Phenomics (2022) 2:18–32
https://doi.org/10.1007/s43657-021-00032-z

REVIEW

Recent Progresses in Electrochemical DNA Biosensors for MicroRNA Detection

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Received: 1 June 2021 / Revised: 28 September 2021 / Accepted: 11 October 2021 / Published online: 21 January 2022

Abstract

MicroRNAs (miRNAs), as the small, non-coding, evolutionary conserved, and post-transcriptional gene regulators of the genome, have been highly associated with various diseases such as cancers, viral infections, and cardiovascular diseases. Several techniques have been established to detect miRNAs, including northern blotting, real-time polymerase chain reaction (RT-PCR), and fluorescent microarray platform. However, it remains a significant challenge to develop sensitive, accurate, rapid, and cost-effective methods to detect miRNAs due to their short size, high similarity, and low abundance. The electrochemical biosensors exhibit tremendous potential in miRNA detection because they satisfy feature integration, portability, mass production, short response time, and minimal sample consumption. This article reviewed the working principles and signal amplification strategies of electrochemical DNA biosensors summarized the recent improvements. With the development of DNA nanotechnology, nanomaterials and biotechnology, electrochemical DNA biosensors of high sensitivity and specificity for microRNA detection will shortly be commercially accessible.

Keywords miRNA · Electrochemical detection · Amplification strategies · DNA biosensor

Introduction

MicroRNAs (miRNAs) are small, non-coding RNAs with a length of 19–25 nucleotides, which were first discovered in a study of gene *lin-14* in *C. elegans* (Lee et al. 1993). Although with small length, miRNAs have important functions in transcriptional and post-transcriptional regulation of gene expression. Recent researches have indicated that miRNAs play essential roles in various physiological or pathological processes such as cell differentiation, proliferation, and apoptosis (Li and Gregory 2008; Bartel 2009; Qiu et al. 2021). Aberrant expression of miRNAs has been implicated in numerous diseases such as human cancers, cardiovascular diseases, viral infections, kidney diseases and nervous system diseases (Esquela-Kerscher and Slack 2006; Calin and Croce 2006; van Rooij et al. 2006; Jones et al. 2014). miRNAs in the blood are highly stable and hardly affected by RNAase, pH or temperature (Mitchell et al. 2008; Chen et al. 2008). Due to these advantages, miRNAs have been regarded as promising biomarkers of various human diseases. Rapid and sensitive detection of miRNAs can be applied to clinical diagnosis providing critical information on disease status (Chen et al. 2008; Garg and Sharp 2016; Choi 2020). However, the intrinsic properties of miRNAs, such as low abundance, short and highly homologous sequences, make it practically challenging to develop a rapid, low-cost, and sensitive miRNA detection platform.

In the past decade, extensive research efforts have been made towards rapid and sensitive miRNA detection techniques (Labib et al. 2016; Koo et al. 2019; Jet et al. 2021). Conventional techniques for miRNA detection include Northern blotting, RT-PCR and microarrays (Lagos-Quintana et al. 2001; Kroh et al. 2010; Koscianska et al. 2011;

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Zhou et al. 2011). Some published works have evaluated the advantages and disadvantages of these conventional techniques (Keshavarz et al. 2015; Chen et al. 2018a; Kilic et al. 2018). Northern blotting is the golden standard, but it is time-consuming and limited by low sensitivity. At the same time, the application of RT-PCR is restricted by sophisticated instruments and false-positive problems due to the short primers involved in the miRNA amplification step of the PCR process. Microarray is a semi-quantitative detection method with low sensitivity and selectivity, which limits its practical application. Electrochemical biosensors have been widely integrated and hyphenated with sampling, fluidic handling, separation and other engineering detection scenarios (Boriachek et al. 2018; Masud et al. 2019b; Kalambate et al. 2020). Besides, electrochemical biosensors exhibit numerous advantages such as high sensitivity and selectivity, reliable reproducibility, easy use for continuous on-site analysis, minimal sample preparation, relatively low cost, and short time of response. Since the first reported electrochemical biosensor for miRNAs detection (Gao and Yang 2006), publications concerning electrochemical miRNA biosensors have increased rapidly (Fig. 1, Web of Science).

