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Mini review

Electrochemical Biosensors for MicroRNA Detection using Duplex-Specific Nuclease based Signal Amplification Strategies

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MicroRNAs (miRNAs) play an important function in physiological and pathological processes. They have attracted extensive attention and become the important breakthrough in tumor diagnosis and clinical treatment. In recent years, many sensitive and accurate detection techniques in vitro have been proposed for the quantification of miRNAs. Duplex-specific nuclease (DSN) displays considerable cleavage preference for DNA in the DNA/RNA hybrid. This paper reviewed the progress in electrochemical detection of miRNAs based on the DSN-assisted signal amplification.

Keywords: microRNA; electrochemistry; duplex-specific nuclease; signal amplification

1. INTRODUCTION

MicroRNAs (miRNAs), a group of endogenous and non-coding RNAs, play an important function in physiological and pathological processes. Compared with traditional RNA, miRNAs can resist the degradation of ribonuclease A (RNase A), repeated freezing and thawing, and extreme pH conditions. Different diseases have different miRNAs expression profiles. Thus, miRNAs are a kind of potential biomarkers for the diagnosis of diseases [1]. At present, there is an urgent need for rapid, sensitive and specific detection of low-abundance miRNAs.

Since Heeger's group first reported the reagentless electrochemical genesensor, a variety of methods for detection of different sequence-specific nucleic acids have been developed [2, 3]. The conformational change of redox-labeled nucleic acids would lead to the increase or decrease in current due to the change of the distance between the labels and the sensor surface [4]. MiRNAs from different cancer cells can be simultaneously determined by employing electroactive labels with distinct

voltammograms. Although these approaches displayed some advantages, low sensitivity hampered their further applications. To address these challenges, plenty of signal amplification strategies have been designed with the aid of nanomaterials, enzymes or mimics and nucleases with different functions. Among them, duplex-specific nuclease (DSN), isolated from the Kamchatka crab by Shagin's group, displays considerable cleavage preference for DNA in the DNA/RNA hybrid and possesses good discrimination between perfectly matched and non-fully matched duplexes [5]. In recent years, many DSN-based signal amplification methods have been developed to detect miRNAs with high sensitivity, such as electrochemistry, colorimetry, fluorescence, surface enhanced Raman spectroscopy and so on [6-13]. This paper mainly focused on the recent progress in DSN-based electrochemical techniques for miRNAs detection.

2. DSN-BASED ELECTROCHEMICAL METHODS

2.1 Electrostatic interaction

Electrochemical impedance spectroscopy (EIS) is a simple, convenient and sensitive electrochemical technique for bioassays [14-16]. The phosphate groups in DNA or RNA can prevent the electron transfer of $[Fe(CN)_6]^{3-/4-}$ on electrode surface. Based on this fact, Ren et al. constructed a label-free DSN-based electrochemical biosensor for miRNA detection (Fig. 1) [17]. Firstly, the thiolated DNA capture probes were immobilized on a gold electrode to capture target miRNA. After DNA in the DNA/miRNA hybrid was cleaved by DSN, miRNA would be released to the solution and then hybridized with other DNA molecules immobilized on electrode surface, thus producing an isothermal amplification cycle. Moreover, Li and co-workers proposed an immobilization-free electrochemical strategy for the determination of miRNA let-7a [18]. In this work, 1, 6-hexanedithiol (HT)-modified gold electrode prevented the electron transfer of $[Fe(CN)_6]^{3-/4-}$. Adsorption of bare gold nanoparticles (AuNPs) on the HT-modified electrode surface facilitated the electron transfer. When the AuNPs were enclosed by DNA probes, they would lose the ability to bind the HT molecules. When the DNA probes were hybridized with target miRNA, AuNPs would be released and then adsorbed on the electrode to facilitate the electron transfer. This method achieved a detection limit of 16 fM.

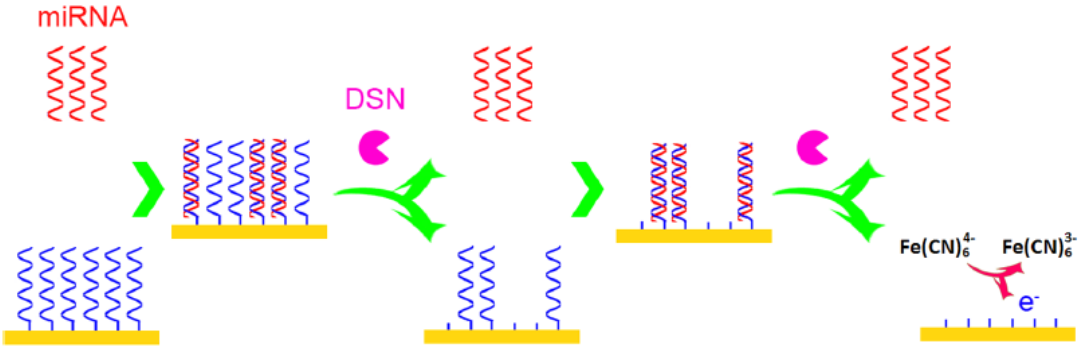

Figure 1. Schematic illustration of the working principle of the label-free electrochemical biosensor. Reprinted with permission from reference [17]. Copyright 2013 American Chemical Society.

