

Mini Review

Recent Development of the Electrochemical Sensors for miRNA Detection

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MicroRNAs (miRNAs) are a family of non-protein-coding, endogenous, small RNAs that play a significant role in gene expression and biological processes. The expression level and types of miRNAs are strictly related to the early diagnosis, targeted therapy, and prognosis of diseases. miRNAs are therefore considered as ideal biomarkers of some diseases, especially cancers. It is thus important and necessary to detect miRNAs using a sensitive, accurate, specific, simple and fast method. Electrochemical sensors have attracted wide attention for sensing biomolecules because of the unique properties, such as simple operation, low cost, fast response, good stability and high sensitivity. In this review, we mainly summarize recent developments in electrochemical miRNA biosensors. We cover a diverse range of electrochemical miRNA sensors, including early electrochemical miRNA sensors, as well as the improvement of electrochemical miRNA sensors and the detection of two miRNAs. While electrochemical miRNA sensors still suffer from the inability to achieve high-throughput detection and cumbersome construction of the entire sensor, their shortcomings will reorient the development of electrochemical detection of miRNA that will make its application possible in the field of biomedical research and clinical analysis.

Keywords: miRNAs, electrochemical sensors, signal amplification, probe immobilization, detection of two miRNAs

1. INTRODUCTION

MicroRNAs (miRNAs) are a type of endogenous, non-coding, small RNA with a length of about 19-25 nucleotides. The first discovery can be traced back to 1993 when Lee found a non-coding single-stranded small molecule RNA1 (lin-4) while studying the developmental pathway of *C. elegans*[1]. This small RNA plays a role in regulating development. MiRNAs participate in a series of important life processes, such as cell proliferation, differentiation, apoptosis, and metabolism among

others[2-5]. Importantly, they perform many of regulatory functions in cells, mainly by cleaving mRNA and inhibiting the translation of mRNA that regulates gene expression. When the sequences of an miRNA and the 3'UTR region of an mRNA are completely paired and combined, mRNA degradation will be induced. If only part of the sequences are paired and combined, mRNA translation will be inhibited[2, 6]. For example, lin-4, which was first discovered in *Caenorhabditis elegans*, inhibits the translation of mRNA and ultimately affects the development of nematodes by further inhibiting protein synthesis [1]. miRNAs that regulate gene expression through inhibiting translation of mRNA are also currently the most frequently discovered.

Initially, an miRNA is a fragment of RNA containing a stem-loop structure. The long chain of RNA is called primary miRNA (pri-miRNA) and is transcribed inside the cell nucleus by RNA polymerase II as shown in Fig. 1[7, 8]. Afterwards, the pri-miRNA is cut into a precursor miRNA (pre-miRNA) of up to approximately 70 nucleotides by Drosha, the RNase III endonuclease. Pre-miRNA is then transported from the nucleus to the cytoplasm *via* the transport protein Exportin-5 (Exp-5). Under the action of an RNase III enzyme known as Dicer, the pre-miRNA is cleaved into mature miRNA with a length of 19 to 25 nucleotides. Finally, mature miRNA generally binds to the RNA-induced silencing complex (RISC) to form a RISC complex, which inhibits or cleaves the mRNA by binding to the 3'UTR region of the mRNA, thus performing its regulatory function.

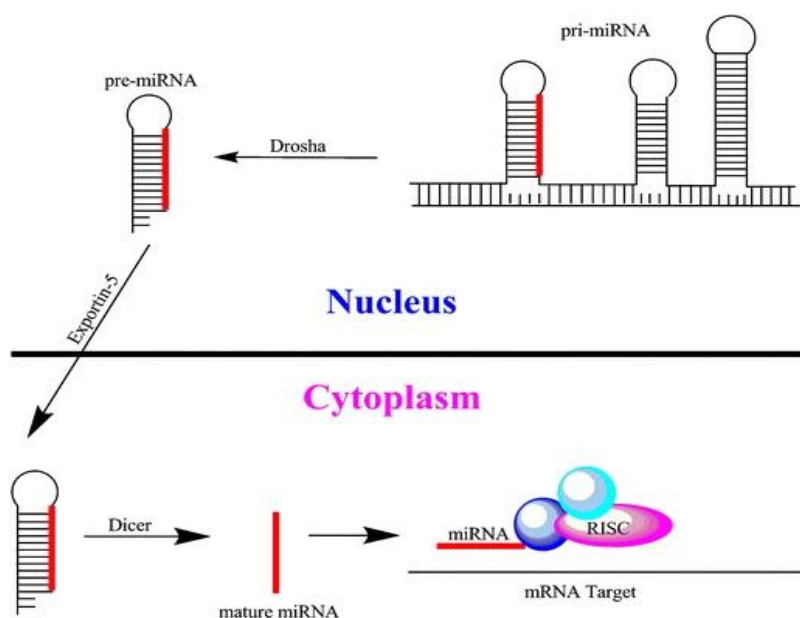


Figure 1. The process of miRNA biogenesis showing how mature miRNA is formed in the cell[9]. Copyright 2009 Springer nature.

With the discovery of various miRNAs and their unique regulatory roles in cells, miRNAs have attracted increasing attention due to their close relation to a variety of diseases and disorders[10]. miRNAs are abnormally expressed in many diseased cells, such as those implicated in cancer[11, 12], neurological disorders[13] and cardiovascular disorders[14], among others. Thus, miRNAs have been regarded as a novel type of important biomarker for the diagnosis, prognosis and therapy of related

diseases. It is therefore important to perform sensitive, specific, simple and fast detection of miRNA. However, the detection of miRNAs is challenging due to their small strands, sequence homology among family members, and low abundance in total RNA samples[2, 15]. While there are many methods currently available for miRNA detection -- including some classic biological methods like northern blotting[16, 17], microarray[18, 19], real-time quantitative polymerase chain reaction (RT-qPCR)[20, 21] -- their intrinsic limitations hinder their application; these include low sensitivity, long processing times, poor specificity and the need for professionally trained operators[22-24]. These shortcomings make it extremely difficult to detect miRNA in real samples and thus to apply miRNA detection in clinical analysis. To overcome the shortcomings of these traditional methods, some non-biological methods have been developed recently, including colorimetric[25], fluorescence[26], surface-enhanced Raman scattering (SERS)[27], bioluminescent[28] and electrochemical sensors[29, 30], and others. Electrochemical sensors have attracted increasing attention because of their simple operation, low cost, fast response, good stability and high sensitivity (Table 1).