DNA carries genetic information and is considered as the foundation material of biological heredity (Varshney et al. 2020). Researchers have employed DNAs as non-biological engineering materials to construct biosensors due to the advantages of its programmability, biocompatibility and stability (Song et al. 2008; Masud et al. 2017; Stanciu et al. 2021). Electrochemical DNA biosensors were constructed using DNAs as recognition probes to hybridize with the target molecules (Zeng et al. 2020). Such biosensors have held great promise as devices suitable for point-of-care diagnosis and multiplexed platforms for fast, inexpensive and

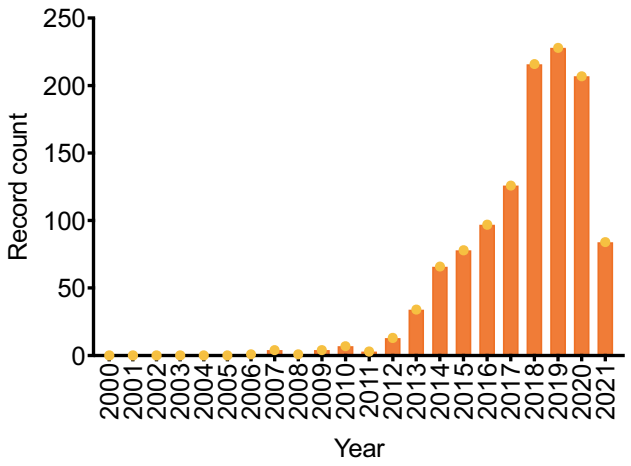

Fig. 1 Publications on electrochemical biosensors for miRNA detection since 2000 (source—Web of Science, June 2021). Keywords: "electrochemical sensor OR electronic" and "miRNA OR microRNA OR miR-"

straightforward miRNA analysis (Miao and Tang 2020a). These features made electrochemical DNA biosensors as desired solutions for phenomics diagnosis (Chen et al. 2018a; Soda et al. 2019; Masud et al. 2019a; Wu et al. 2020). Chen et al. (2018a) summarized the signal amplification strategies for miRNA detection that is not specific for electrochemical biosensors. Huang et al. (2018) reviewed DNA nanostructure-based electrochemical, optical biosensors, and application in the tumor field. Jet et al. (2021) analyzed miRNA detection methods, especially for sensitive multiplex detection, and they indicated the importance of multiplex miRNA sensing in disease-related signature discovery. Electrochemical miRNA biosensors with high sensitivity, high selectivity, and reliable reproducibility hold promise to multiplex detection. Besides, Hasan et al. (2021) reviewed the application of electrochemical biosensors in cancer-related biomarkers detection.

Herein, we summarized and discussed the recent development of electrochemical DNA biosensors for miRNA detection. We also reviewed the reaction modes of miRNA detection and signal amplification strategies of the electrochemical DNA biosensors. Currently, the detection limit at Femto- or Atto-molar of miRNA has been achieved feasibly by electrochemical DNA biosensors (Xu et al. 2020; Miao and Tang 2020b). With the development of DNA nanotechnology, nanomaterials, and biotechnology, miRNA detection of high sensitivity and specificity will be commercially accessible.

Working Principles of Electrochemical DNA Biosensors

The complementary base pairing proposed by Watson and Crick (1953) is the foundation of electrochemical DNA biosensors. The DNAs could be utilized as probes to form the self-assembled monolayers on the surface of an electrode through covalent bonds or physical adsorption (Degliangeli et al. 2014). The electrochemical DNA biosensors include electrodes, probes, and electronic reporters (Keshavarz et al. 2015). The electrode acts as the electrochemical transducer that converts biochemical signals to an electronic signal (Chaubey and Malhotra 2002). Excellent electrodes require high electrical conductivities and substrate affinity, stable electrochemical and physical properties, with a wide working range and low costs (Sonuç Karaboğa et al. 2016). According to previous reviews (Isin et al. 2017; Sharma et al. 2018; Abraham et al. 2020), the electrodes can be divided into metal-based electrodes (gold, platinum, aluminum and copper), metal oxides (MnO_2, Fe_3O_4 and CeO_2), and carbon-based electrodes (glassy carbon, pencil graphite, carbon fiber, carbon ceramic and boron-doped diamond). Different modification strategies on bare electrodes have

been developed to improve the electrical conductivity and selectivity, including modifications with conducting materials and capture probes (Sharma et al. 2018). For instance, Cui et al. (2019) used iron embedded nitrogen-rich carbon nanotubes (FeCN) to modify the glassy carbon electrode (GCE), then the conductivity was increased and the current of thionines (12.30 mA) was significantly enhanced when compared with the bare GCEs (2.022 mA) that based on different pulse voltammetry (DPV) curves. They modified gold nanoparticles (AuNPs) on the FeCN modified GCE followed by thiolated capture probes immobilization via the Au–S bond to realize selective and sensitive detection of miRNA-486-5p of A549 cells (Cui et al. 2019).