As a common indicator of electrochemistry, positively charged $[Ru(NH_3)_6]^{3+}$ can interact with negatively charged phosphate groups in the DNA chain. Miao and co-workers reported an eletrochemical miRNA biosensor on the basic of DSN recycling amplification and DNA/AuNP recruitment [19]. The ssDNA1 probe on electrode surface can capture DNA2/AuNP, thus allowing for the adsorption of large numbers of $[Ru(NH_3)_6]^{3+}$ through the electrostatic interaction. When the ssDNA1 probe was hybridized with miRNA, it would be cleaved by DSN. In this case, DNA2/AuNP can not be captured by the sensor electrode. This method has a linear range of 0.1 fM ~ 100 pM and a detection limit down to 50 aM. Bo and co-workers reported a biometric solution for measuring miRNAs based on the triple signal amplification of DSN-assisted digestion and bridging DNA-AuNPs [20]. As shown in Fig. 2, three AuNPs can be linked by the bridge DNA to generate the bridging DNA-AuNPs. The hairpin-like DNA probe on electrode surface is unable to capture the DNA-AuNPs nanocomposites. When the hairpin was opened by hybridization with target miRNA, DNA in the DNA/RNA hybrid would be digested by DSN. The remained DNA segment on electrode surface can hybridize with the fourth sequence on the bridge DNA-AuNPs. The positively charged $[Ru(NH_3)_6]^{3+}$ can be captured by the DNA-AuNPs through electrostatic interactions, thus producing a high electrochemical signal. As a result, the method shows a wide linear range (10^{-17} ~ 10^{-11} M) and a low detection limit (6.8 aM).

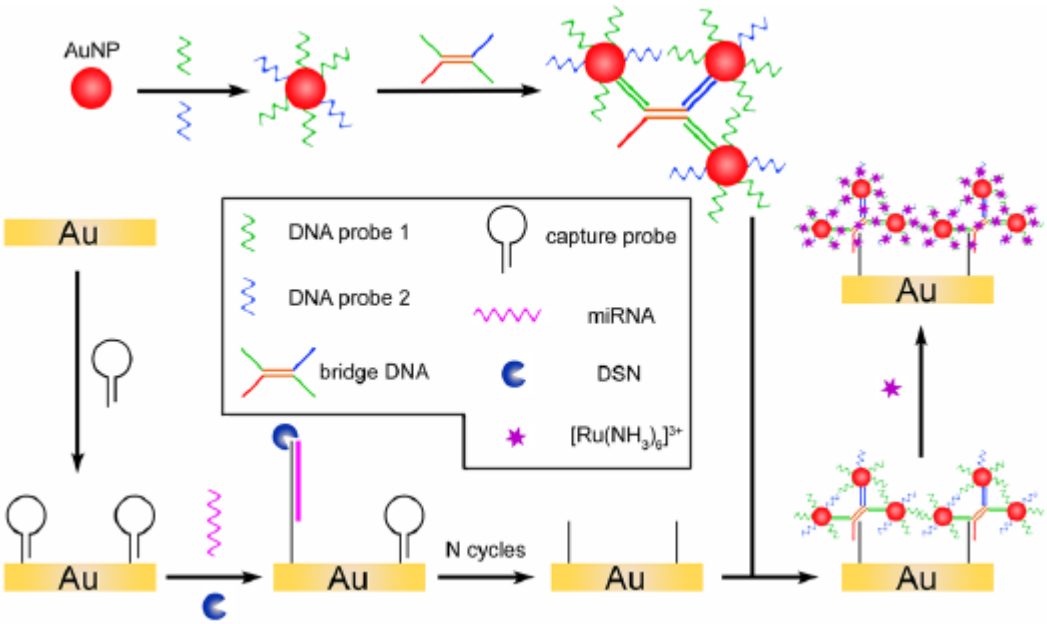

Figure 2. Illustration of the electrochemical approach for triple amplified detection of miRNA. Reprinted with permission from reference [20]. Copyright 2018 American Chemical Society.

Yu et al. proposed a sensing scheme for determining miRNA-141 (miR-141) based on cascading signal amplification (Fig. 3) [21]. The biosensor was established by two amplification parts: DSN-assisted target recycling and catalytic hairpin assembly (CHA). Many DNA connectors were generated after the target recycling process. Then, the CHA was initiated by the DNA connector with hairpin-AuNPs as the sensing units. The AuNPs networks on electrode surface facilitated the adsorption of $[Ru(NH_3)_6]^{3+}$ (RuHex). The detection limit is down to 25.1 aM. In addition, Guo et al. developed a selective and sensitive electrochemical biosensor to determine miRNA-196a by cyclic

enzymatic signal amplification (CESA) and template-free DNA extension reaction [22]. Benefiting from this dual amplification mechanism, the method shows a detection limit as low as 15 aM and a linear range of 0.05 fM ~ 50 pM.