Table 1. Comparison of different methods for miRNA detection

Methods	Advantages	Disadvantages
Northern blotting	ability to discover new miRNAs	low sensitivity
Microarray	small sample volume required and high-throughput detection	non-quantitative nature, more experimental validation needed
RT-qPCR	accurate and sensitive	high cost, highly purified primers and professionally trained operators needed
SERS	excellent reproducibility, single-nucleotide specificity	inability to differentiate sequences with overlapping peaks
Fluorescence	high sensitivity, rapid assay	external excitation source required to excite the fluorophores
Bioluminescence	high sensitivity	inability to be applied <i>in vivo</i>
Electrochemical sensors	high sensitivity, low cost, fast response, miniaturization	inability to realize high-throughput detection

2. ELECTROCHEMICAL miRNA SENSORS

Recently, great progress has been made in the development and applications of electrochemical biosensors for the detections of monosaccharides, enzymes, proteins, nucleic acids and other biological macromolecules[31]. Generally, an electrochemical biosensor mainly uses a solid electrode as the basic electrode on the surface of which the bioactive molecule acting as a molecular identifier is fixed. The target molecule is captured on the electrode surface upon its specific recognition by the molecular

identifier, and this interaction can be measured as changes in electrical signals such as current, potential, resistance, etc. to achieve qualitative and quantitative analyses of target analytes[32]. Due to their attractive properties such as miniaturization, simple operation, low cost, fast response and high sensitivity, electrochemical sensors have increasingly shown great potential in the detection of miRNA.

2.1 Early electrochemical miRNA sensors

At present, electrochemical sensors based on miRNA detection mostly rely on the immobilization and hybridization of probes. For a typical electrochemical miRNA sensor, a capture probe (CP) complementary to the target miRNA is first immobilized on a solid electrode. In the presence of target miRNA, the CP hybridizes with the target miRNA. This hybridization event is converted into a measurable electrochemical signal with the signal intensity proportional to the concentration of miRNA, resulting in both qualitative and quantitative analysis of the miRNA[33]. For example, Gao reported an electrochemical biosensor that directly labeled miRNA with a redox active and catalytic moiety, Ru(PD)₂Cl₂ (PD = 1,10-phenanthroline-5,6-dione), for the sensitive detection of miRNA[34]. Here, the capture probe was first immobilized on an electrode, then specifically hybridized with target miRNAs labeled with Ru(PD)₂Cl₂. Due to the excellent electrocatalytic activity of Ru(PD)₂Cl₂ for hydrazine oxidation, a good level of performance was obtained for miRNA detection with a limit of detection (LOD) as low as 0.2 pM (Fig. 2A).

In contrast, a label-free miRNA electrochemical biosensor was reported based on targeted miRNA-specific recognition and duplex-specific nuclease (DSN) selectively cleaving hybrid double-strands[35]. As illustrated in Fig. 2B, a single layer of a thiolated deoxyribonucleic acid (DNA) capture probe is first fixed on a gold electrode as a target miRNA capture interface. Subsequently, with target miRNA present, the specific hybridization between the capture probe and target miRNA forms miRNA-CP double strands. All such DNA-miRNA duplexes are then cleaved from the biosensor by DSN, leading to the release of miRNA back into the sample solution for further hybridization, which continues until the capture probe is exhausted. A highly selective detection of miRNA is then performed through electrochemical impedance spectroscopy (EIS). By comparison, label-free methods can directly detect miRNAs in serum without the cumbersome steps of miRNA extraction and purification. There are many more studies on the construction of early electrochemical miRNA biosensors, some of which are listed in Table 2.

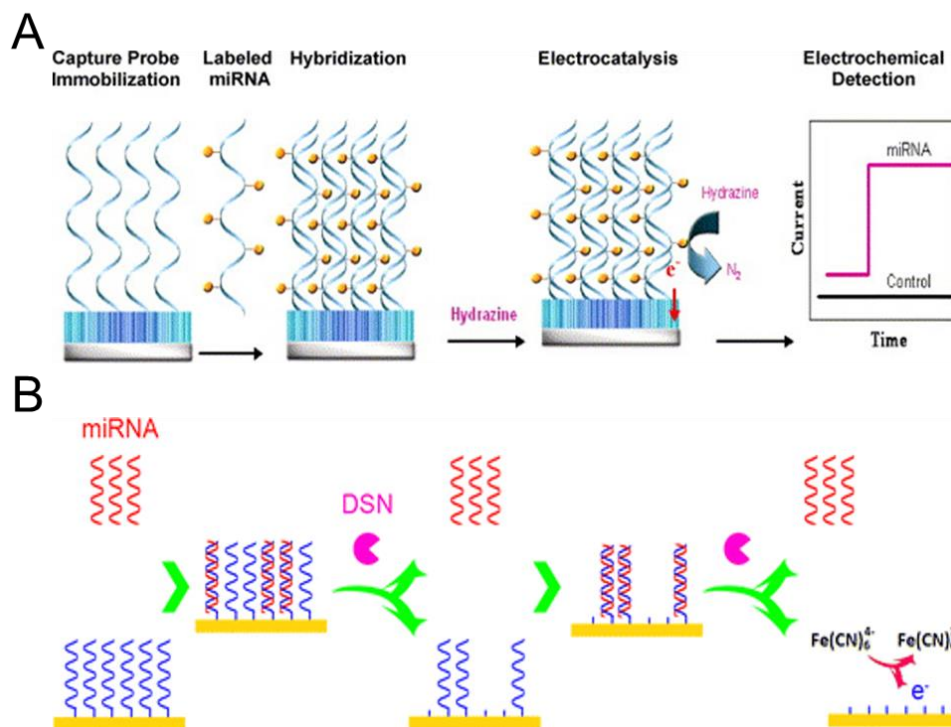


Figure 2. (A) Schematic representation of an miRNA assay using electrocatalytic labels[34]. Copyright 2007 Elsevier. (B) Schematic illustration of the working principle of the label-free electrochemical biosensor[35]. Copyright 2013 American chemical society.