The probes could hybridize with DNAs or RNAs, of which the sequences are complementary to adjacent areas of the probes (Xu et al. 2020). The developed DNA biosensors are usually immersed in the detection samples containing targets (Boriachek et al. 2018). Once target miRNAs exist in the sample solution, the DNA probes immobilized on the surface of the electrode will hybridize with the target miRNAs, forming DNA–RNA double helix structures. The hybridization process will lead to signal variation from the electroactive indicators or electrode properties such as conductivity. The concentration of the detected miRNAs is correlated with the signal change. The sensitivity and selectivity of the electrochemical DNA biosensors are greatly influenced by hybridization modes and signal amplification strategies (Boriachek et al. 2018; Wu et al. 2020).

Three types of reaction modes for hybridizing DNA recognition probes and target miRNAs were commonly used to fabricate electrochemical DNA biosensors, including direct reaction, sandwich reaction and competitive reaction (Fig. 2). Direct reaction mode is the primary reaction mode, meaning the recognition probes with reporters directly interacted with the target miRNAs without other assistance requirements (Degliangeli et al. 2014). Sandwich reaction mode refers to a type of reaction in which a recognition probe is first combined with part of the target miRNA to form a DNA–RNA complex. Then the last part of the miRNA is hybridized with another signal probe to form a complex, which is similar to a sandwich structure (Chen et al. 2018b). The competitive reaction is a reaction mode based on the competition process, relying on the target miRNAs competing with labeled miRNAs for hybridization with the recognition probes (Zouari et al. 2017). According to electrochemical techniques and parameters utilized to capture and readout the electronic change, the electrochemical biosensors can be classified into five primary types, including voltammetry, impedance, electrical conductivity, potential, and field-effect transistor, which have been thoroughly introduced in a previous review (Labib et al. 2016).

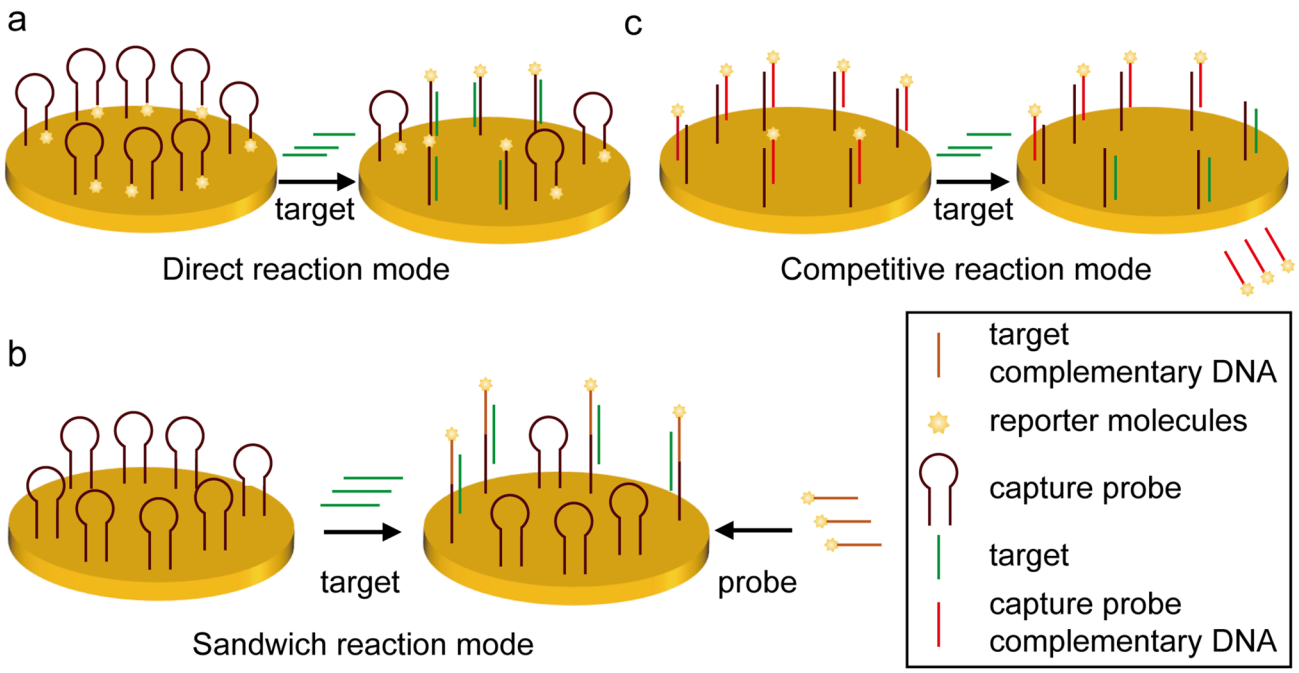

Fig. 2 Schematic diagram of three types of reaction modes for hybridization of DNA recognition probes and target miRNAs