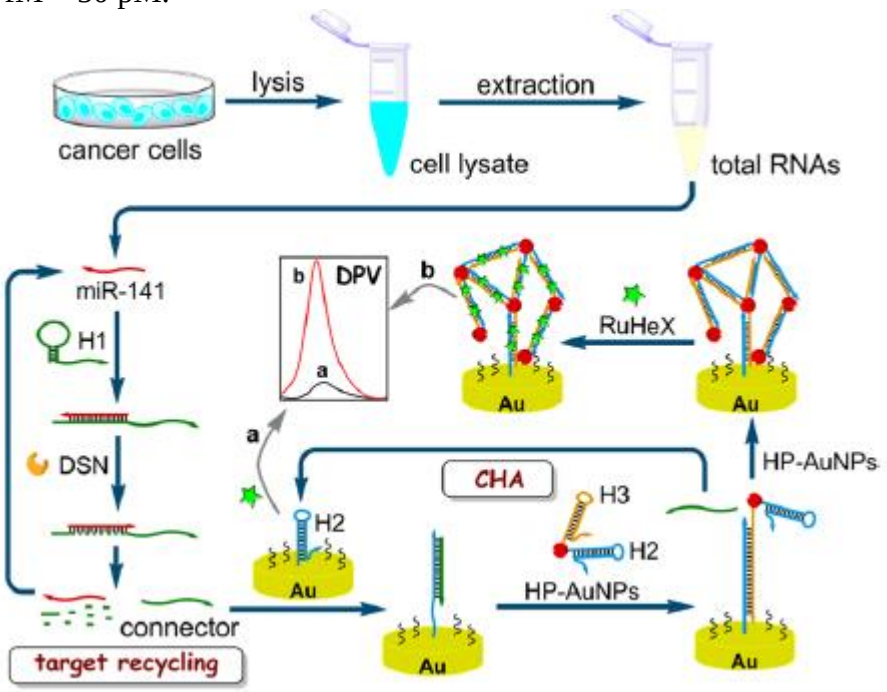

Figure 3. Schematic illustration of cascade amplification of DSN-assisted target recycling and CHA reaction. Reprinted with permission from reference [21]. Copyright 2018 American Chemical Society.

2. 2 Redox labels

Electroactive small molecules are the commonly used signal reporters to label recognition elements (e.g. DNA and antibody). Yuan's group reported a multiplexed electrochemical biosensor for the detection of miRNA-141 and miRNA-21 from cancer cells through the DSN-assisted target recycling signal amplification [23]. As illustrated in Fig. 4, thiolated hairpin probes labeled with methylene blue (MB) and ferrocene (Fc) were assembled on the gold electrode surface via the formation of Au-S bonds. In the absence of target miRNAs, the probes with hairpin structures kept intact and produced two distinguished redox peaks. Once the target miRNAs were added, the hairpin probes were opened and the DNA sequences in the DNA/RNA duplexes were cleaved by DSN. Then, the miRNAs were released and initiated cyclic cleavage of MB/Fc-labeled DNA probes, thus leading to the removal of massive MB/Fc labels from the sensing surface and resulting in remarkably suppressed current peaks at different potential positions. Detection limits of this method for miRNA-141 and miRNA-21 were estimated to be 4.2 and 3.0 fM, respectively. Recently, Wang and coworkers reported a DSN-assisted electrochemical biosensor for miRNA detection by using Au nanoparticles/carbon nitride nanosheet nanocomposite to modify the electrode for further improving the sensitivity [24]. By using DSN for signal amplification and positively charged AuNPs as the carriers of Fc-labeled DNA probes, we reported the electrochemical detection of miRNA-21 [25]. In short, DNA probes anchored on electrode surface can capture positively charged AuNPs and recruit a

large number of Fc-labeled DNA probes from solution through the electrostatic interactions, thus producing a strong electrochemical signal. When the DNA probes on electrode surface were hybridized with miRNA-21 and then digested by DSN, the assembly of AuNPs and Fc-labeled DNA probes on electrode surface was limited. This led to a significant decrease in the electrochemical signal. The current is inversely proportional to the content of miRNA in the range of 0 ~ 25 fM.

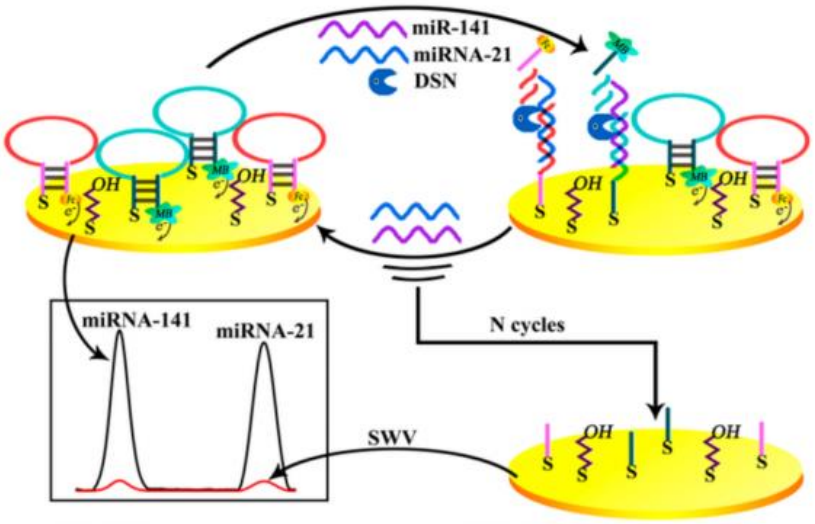

Figure 4. Schematic ilustration of multiplexed and amplified electrochemical detection of miRNA-141 and miRNA-21 by coupling different redox labels with DSN-assisted target recycling signal amplification. Reprinted with permission from reference [23]. Copyright 2014 American Chemical Society.