Table 2. Early electrochemical miRNA biosensors

Sensor	MiRNA	Technique	Linear range	DOL	Ref.
DSN	let-7b	EIS	2.0 fM - 2.0 pM	1.0 fM	[35]
Ru(PD) ₂ Cl ₂	let-7b	Amperometric	0.50 - 400 pM	0.20 pM	[34]
GOx	let-7b	Amperometric	20 fM - 10 pM	10 fM	[36]
GOx	let-7b	Amperometric	8.0 fM - 10 pM	4.0 fM	[37]
Pd NPs	miRNA-155	CV	5.6 pM - 560 pM	1.87 pM	[38]
biotin-labeled DNA-AuNPs	miRNA-21	Amperometric	0.01 pM - 7 pM	6 fM	[39]

Note: Pd nanoparticles (Pd NPs), Glucose oxidase (GOx), Cyclic voltammetry (CV)

2.2 Improvements in electrochemical miRNA sensors

Although some of early electrochemical methods for miRNA detection have some advantages, such as fast response, low cost and simple operation, the fatal shortcoming of low sensitivity greatly limits their applications. Poor sensitivity will make it extremely hard to detect miRNA in real samples

and thus hinder application in clinical analysis. Accordingly, more and more highly sensitive, specific and stable methods have been developed[40]. The improvements to electrochemical miRNA sensors will be introduced here in the context of three aspects: application of nanomaterials, target amplification and probe immobilization on the electrode surface.

2.2.1 Nanomaterials

In the 21st century, the rapid development of nanomaterials and nanotechnology has yielded great advantages in many fields, such as medical imaging and diagnosis, drug delivery, gene therapy, biosensors, etc. In the field of biosensors, nanomaterials usually function well as recognition elements because of their unique optical, electrical, mechanical, and magnetic properties and chemical activity. Additionally, the nano-scale range of molecular motion help improve, even revolutionize the molecular operating system through better integration of the excellent performance of nanomaterials into the molecular operation, thereby [41-44]. In view of the above characteristics, interest in the fabrication of nanomaterial-based electrochemical miRNA sensors has been constantly increasing, with breakthroughs being made continuously.

Application of metal nanomaterials in electrochemical miRNA sensors

Metal nano-materials have become some of the most commonly used nano-materials in electrochemical biosensors due to their good electron transfer properties. Nano-gold, nano-silver, nano-platinum, and others have been used, however, nano-gold is the most widely used owing to its stable chemical properties, good biocompatibility and easy surface modification[45]. Tian reported a simple, label-free electrochemical miRNA biosensor using toluidine blue (TB) as a redox indicator and Au nanoparticles (NPs) superlattice as a carrier material[46]. Briefly, the electrode was modified with Au NPs superlattice to increase the surface area and conductivity, then capture probe ssRNA was immobilized on the electrode. Based on the specific hybridization between ssRNAs and target miRNAs, the hybridization indicator TB was finally embedded in the formed double strand for ultra-sensitive detection of miRNA (Fig. 3A).

Application of oxide nanomaterials in electrochemical miRNA sensors

In addition to having good surface hydrophilicity and biocompatibility, various nano-oxides have some special effects, for example, the photoelectric effect of TiO₂ or the magnetic effect of nano-Fe₃O₄, etc, producing some unexpected influence in the construction of electrochemical biosensors[47]. Shen took advantage of the magnetic effect of nano Fe₃O₄ to synthesize the magnetic DNA nanospheres labeled with electrochemical activity indicators and constructed an electrochemical sensor that can simultaneously detect miRNA21 and miRNA155[48]. As shown in Figure 3B, a layer of Au NPs was first used to modify the surface of the Fe₃O₄ nanospheres (Fe₃O₄@Au NSs) to further increase its surface area and serve as a carrier for DNA loading. DNAs labeled with electrochemically

active indicators were bound to the surface of $\text{Fe}_3\text{O}_4@\text{Au}$ via a hyperbranched hybridization chain reaction (H-HCR) to form magnetic DNA nanospheres. In the following step, the magnetic DNA-labelled $\text{Fe}_3\text{O}_4@\text{Au}$ NSs are immobilized on a gold stir bar through interaction with complementary DNA (cDNA) and act as signal probes to capture the target via the hybridization of DNA and RNA. As a result, the magnetic DNA-labelled $\text{Fe}_3\text{O}_4@\text{Au}$ NSs are released from the gold stirring rod, and finally magnetically enriched to trigger signal amplification, facilitating the electrochemical detection of miRNA.

Application of carbon-based nanomaterials in electrochemical miRNA sensors

Like metal nanomaterials, carbon-based nanomaterials also have excellent electron transfer properties, high loading capacity of biomolecules and good biocompatibility. At present, common carbon-based nanomaterials, such as carbon nanotubes and graphene, etc., have been widely used to construct electrochemical miRNA biosensors[49, 50].