Signal Amplification Strategies of Electrochemical DNA Biosensors

Due to the low abundance of miRNAs in actual samples and high homogeneities of different miRNAs, signal amplification strategy could reduce the limit of detection (LOD), which is an essential factor for evaluating biosensor sensitivity of miRNA and detective interference signals (Chen et al. 2018a). Numerous signal amplification strategies have been developed for electrochemical DNA biosensors. The typical strategies used in miRNA detection included nanomaterial-based amplification, enzyme amplification (Chen et al. 2018a), hybridization chain reaction (HCR) (Zeng et al. 2020) and catalytic hairpin assembly (CHA) (Chen et al. 2018b). Multiple signal amplification strategies may be combined to realize ultrasensitive detection of miRNAs.

Nanomaterials-Based Signal Amplification Strategy

With the development of nanotechnology, nanomaterials have been widely used to construct electrochemical biosensors due to the property of a high surface-to-volume ratio. Common nanomaterials mainly include gold nanoparticles (AuNPs) (Koo et al. 2016), carbon nanomaterials (Li et al. 2014), silver nanoparticles (Salahandish et al. 2018), copper nanoparticles (Tian et al. 2018a) and metal–organic frameworks (MOFs) (Chang et al. 2019). Here, we summarized and compared the nanomaterials utilized in electrochemical miRNA biosensors (Table 1).

AuNPs have a large surface-to-volume ratio, high stability and excellent biocompatibility, and are suitable for serving as substrates or mediators in miRNAs detection (Degliangeli et al. 2014; Liu et al. 2014; Han et al. 2019; Bao et al. 2020). A dual-mode electrochemical biosensor was developed for miRNA-21 detection based on AuNPs@MoS_2 (Su et al. 2017). As shown in Fig. 3a, AuNPs@MoS_2 film was modified on the surface of the electrode as a nanoamplifier. DNA recognition probes were immobilized on the AuNPs@MoS_2 decorated electrode to hybridize with the target miRNAs. Once target miRNAs existed in the detection system, the formed sandwich structures would upregulate the EIS and DPV signals with increased miRNAs. Tian et al. (2018a) applied AuNPs superlattices to amplify electrochemical signals. AuNPs superlattices were modified on the electrode surface to enlarge the specific surface area and electrical conductivity. The biosensors displayed good selectivity and repeatability with a LOD of 78 aM. Conventional single-stranded (ssDNA) probes and stem-loop DNA probes can hardly achieve high sensitivity in miRNA detection. 3D DNA tetrahedron probes were developed by assembling four designed oligonucleotides, and the novel structure could control the probe density and obtain upright orientation. AuNPs were combined with 3D DNA tetrahedron probes for miRNA detection (Liu et al. 2015). The sensing part of the unique 3D DNA probe was a stem-loop structure constructed on the top of DNA tetrahedron bearing three thiol groups as anchors on the gold electrode. Electroactive ferrocene was utilized to generate an electrochemical signal, and AuNPs electrodeposition on the gold electrode was introduced to improve the sensitivity. Tavallaie et al. (2018) modify gold-coated magnetic nanoparticles (Au@MNPs) with methylene-blue-labeled probe DNAs complementary to target miRNAs. After DNA probes hybridizing with targeted miRNAs, Au@MNPs were magnetically collected on the gold electrode and reconfigured electrical signal to quantify the target miRNAs (Tavallaie et al. 2018). The developed biosensor first measured miRNA from 10 aM down to 1 nM in unprocessed blood samples (Tavallaie et al. 2018).

Carbon nanomaterials including graphene and carbon nanotubes are also frequently used to construct electrochemical DNA biosensors due to the advantages of hardness, conductivity and heat resistance (Li et al. 2014; Isin et al. 2017; Wang et al. 2019a, b). Kong et al. (2016) used three-dimensional (3D) graphene films growth in situ on the gold substrate for biosensor fabrication. Since the 3D graphene films exhibited excellent properties such as enlarged surface area, high conductivity and solid binding strength, the sensitivity and specificity of the developed electrochemical biosensor have been significantly improved. As shown in Fig. 3b, Deng et al. (2018) employed the shortened multi-walled carbon nanotubes (MWCNTs) with high-loaded thionin as an amplified signal indicator for miRNA detection. DNA probes were inserted into an alkane monolayer-assembled and AuNPs-modified electrode. By combining large numbers of Thi as signal indicators decorated on MWCNTs, an increased current response was achieved due to the large effective surface area of MWCNTs. The LOD of the biosensor was 0.032 pM.