2.3 Enzymatic amplification

As we all know, enzyme labels are widely used in the field of bioassays because of their high efficiency, good specificity, and strong diversity. Enzymatic amplification strategies have been proposed for the determination of various target analytes [26, 27]. In recent decades, a large number of DSN-based miRNA biosensors have been developed with enzymatic signal amplification. For example, we developed a highly sensitive electrochemical miRNA biosensor by combining DSN-assisted target cycling strategy and ALP plus redox-cycling reaction [28]. Huang's group proposed a sensitive electrochemical biosensor for miRNA detection on basis of CHA and enzyme signal amplification [29]. Tungsten oxide (WO_3)-Gr composites were synthesized through a hydrothermal method and used as the electrode modifiers. The target miRNA could circularly trigger the hybridization of two hairpin-like DNA probes. Then, the captured alkaline phosphatase (ALP) would produce a large number of ascorbic acid (AA), inducing the electrochemical-chemical-chemical (ECC) redox cycling between ferrocene methanol (FcM) and tris (2-carboxyethyl) phosphine (TCEP) and generating a strong electrochemical response. At the same time, they also employed AuNPs/hollow molybdenum disulfide microcubes as the electrode substrate for miRNA detection coupled with DSN amplification and ALP-triggered ECC redox cycling [30]. Zhang's group integrated redox reaction of AA/iodine with DSN-assisted target recycling for miRNA-21 detection [31]. In this work, AA, the product of the ALP-catalyzed hydrolysis of ascorbic acid 2-phosphate (AAP), induced a redox reaction in the presence of iodine, leading to a strong electrochemical signal. This method exhibits a low

detection limit (34 aM) and good selectivity.

Aiming to enhance the ratio of enzyme to target in each hybridization, nanoparticles with high surface-to-volume ratio and excellent electrical properties were widely used to carry recognition elements and enzyme labels. Shen's group reported a triple signal amplification electrochemical biosensor for sensitive detection of miRNA-21 [32]. After biotin-labeled DNA probes were captured by the products of DSN cleavage, streptavidin (SA)-coated AuNPs were bound to the electrode surface by the specific biotin-SA interaction and then many biotin-labeled horseradish peroxidase (HRP) molecules were attached on the electrode surface. HRP-catalyzed reduction of H_2O_2 in the presence of 3,3',5,5' tetramethylbenzidine (TMB) generated an amplified electrochemical signal.

G-quadruplex-hemin DNAzyme with HRP-like activity can catalyze the H_2O_2-mediated oxidation or reduction and has been widely applied to construct various electrochemical biosensors. Based on 2'-O-methyl modified DNAzyme and DSN-assisted target recycling, Chen's group developed a label-free electrochemical biosensor for miRNA-21 detection [33]. In the presence of target miRNA, DSN-catalyzed hydrolysis caused the release of a large number of 2'-O-methyl modified G-rich sequences on the electrode surface. Then, the 2'-O-methyl modified G-rich sequences could bind hemin to form the DNAzyme and catalyze the oxidation between H_2O_2 and TMB accompanied by an increased electrochemical current signal. Gao's group reported a sensitive impedimetric detection of miRNAs based on multiple-DNAzyme-decorated AuNPs [34]. After the hybridization, DNAzyme catalyzed the biocatalytic oxidation of 4-chloro-1-naphthol (CN) to the insoluble products that deposited onto the electrode surface. The resulting insulating layer blocked the efficient electron transfer of redox mediators. Thus, the electron-transfer resistance (R_{et}) of the biosensor increased with the increasing amount of insoluble products, which is linearly related to the target concentration. Zhang's group combined tetrahedral DNA nanostructures with DSN and DNAzyme for miRNA-21 detection in serum (Fig. 5) [35]. In this method, three-dimensional (3D) DNA nanostructure was employed to modify the electrode to remain the binding ability with the target. DNAzyme catalyzed the reduction of H_2O_2 that was generated via the oxidation process of L-cysteine under aerobic conditions. However, in the presence of miRNA-21, the DSN-assisted hydrolysis of DNA/RNA would significantly reduce the current intensity. The biosensor exhibited a wide linear response (0.1 ~ 100 fM) and a low detection limit (0.04 fM). Recently, Zhou et al. designed a dual amplification strategy for the detection of miRNAs with click chemistry-mediated DSN-assisted target recycling and functionalized fullerene (FC60) [36]. In this experiment, amino- and thiol-labeled FC60 with a large surface active sites and better biocompatibility was employed to modify the gold electrode. The Cu(I)-catalyzed azide-alkyne cyclo-addition was used to connect two G-quadruplex-contained sequences. The dual amplification method exhibits a better analytical performance than that of the method without FC60. The linear range was found to be 0.1 pM ~ 100 nM.

Xie's group used double-loop hairpin (DHP) probe and doxorubicin-loaded AuNPs to develop an electrochemical miRNA detection method [37]. DHP consisted of a miRNA recognition sequence, an output segment and output's complementary fragment protected MB from digestion and improved the discrimination of miRNA. AuNPs were employed to load doxorubicin (Dox, an electroactive indicator) and DNA to magnify the electrochemical signal and improve the sensitivity. After the hybridization and hydrolysis, Dox-AuNPs were liberated away from the electrode, resulting in a

decreased current. Owing to the large surface area and good electronic transport properties, carboxylate-reduced graphene oxide (COOH-rGO) was also employed by Huang's group to load electroactive MB molecules through the strong electrostatic attraction [38]. Meanwhile, COOH-rGO stacked on the cDNA sequences by the π–π stacking interaction. Thus, under the DSN-assisted target recycling, a lot of cDNA probes were cleaved and the amount of MB-COOH-rGO captured on the electrode decreased, which was accompanied by the decrease of the DPV signal. Kong's group proposed a double signal amplification strategy for ultrasensitive electrochemical detection of miRNA based on DSN and quantum dot-DNA nanocomposites [39]. After the DSN hydrolysis, triple CdTe-labeled DNA nanocomposites were bound to the electrode, producing a strong DPV signal.