Bao integrated a gold nanoparticle/polypyrrole-reduced graphene oxide (Au/PPy-rGO), catalyzed hairpin assembly (CHA) and HCR multi-signal amplification strategies to detect miRNA [51]. Fig. 3C shows that a layer of PPy-rGO film was first electropolymerized on a glassy carbon electrode (GCE), followed by the electrodeposition of a layer of gold on PPy-rGO modified GCE. Next, the thiolated capture probe (SH-CP) was self-assembled on the modified electrode by forming Au-S bonds. The H1 hairpins are opened first with the target miRNA, H2 hairpins hybridize with the opened H1, and the miRNA is simultaneously released into the next cycle, triggering the dynamic assembly of the two hairpin substrates (H1 and H2). Subsequently, HCR is completed in a mixed solution (hairpins H3 and H4). Finally, a large amount of methylene blue (MB), the signal indicator that electrochemically respond to detection of miRNA, is embedded in the small grooves of long double-stranded DNA (dsDNA) polymer.

Xiao also designed an ultra-sensitive electrochemical sensor based on carboxylate-reduced graphene oxide (COOH-rGO) to enhance signals combined with DSN-assisted target recycling for the detection of miRNA-21[52]. Briefly, the captured DNA (cDNA) is first self-assembled on a bare gold electrode (GE) where 6-mercaptohexanol (MCH) is immobilized on the cDNA/GCE to prevent non-specific binding. In Route a, the existence of π - π stacking interactions between the hexagonal cells of COOH-rGO and the planar structure of the cDNA makes COOH-rGO stack continuously on the cDNA sequence. A large number of MB molecules can thus be simultaneously bound, producing an electrochemical signal for miRNA-21 detection.

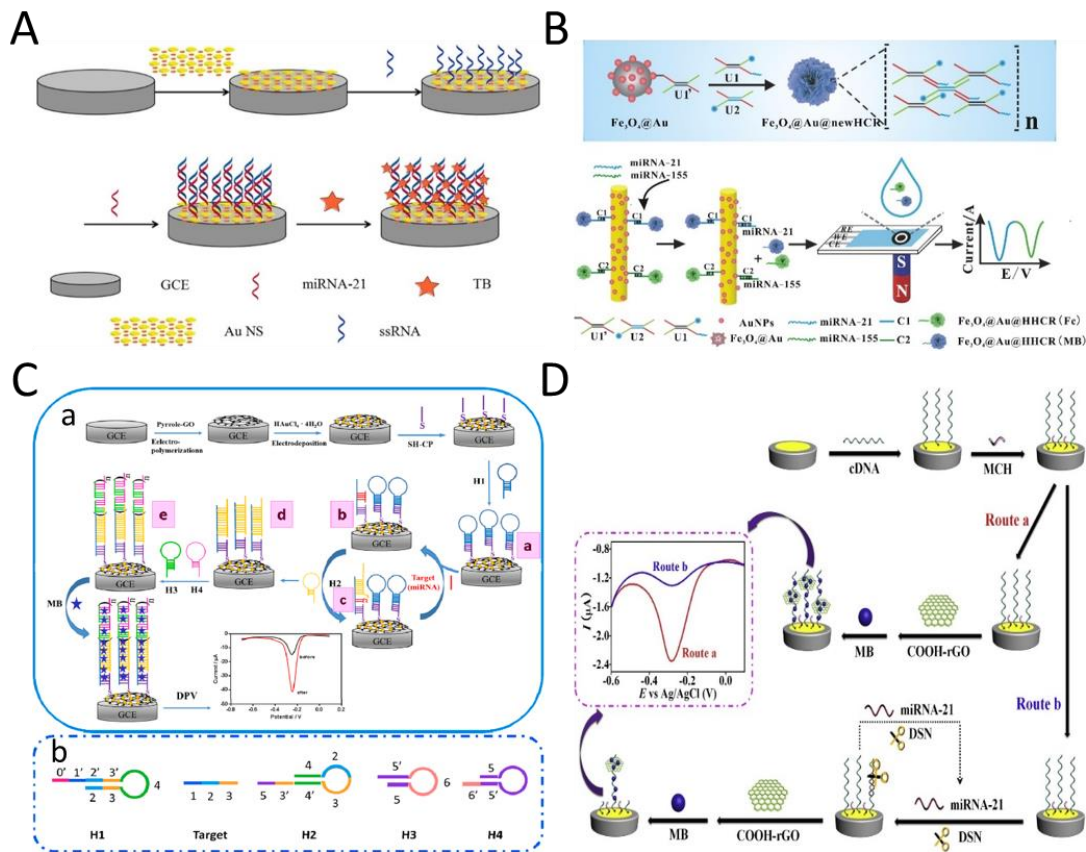


Figure 3. (A) The electrochemical sensing strategy using AuNPs superlattice as a supporting material[46]. Copyright 2018 Elsevier. (B) Schematic illustration of the preparation of H-HCR self-assembled magnetic DNA nanospheres ($\text{Fe}_3\text{O}_4@Au@HHCR$)[48]. Copyright 2020 Elsevier. (C) Schematic illustration of the electrochemical miRNA biosensor based on the enzyme-free signal amplification of CHA and HCR[51]. Copyright 2019 Elsevier. (D) Fabrication process of an electrochemical biosensor and its application for miRNA-21[52]. Copyright 2019 Elsevier.

In Route b, the coexistence of target miRNA-21 and DSN makes miRNA-21 specifically hybridize upon recognition by cDNA to form a cDNA/miRNA-21 hybrid which is subsequently cleaved by DSN, releasing miRNA-21. The specific hybridization of the released miRNA-21 with another cDNA occurs cyclically, resulting in a large reduction in cDNA and significant DSN-assisted target recovery. Consequently, MB hardly attaches to the electrode surface, resulting in a significant and dramatic drop in the differential pulse voltametric (DPV) signal. (Fig. 3D).

