. He. *Biosens. Bioelectron.* 53 (2014) 399.
43. N. Xia, Y. Zhang, X. Wei, Y. Huang, L. Liu. *Anal. Chim. Acta* 878 (2015) 95.
44. Y. Si, Z. Sun, N. Zhang, W. Qi, S. Li, L. Chen, H. Wang. *Anal. Chem.* 86 (2014) 10406.
45. G. C. Han, X. Z. Feng, Z. C. Chen. *Int. J. Electrochem. Sci.* 10: (2015) 3897.
46. G. Pelossof, R. Tel-Vered, J. Elbaz, I. Willner. *Anal. Chem.* 82 (2010) 4396.
47. G. Xiang, D. Jiang, F. Luo, F. Liu, L. Liu, X. Pu. *Sens. Actuators B: Chem.* 195 (2014) 515.
48. Y. Yu, Z. Chen, L. Shi, F. Yang, J. Pan, B. Zhang, D. Sun. *Anal. Chem.* 86 (2014) 8200.
49. N. Xia, L. Liu, Z. Sun, B. Zhou. *J. Nanomater.* 2015 (2015) 892674.
50. H. Deng, W. Shen, Y. Ren, Z. Gao. *Biosens. Bioelectron.* 60 (2014) 195.
51. J. Wan, X. Liu, Y. Zhang, Q. Gao, H. Qi, C. Zhang. *Sens. Actuators B: Chem.* 213 (2015) 409.
52. Y. Ren, H. Deng, W. Shen, Z. Gao. *Anal. Chem.* 85 (2013) 4784.
53. C. Yang, B. Dou, K. Shi, Y. Chai, Y. Xiang, R. Yuan. *Anal. Chem.* 86 (2014) 11913.
54. L. Liu, Y. Gao, H. Liu, N. Xia. *Sens. Actuators B: Chem.* 208 (2015) 137.
55. X. Zhang, D. Wu, Z. Liu, S. Cai, Y. Zhao, M. Chen, Y. Xia, C. Li, J. Zhang, J. Chen. *Chem. Commun.* 50 (2014) 12375.
56. M. Wang, Z. Fu, B. Li, Y. Zhou, H. Yin, S. Ai. *Anal. Chem.* 86 (2014) 5606.
57. P. Miao, B. Wang, Z. Yu, J. Zhao, Y. Tang. *Biosens. Bioelectron.* 63 (2015) 365.
58. Y. Peng, J. Jiang, R. Yu. *Anal. Methods* 6 (2014) 2889.