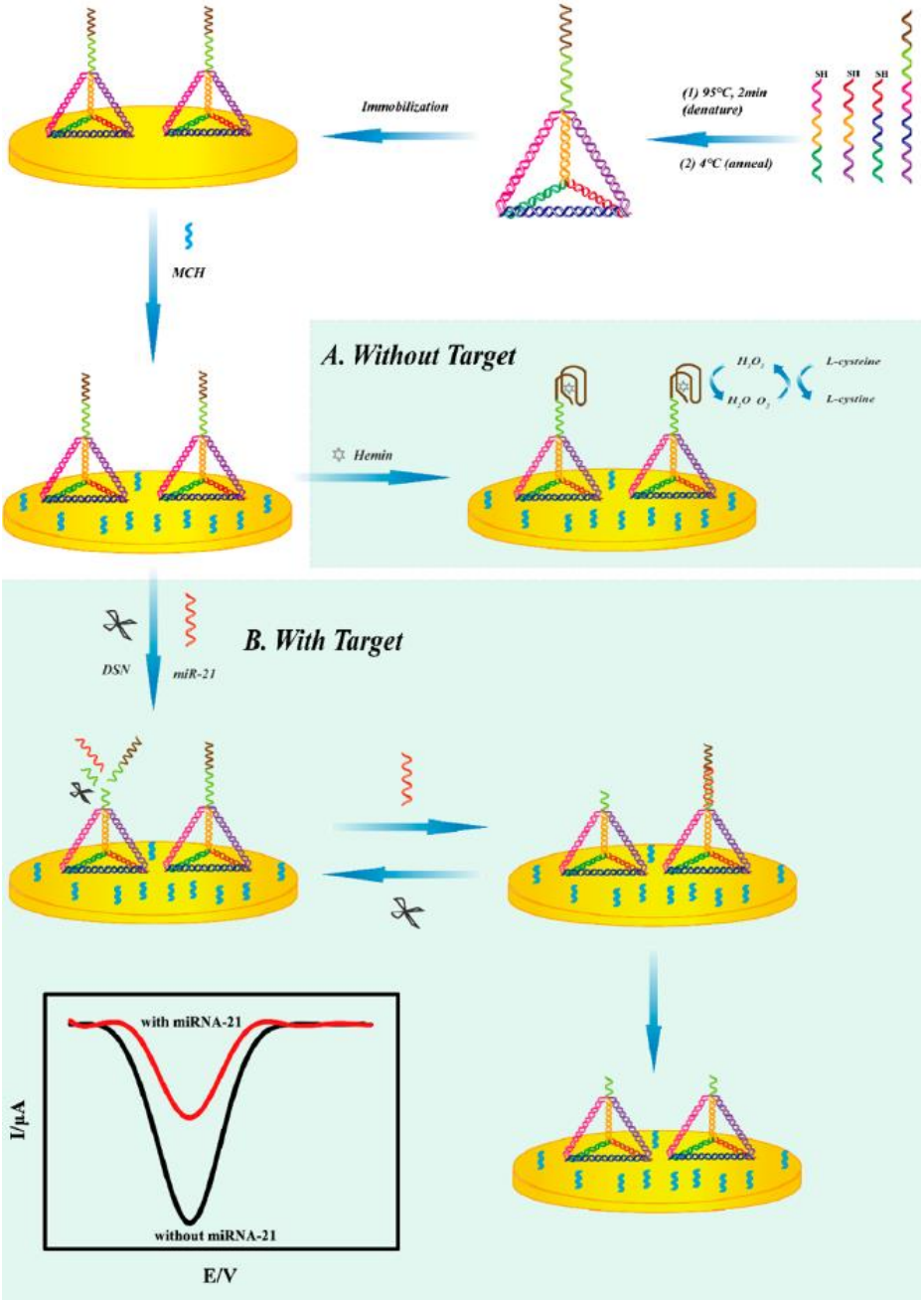

Figure 5. Schematic illustration of the electrochemical biosensor for the detection of miRNA-21 based on tetrahedral DNA nanostructures and G-quadruplex-hemin conformation. Reprinted with permission from reference [35]. Copyright 2019 American Chemical Society.

Che's group developed a dual-mode biosensor for miRNA detection by using copper- and cobalt-codoped CeO_2 (CuCo-CeO_2 NSs) nanospheres as highly efficient electrocatalysts (Fig. 6) [40]. In this work, CuCo-CeO_2 NSs enhanced the redox signal and electrocatalytic properties because of their numerous extra oxygen vacancies. In the presence of miRNA-141, the hairpin probe was transformed into the "active" DNAzyme and then specifically cleaved by Mg^{2+} after the hybridization. Like DSN-assisted target cycling amplification, the released miRNA-141 could bind to other hairpin probes to drive another cycle of activation and cleavage. Finally, a large amount of DNA fragments were released from the electrode surface and numerous DNA-labeled CuCo-CeO_2 NSs were immobilized. Due to the improved electrocatalytic activity toward H_2O_2, CuCo-CeO_2 NSs could directly act as the redox mediators, generating a detectable electrochemical signal by DPV. Besides, CuCo-CeO_2 NSs with the superior electrocatalytic activity promoted the oxidization of DAB into insoluble precipitates (IPs), leading to the increase of R_{et}. The detection limit for both DPV and EIS methods was 33 aM, which is lower than the conventional single sensing methods.