The application of nanomaterials greatly improves the performance of electrochemical miRNA sensors in the following ways: I) nanomaterials with excellent conductivity are used to modify the electrode surface, which can greatly improve the electron transferability on the electrode surface and enhance the electrical signal; II) compared to non-nano materials, nano materials possess a large specific surface area, which is conducive to absorbing a large number of probes and in combination with a large number of electrochemically active indicators; III) some nanomaterials exhibit many unique properties. For example, the magnetic effect of nano- Fe_3O_4 mentioned above is usually used to

collect signals through magnetic enrichment. Because of the excellent performance of nanomaterials, it is believed that nanomaterials will be more and more widely used in electrochemical miRNA sensors.

2.2.2 Signal amplification

The poor sensitivity of the early electrochemical miRNA sensors challenged their practical use in miRNA detection for many years[53, 54]. Therefore, a growing number of methods have been developed to improve the detection signal. The specific catalytic ability of enzymes has especially been used to assist in signal amplification, but enzymes are prone to variations in the surrounding environmental conditions such as temperature and pH[55]. This shortcoming necessitated the development of several target amplification methods applied under mild conditions, such as HCR[56-58], CHA[59, 60] and rolling circle amplification (RCA)[61, 62].

HCR

HCR, as a signal amplification strategy, has the advantages of simple reaction processing, mild conditions, free enzymes and high amplification efficiency and has thus been widely used in electrochemical miRNA sensors. Wang developed an electrochemical sensor with overlapping Y-shaped dsDNA synthesized by HCR and in situ, precise preparation of copper nanoclusters for miRNA-21 analysis[63]. As shown in Fig. 4A, a double-stranded DNA complex is first formed based on the specific hybridization between initiates A and B via a base pairing interaction, while the initiate B in the double strand can be replaced by target miRNA-21 through a strand displacement reaction. The initiate A is degraded into fragments by the action of exonuclease T7 (EXO T7), and miRNA-21 and EXO T7 are thus released and enter into the next cycle in this procedure. As the by-product in this cycle, the produced initiate B further interacts with capture probe 1 and capture probe 2 on the electrode to form Y-shaped DNA. Then, with the participation of hairpins 1 and 2, HCR is triggered to form a special overlapping Y-shaped branching dsDNA, which is then used as a template for in situ synthesis of copper nanoclusters. Cu(0) is oxidized into Cu²⁺ by HNO₃ and released into the solution. The oxidative peak current of copper is obtained through the differential pulse stripping voltammetry (DPSV) technique for the detection of miRNA21.

RCA

In a linear RCA reaction, short primers can be extended to long single-stranded DNA (ssDNA) with many tandem repeats via the participation of the complementary circular template to improve the detection signal. Zhang developed an ultrasensitive and label-free electrochemical biosensor for miRNA detection based on RCA-mediated Pd NPs[61]. In this sensor, a hairpin probe (HP) is first self-assembled on the gold electrode through the Au-S bond. The designed HP here has two recognition sequences: A1, which is complementary to miRNA-21 and A2, which serves as a primer for the RCA reaction. In the presence of miRNA-21, the stem-loop structure of the HP will be

expanded due to its hybridization with miRNA-21, resulting in complete exposure of the A2 region. In the presence of T4 ligase and the circular template (CT), the exposed A2 region will hybridize to the CT and trigger the RCA reaction under the action of phi29 DNA polymerase and deoxyribonucleoside triphosphates (dNTPs). As a result, a large amount of G-rich long ssDNA is produced that can be used as an effective template for in situ synthesis of Pd NPs. Here, a large amount of Pd NPs are used as electrochemical activity indicators to generate large electrochemical signals. In contrast, the stem-loop structure of the HP is not opened without miRNA-21, and thus RCA is not triggered and subsequent reactions do not produce any obvious electrochemical signals. (Fig. 4B).

CHA

CHA has attracted widespread attention due to its beneficial properties as an enzyme-free, simple process with negligible background signals. Liu developed an enzyme-free electrochemical biosensor for the detection of microRNA-21 through dual signal amplification of CHA and a $\text{Fe}_3\text{O}_4/\text{CeO}_2/\text{Au}$ nano-catalyst [64] where the target microRNA-21 first hybridizes with hairpin H2 to form H2-T dsDNA and subsequently opens the H1 hairpin structure for the formation of H1-H2 dsDNA on the electrode. At the same time, the target is released to participate in the next cycle to generate a large amount of H1-H2 dsDNA for signal amplification. Then, the single-stranded DNA S1 labeled $\text{Fe}_3\text{O}_4/\text{CeO}_2/\text{Au}$ binds to H1-H2 dsDNA. Here, $\text{Fe}_3\text{O}_4/\text{CeO}_2/\text{Au}$ shows a strong catalytic effect on the electrochemical activity indicator MB. With $\text{Fe}_3\text{O}_4/\text{CeO}_2/\text{Au}$ magnetite nanoparticles ($\text{Fe}_3\text{O}_4/\text{CeO}_2/\text{Au}$ MNPs) catalyzing the intercalation of MB into dsDNA, a strong current signal can be obtained. Eventually, the sensitive and stable detection of miRNA-21 was achieved based on this enzyme-free target recycling and dual signal amplification strategy (Fig. 4C).