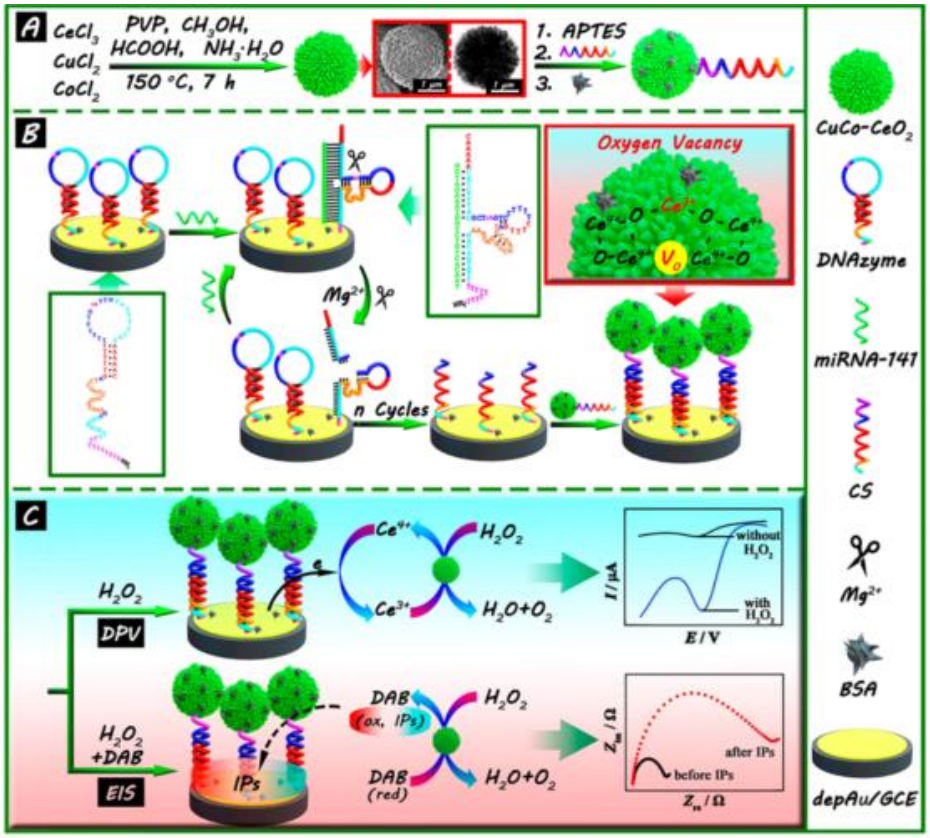

Figure 6. Schematic illustration of (A) synthesis of the CuCo-CeO_2 NSs, (B) Mg^{2+}-induced DNAzyme-assisted target recycling amplification strategy and (C) dual-mode sensing principle for miRNA-141 determination. Reprinted with permission from reference [40]. Copyright 2019 American Chemical Society.

2.4 Immobilization-free methods

Although electrochemical biosensors have made great progress in the detection of miRNAs, most of the electrochemical methods are heterogeneous and require the immobilization of precursors

on electrode surface [41]. However, there are still some problems in the immobilization-based strategy such as tedious process and long time. Because the recognition reaction takes place between the solution and the electrode interface, the steric hindrance of electrode surface and the decrease of the degree of freedom of precursor molecular configuration make the heterogeneous biosensors have low binding efficiency and poor enzymatic kinetics. For this view, a few immobilization-free methods have been developed for miRNA detection based on the signal amplification of DSN. For example, Castañeda and co-workers proposed a simple method for miRNA detection through the PtNPs-electrocatalytic amplification (Fig. 7) [42]. The PtNPs were passivated when they were modified with the DNA probes. Once the probes were hybrized with target miRNA, DNA in the DNA/RNA hybrids would be digested in the presence of DSN, which resulted in the exposure some PtNP surface and the reactivation of the electrocatalytic properties. This work also provided an opposite example that electrocatalytic amplification with DNA-protected metal nanoparticles is ineffective for biosensing applications. Moreover, Wang et al. have constructed an electrochemical biosensor on the basis of interaction of DNA-modified AuNPs and silver nanoparticles (AgNPs) [43]. DNA probes on the surface of AuNPs can hybridize with target miRNA and then be digested cyclically by DSN. AgNPs can be captured by the pure AuNPs-modified electrode due to the elimination of electrostatic repulsion and steric hindrance effect. The detection of miRNA was achieved by silver stripping peak. A detection limit of 0.62fM was obatined with a linear range of 1 ~ 1000 fM.