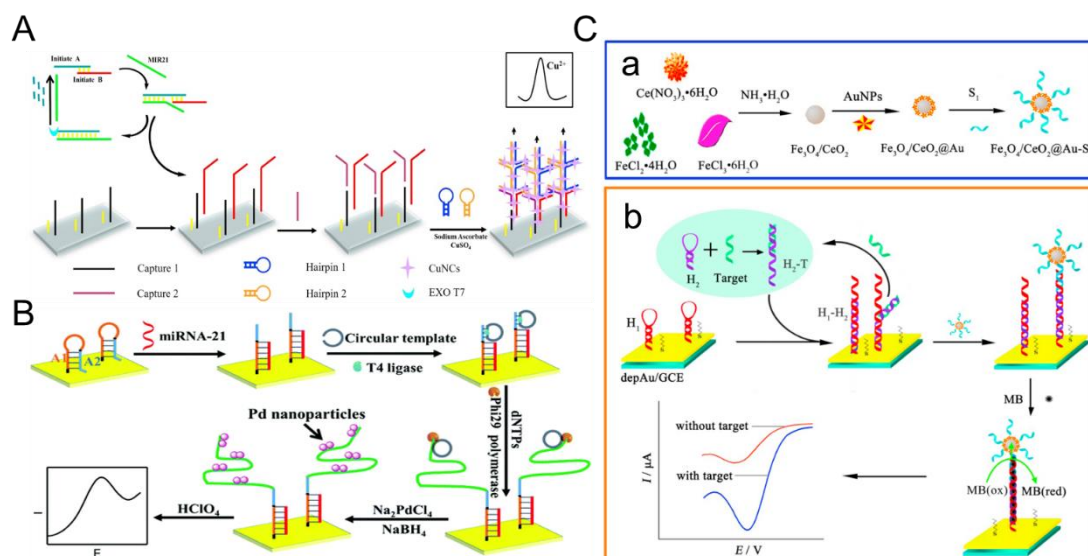


Figure 4. (A) Schematic illustration of the proposed integrated aptasensor[63]. Copyright 2017 Elsevier. (B) Schematic of the electrochemical detection of miRNA-21 based on the RCA product-mediated synthesis of Pd NPs[61]. Copyright 2019 Royal Society of Chemistry. (C) Schematic diagram of the fabrication process of the $\text{Fe}_3\text{O}_4/\text{CeO}_2/\text{Au}$ MNPs-based miRNA sensor[64]. Copyright 2018 Elsevier.

2.2.3 The immobilization of probes on the electrode surface

Probe immobilization optimization

Probe immobilization methods suffer from the inability to control the density and orientation of the surface recognition probes on electrodes, leading to poor electrochemical performance as a result of tangles between probes and the negative effects of nonspecific interactions. Due to their excellent properties of mechanical rigidity, structural stability, and controllable probe density and orientation, DNA tetrahedron nanostructures (DTN) have been widely used for optimizing probe immobilization to construct excellent biological sensing platforms and improve the recognition efficiency of biomolecules [65, 66].

Zhu constructed a DTN-based electrochemiluminescence-electrochemical (ECL-EC) ratiometric electrochemical biosensor for ultra-sensitive detection of miRNA-133a[67]. In this work, three vertices of the DTN with thiol groups were self-assembled in a highly ordered manner on a gold electrode through Au-S bonds. A hairpin DNA (miRNA-helper) with a linear sequence of 25 nucleotides at the 5' end and marked with MB was carefully designed to be complementary to the capture sequence of DTN for the electrochemical detection of short miRNA-133a. When the miRNA-133a is present in solution, the miRNA-helper of the stem-loop structure opens owing to hybridization with miRNA-133a. Then, HCR is triggered by hairpin probes H1 and H2 labeled with $\text{Ru}(\text{bpy})_3^{2+}$. The ECL signal of $\text{Ru}(\text{bpy})_3^{2+}$ increases proportionally with increasing miRNA-133a, while the EC signal of MB has nothing to do with the concentration of the target miRNA, presenting only the real-time state of the electrode surface. Therefore, the highly sensitive and repeatable quantitative analysis of miRNA-133a can be performed according to the ratio of $\text{ECL}_{\text{Ru}}/\text{Current}_{\text{MB}}$. (Fig. 5A).

Also, the ultrasensitive detection of miRNA-21 in serum was achieved based on DTN and G-quadruplex/heme conformation [68]. Briefly, DTN with a complementary segment to the target miRNA sequence was first synthesized by self-assembly and immobilized on the gold electrode through Au-S bonds. Without miRNA-21, the introduced heme combines with the G quadruplex on DTN to form G-quadruplex/hemin, which catalyzes the reduction of H_2O_2 to H_2O , and the oxidization of hemin (Red) to hemin (Ox) at the same time(Fig. 5B).

Probe-free

Some inherent disadvantages of the probe-based methods, such as the complex and time-consuming immobilization process, the limited reaction area, and the presence of local steric hindrances, significantly limit their wide use in miRNA detection due to the resulting low recognition efficiency[69]. Recently, several probe free electrochemical sensors have been reported for miRNA detection. For example, Hou developed an HCR-based, enzyme-free, label-free homogeneous electrochemical sensor for highly sensitive and specific miRNA detection[56]. Two DNA HPs (HP1 and HP2) were first designed. HP1 has sequences complementary to the target miRNA (let-7a) and HP2. Without the target miRNA, only a small amount of MB can go into the stems of HP1 and HP2, leading to a large electrochemical signal due to the release of MB on the surface of the indium tin

oxide (ITO) electrode. However, after hybridizing with the target miRNA, HP1 is opened, and the toe-mediated strand displacement reaction consequently triggers the autonomous cross-opening of the two HPs to form long double-stranded DNA. As a consequence, a large amount of MB is intercalated into the long double-stranded DNA and G quadruplex with only a small amount of free MB in the solution. The decrease of the electrochemical signal is proportional to the concentration of the target miRNA. Through this “signal-off” mode, a homogeneous electrochemical strategy based on HCR amplification was successfully realized for a sensitive miRNA assay without using probes or enzymes (Fig. 5C).

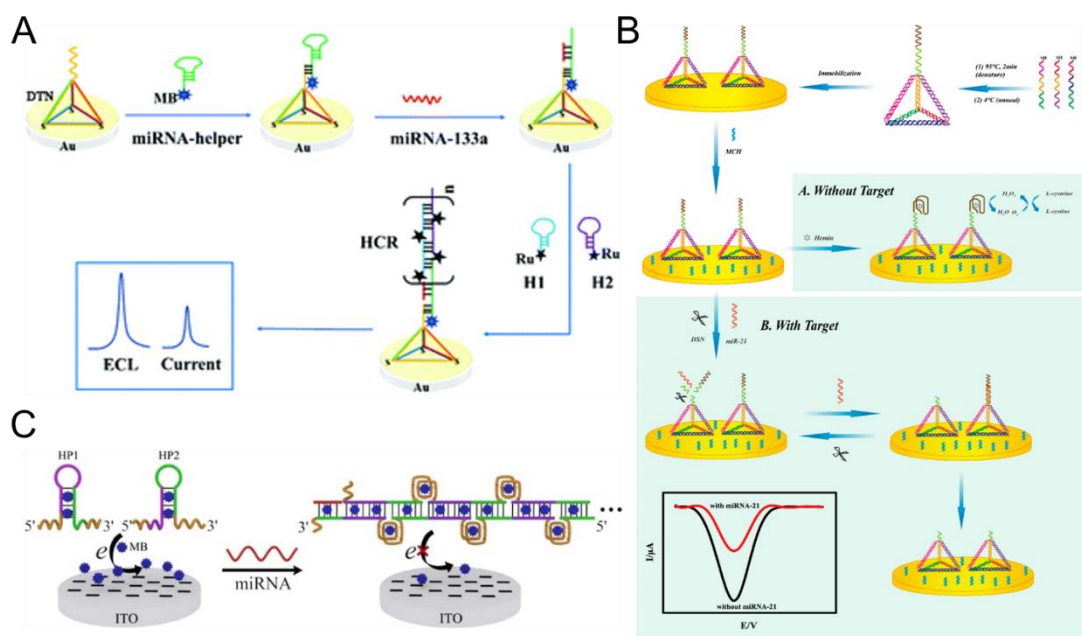


Figure 5. (A) Schematic illustration of the DTN-based ECL–EC ratiometric biosensor for ultra-sensitive detection of microRNA-133a [67]. Copyright 2019 Royal Society of Chemistry. (B) Schematic illustration of the electrochemical biosensor based on DTN and G-quadruplex/heme conformation for the detection of miRNA-21[68]. Copyright 2019 American chemical society. (C) The principle of the label-free and enzyme-free homogeneous electrochemical strategy based on HCR amplification for sensitive miRNA assay[56]. Copyright 2015 American chemical society.

In summary, electrochemical miRNA sensors have been significantly developed and improved in terms of sensitivity, specificity, and stability.

The improvement of electrochemical sensors based on nanomaterials, target amplification, and probe immobilization are not simply independent, but rather work together to build a multi-signal amplification platform to synergistically improve the performance of the sensor. For example, as mentioned above, Liu developed an enzyme-free electrochemical biosensor to detect microRNA-21 by building dual signal amplification using CHA and a $\text{Fe}_3\text{O}_4/\text{CeO}_2/\text{Au}$ nanocatalyst. The application of some multi-signal amplification platforms in electrochemical miRNA sensors are listed in Table 3.

Table 3. Multiple signal amplification platforms in electrochemical miRNA sensors

Platform	MiRNA	Technique	Linear range	DOL	Ref.
HCR and EIM	miRNA-21	ASV	2.5 fM to 25 nM	0.12 fM	[70]
Au/PPy-rGO, CHA and HCR	miRNA-16	DPV	10 fM to 5 nM	1.57 fM	[51]
EATR and Fe ₃ O ₄ /CeO ₂ @Au	miRNA-21	DPV	1 fM to 1 nM	0.33 fM	[64]
RCA and PdNPs	miRNA-21	DPV	50 aM to 100 fM	8.6 aM	[61]
FeCN	miRNA-486	DPV	1 fM to 1 nM	0.853 fM	[71]
DTN and HCR	miRNA-133a	ECL/SWV	50 aM to 10 pM	12.17 aM	[67]
EATR and FC60	miRNA-141	DPV	0.1 pM to 100 nM	7.78 fM	[72]
bisferrocene and CHA	miRNA-21	DPV	0.2 fM to 0.2 nM	0.1 fM	[59]
Cu-NMOF@PtNPs/HRP And TSDR	miRNA-155	SWV	0.5 fM to 0.1 nM	0.3f M	[73]
CS-MoS ₂ and CHA	miRNA-21	DPV	0.1 fM to 0.1 nM	16 aM	[74]
EXO T7 and Cu NPs	miRNA-141	DPV	1fM to 1pM	45 aM	[75]

Note: EIM: enzyme-induced metallization, EATR: enzyme-assisted target recycling, FeCN: iron-embedded nitrogen-rich carbon nanotubes, FC60: fullerene nanoparticles, Cu-NMOF@PtNPs/HRP: nanoscale copper-based metal organic framework assembled by Pt nanoparticles and horseradish peroxidase, TSDR: toehold strand displacement reaction, CS-MoS₂: carbon sphere-MoS₂, Cu NPs: copper nano particles, ASV: anodic stripping voltammetry, SWV: square wave voltammetry.

2.3 The detection of two miRNAs

Although electrochemical miRNAs sensors have many attractive advantages, they also suffer from some shortcomings. For example, the electrochemical miRNA sensors are currently limited to one target detection, which will greatly affect the efficiency of the multiple miRNAs detection. As novel biomarkers, miRNAs do not have a simple one-to-one relationship to diseases[76]. Therefore, it is quite expected and necessary to realize the detection of multiple miRNAs at the same time. In recent years, some electrochemical sensors based on simultaneously detecting two miRNAs have been explored.