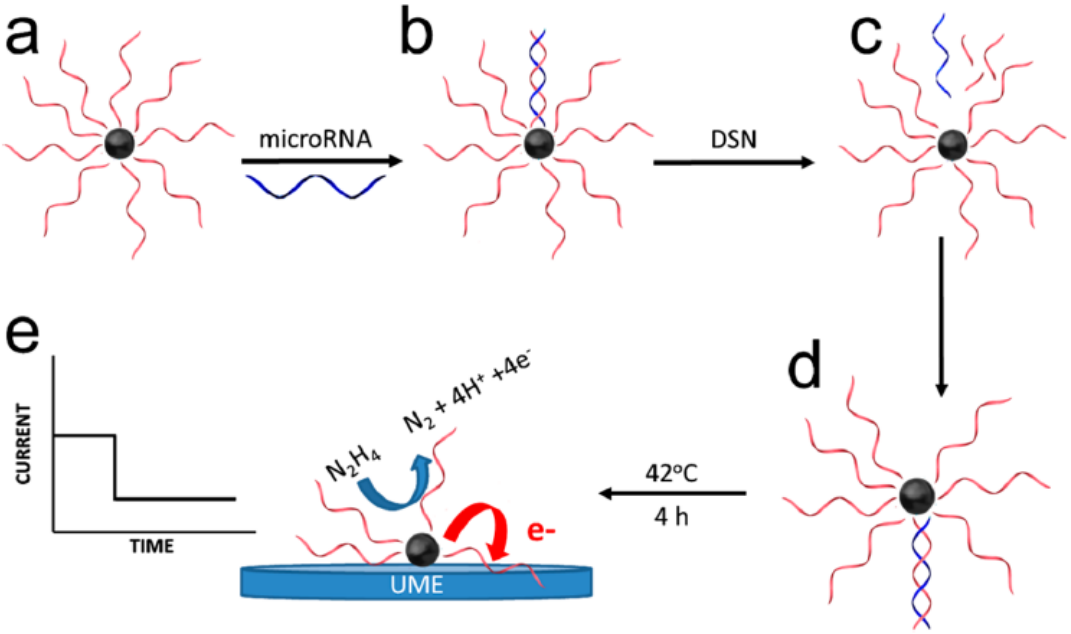

Figure 7. Schematic illustration of DSN-assisted detection of miRNA with electrocatalytic PtNPs. Reprinted with permission from reference [42]. Copyright 2017 American Chemical Society.

Fu's group proposed an efficient electrochemical biosensor which was used to determine miRNA in pancreatic cancer cells and lung adenocarcinoma cells [44]. In their study, MB-modified DNA probe (eMB) can hybridize with miRNA to form a DNA-RNA heteroduplex and DSN assisted the target recycling amplification. This method has high sensitivity with a wide linear range of 0.1 pM ~ 10 nM. Zhang et al. presented an immobilization-free electrochemical impedance biosensor for miRNA detection [45]. The DNA probes in solution were captured by magnetic beads (MBs) and then

concentrated on the surface of magnetic electrode. The repulsive interaction between negatively charged DNA and $[Fe(CN)_6]^{3-/4-}$ produced a large charge-transfer resistance. Taking the advantage of DSN-assisted target recycling, the DNA probes were digested and the negatively charged layer was not formed on the MBs surface. This led to a small charge-transfer resistance on the magnetic electrode. The biosensor has a detection limit of 60 aM to miR-21. Miao's group have developed an ultrasensitive "signal-on" self-powered biosensor for miRNA assays based on biofuel cells (Fig. 8) [46]. Electroactive $[Fe(CN)_6]^{3-}$ ions were entrapped inside the porous Fe_3O_4 nanoparticles by DNA. Hybridization of target miRNA to the DNA resulted in the release of $[Fe(CN)_6]^{3-}$. Taking the advantage of the DSN-assisted target recycling, the biosensor exhibits a detection limit as low as 1.4 aM and a linear range of 0.01 ~ 10 fM.

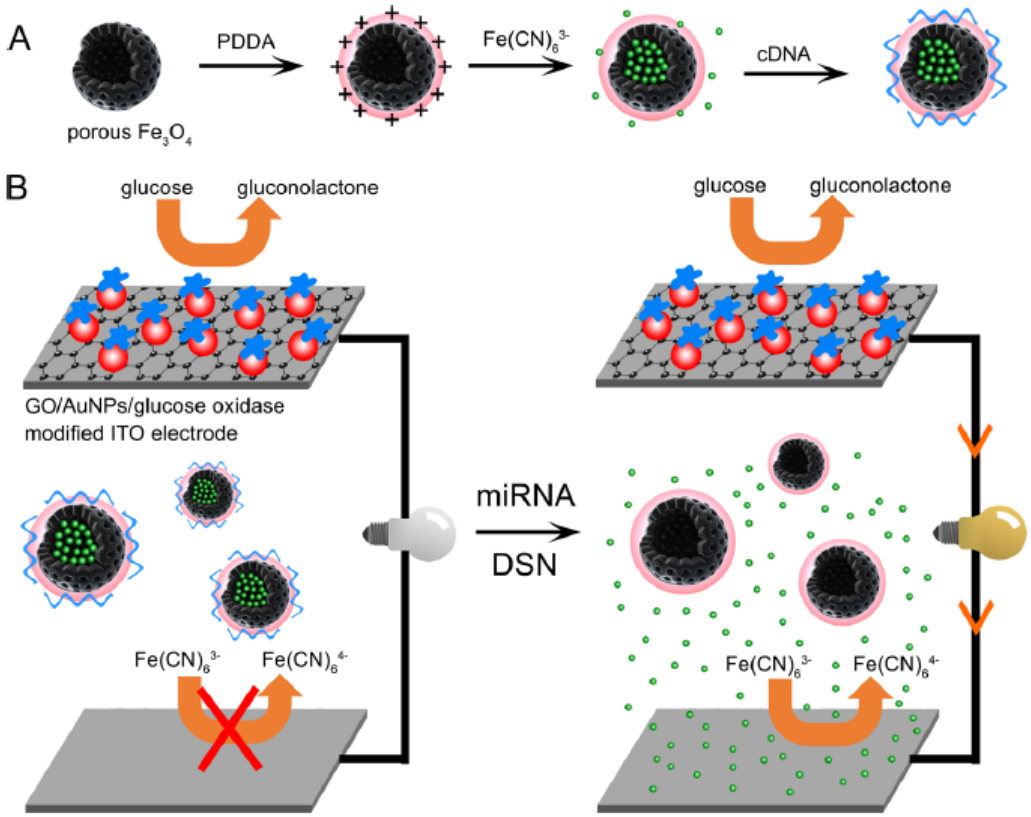

Figure 8. (A) Preparation process of DNA-functionalized porous Fe_3O_4 NPs; (B) Schematic illustration of the working principle of the self-powered biosensor with DSN-assisted amplification. Reprinted with permission from reference [46]. Copyright 2018 American Chemical Society.