For example, Yuan combined two magnetic nanoprobe (DNA1/Fe₃O₄ NPs/Thi and DNA2/Fe₃O₄NPs/Fc) to simultaneously detect miRNA-141 and miRNA-21 based on the response at different potentials with target-triggered HCR strategy [77]. First, hairpin capture probe 1 (HCP1) and hairpin capture probe 2 (HCP2) were self-assembled on the gold electrode. MCH was then immobilized on the electrode to blocks non-specific binding sites. When the target miRNAs are introduced, the HCP1 and HCP2 hairpin structures open owing to hybridization between the target miRNAs and hairpin probes, which further trigger the HCR process and form a long double-strand. As a result, a large amount of magnetic nanoprobe labeled with signal indicator are intercalated into the long double-stranded DNA, resulting in a strong electrochemical signal. The independent electrochemical response of the two magnetic nanoprobe at different potentials makes the

simultaneous and quantitative detection of miR-141 and miR-21 possible. (Fig. 6). More electrochemical biosensors for the simultaneous detection of double miRNAs are listed in Table 4.

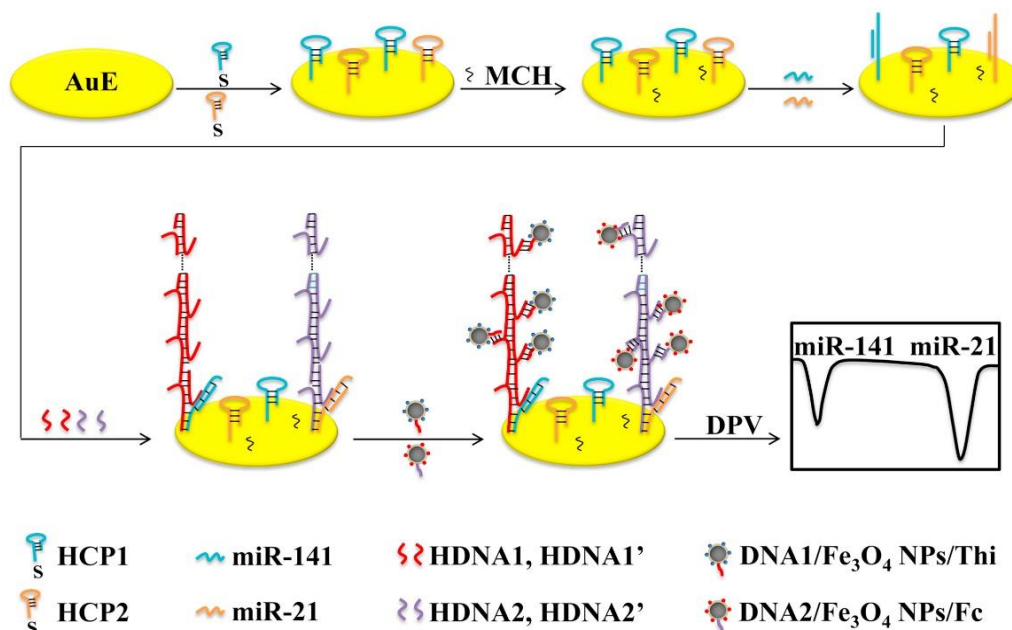


Figure 6. Schematic illustration of simultaneous electrochemical detection of miRNAs based on multifunctional magnetic nanoparticles coupling with HCR[77]. Copyright 2017 Elsevier.

Table 4. Electrochemical biosensors based on double miRNAs detection

Sensor	miRNA	Technique	Linear range	DOL	Ref.
PtCuMOFs, MB and Fc	miRNA-21, miRNA-141	SWV	1 fM to 1 nM	0.1 fM	[78]
HHCR, MB and Fc	miRNA-21, miRNA-155	SWV	5 fM to 2 nM	1.5 fM, 1.8 fM	[48]
DNA1/Fe ₃ O ₄ NPs/Thi and DNA2/Fe ₃ O ₄ NPs/Fc, HCR	miRNA-21, miRNA-141	DPV	1 fM to 1 nM	0.46 fM, 0.44 fM	[77]
DNA circle capture probe, DTN, Fc and MB	miRNA-21, miRNA-155	SWV	0.1 fM to 10 nM	18.9 aM, 39.6 aM	[79]
AuNP@T ₃ C ₂ T _x , DSN, MB and Fc	miRNA-21, miRNA-141	DPV	500 aM to 50 nM	204 aM, 138 aM	[80]

Note: Fc: ferrocene

4. CONCLUSION AND OUTLOOK

In this review, we focus on the application and development of electrochemical sensors in miRNA detection. Due to their miniaturization, simple operation, low cost, fast response and high

sensitivity, electrochemical sensors have attracted increasing attention for the detection of miRNA. Nevertheless, the early electrochemical miRNA sensors suffer from some shortcomings such as insufficient sensitivity and poor specificity, limiting their application to a large extent. Therefore, a large amount of remarkable progress towards the development of electrochemical miRNA biosensors have been made in recent years. This article mainly elaborated the improvements in electrochemical miRNA sensors made in three aspects: the application of nanomaterials, signal amplification, and probe immobilization on the electrode surface. It is clear that the multi-signal amplification platform has gradually become the mainstream strategy for significantly improving the performance of electrochemical miRNA biosensors. However, there are still some shortcomings and deficiencies in miRNA detection. As described below: I) the step of immobilization of the probe on the electrode surface is usually time-consuming and complicated, which makes it hard to control the density and orientation of the surface recognition probes, resulting in low efficiency of hybridization with the target; II) the entire process of miRNA detection is relatively cumbersome and poorly repeatable, hampering their wide application at the point-of-care ; III) the electrochemical sensors currently cannot achieve high-throughput detection for miRNAs, greatly limiting the detection efficiency of multiple miRNAs. However, these shortcomings are believed to focus new trends for future development of electrochemical miRNA sensors, ultimately making possible their application in the field of biomedical research and clinical analysis.

CONFLICTS OF INTEREST

There are no conflicts of interest to declare.

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