2.5 Ratiometric biosensors

The above single-signal electrochemical biosensors are seriously dependent upon laboratory detection conditions, which may limit their development and applications. Ratiometric electrochemical biosensors by the internal calibration mechanism can effectively reduce the background electrical signal and improve the accuracy, reproducibility and sensitivity of electrochemical biosensors [47]. Recently, DSN-based ratiometric electrochemical biosensors have been designed and used for miRNA detection. For example, Zhu et al. have proposed a ratiometric electrochemiluminescent

(ECL)/electrochemical biosensor (Fig. 9) [48]. Thiolated S1 and S3 were hybridized with Fc-labeled S2 and S4 to produce S1/S2 and S3/S4 duplexes on an electrode surface. When miRNA-499 was hybridized with the H1 loop to form the RNA/DNA duplex, the DNA sequence in the hybrid would be cleaved by DSN. A 27-base sequence was then released as the trigger chain (T). At the same time, the target miRNA-499 was also released to start a new cleavage reaction. As a result, a small amount of miRNA-499 promoted the generation of many trigger chains. The T chains can combine with the suspension of H2 and open the hairpin structure to reveal a foothold that can hybridize with H3. Then, the H2/H3 hybrid was generated and the trigger chain was recycled. After that, the extended domain of H2 in the H2/H3 hybrid can be used as the foothold of H4 to form H2/H3/H4. The newly exposed H4 extension hybridized with the sticky end of H5 to release the H2/H3 hybrid and form the H4/H5 hybrid, thus achieving the two-layer CHA cascade amplification. The mixture of H2/H3 and H4/H5 was deposited on the S1/S2 and S3/S4-modified electrode surface. The protrusions of H2 and H4 can replace the Fc-labeled S2 and S4 by branching migration to form S1/H2/H3 and S3/H4/H5. These long dsDNA (S1/H2/H3 and S3/H4/H5) can be used as vectors to embed $Ru(phen)_3^{2+}$ to produce a strong ECL signal. Meanwhile, the electrochemical signal of Fc and the quenching effect on ECL signal are also reduced with the displacement of S2 and S4 on the electrode surface. MiRNA-499 can be determined by the ratio of ECL signal of $Ru(phen)_3^{2+}$ to the electrochemical signal of Fc. With the benefit of DSN-assisted target recycling and CHA amplification, the biosensor achieved high sensitivity and a wide linear range up to 6 orders of magnitude. In addition, Li and co-workers proposed a ratiometric electrochemical biosensor to detect miRNA-21 from tumor cells [49]. The target can trigger the formation of HP1/HP2 duplexes by CHA amplification. This changed the current response of Fc and MB labels. The biosensor has a dynamic range of 5 fM ~ 0.1 nM and a low detection limit of 1.1 fM. Yuan and co-workers reported a sensing scheme for miRNA assays based on a dual-amplification mechanism [50]. DSN triggered the release of Fc tags from the electrode surface and caused the decrease of Fc signal. Then, the Thi signal increased with the capture of numerous DNA/AuNPs/Thi by HCR and nanoprobes amplification. By measuring the ratiometric current (I_{Thi}/I_{Fc}), this method can detect miRNA-141 with a detection limit down to 11 aM.

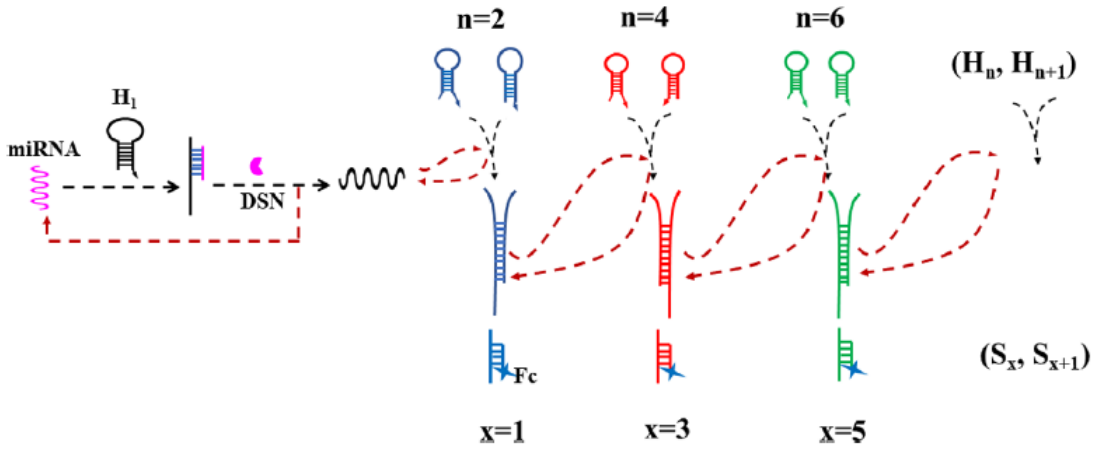

Figure 9. Schematic illustration of DSN-assisted target recycling and multilayer CHA amplification cascade. Reprinted with permission from reference [48]. Copyright 2020 American Chemical Society.

5. CONCLUSION

MiRNAs have been the persistent pursuits of researchers in this field to realize simple and accurate determination of their levels. Although the sensitivity, convenience and rapidity of electrochemical biosensors for miRNA detection have been improved by the signal amplification of DSN, the currently reported methods can not be directly used in clinical diagnosis and monitoring of cancer progress probably due to their poor stability. Therefore, the development of simple, sensitive, stable and low-cost methods for real-time quantification of miRNAs is still urgently desired.

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