MOFs are the open crystalline frameworks with permanent porosity constructed by joining metal-containing units with organic linkers using strong metal–oxygen-carbon bonds (Yaghi et al. 2003) and have open metal sites in the skeleton. They have received increased attention in the field of sensors, especially for electrochemical biosensors, due to their unique merits, including large surface areas, modulated pores, and mimetic catalysis properties (Furukawa et al. 2013; Hu et al. 2021a, b; Zhang et al. 2021). Chang et al. (2019) utilized functionalized UIO-66-NH_2 loading with two different electroactive dyes, methylene blue (MB) and 5,5′-tetramethylbenzidine (TMB), to simultaneously detect two tumor biomarkers of let-7a and miRNA-21. They

Table 1 Comparison of nanomaterials used in electrochemical DNA biosensors for signal amplification

Nanomaterial	miRNAs	Detection	Linear range	LOD	References
Three-dimensional graphene films (3DGFs)	miR-155	SWV	0.01 nM–1.0 μM	5.2 pM	Kong et al. (2016)
Graphene quantum dots (GQDs)	miR-155	i–t curve	1 fM–100 pM	0.14 fM	Hu et al. (2016)
Graphene oxide (GO)	miR-34a	DPV	5 μg/mL–35 μg/mL	7.52 μg/mL	Wang et al. (2019a)
Multi-walled carbon nanotubes (MWCNTs)	miR-24	DPV	1 pM–1 nM, 1 nM–10 nM	1 pM	Li et al. (2014)
Thionin (Thi) on shortened multi-walled carbon nanotubes (S-MWCNTs/Thi)	miR-21	DPV	0.1 pM–12000 pM	0.032 pM	Deng et al. (2018)
AuNPs-decorated MoS2 nanosheet (AuNPs@MoS_2)	miR-21	DPV	10 fM–1 nM	0.78 fM	Su et al. (2017)
AuNPs-decorated MoS2 nanosheet (AuNPs@MoS_2)	miR-21	EIS	10 fM–1 nM	0.45 fM	Su et al. (2017)
MoS2 nanosheet functionalized with thionine and gold nanoparticles (MoS2-Thi-AuNPs)	miR-21	SWV	1.0 pM–10.0 nM	0.26 pM	Zhu et al. (2017)
Hierarchical flower-like gold nanostructures (HFGNs)	miR-21	SWV	1 fM–10 pM	1 fM	Su et al. (2016)
Ferrocene (Fc)-capped gold nanoparticle/streptavidin conjugates	miR-182	CV	5 fM–100 fM	0.14 fM	Lu et al. (2016)
AuNPs superlattice	miR-21	CV	100 aM–1 nM	78 aM	Tian et al. (2018b)
Biotin-MB-AuNPs	miR-21	EIS	1 pM–1000 pM	0.3 pM	Azzouzi et al. (2017)
CuCo−CeO_2 nanospheres	miR-141	EIS	0.1 fM–10 nM	33 aM	Xue et al. (2019)
CuCo−CeO_2 nanospheres	miR-141	DPV	0.1 fM–10 nM	33 aM	Xue et al. (2019)
Gold nanoparticles (AuNPs)	miR-21	SSWV	0.5 pM–1000 pM	0.3 pM	Azzouzi et al. (2019)
Silver nanoparticles (AgNPs)	miR-141	SSWV	50 pM–1000 pM	10 pM	Azzouzi et al. (2019)
APBA–biotin–AuNPs	miR-21	i–t curve	10 fM–5 pM	3 fM	Liu et al. (2014)
Citrate-capped AgNPs	miR-21	LSV	0.1 fM–50 fM	20 aM	Liu et al. (2017)
Pyrrolidinyl peptide nucleic acid (acpcPNA)/polypyrrole (PPy)/silver nanofoam (AgNF)	miR-21	CV	0.20 fM–1.0 nM	0.20 fM	Kangkamano et al. (2018)
Copper nanocluster (CuNC)	miR-101	i–t curve	25 fM–300 fM	8.2 fM	Wang et al. (2017)
Cu-based metal–organic frameworks (Cu-MOFs)	miR-155	DPV	1.0 fM–10 nM	0.35 fM	Wang et al. (2018)
PdNPs@Fe-MOFs	miR-122	EIS	10 fM–100 nM	10fM	Li et al. (2018)
PdNPs@Fe-MOFs	miR-122	i–t curve	0.01 fM–10 pM	0.003 fM	Li et al. (2018)
Tungsten oxide-graphene composites (WO_3-Gr)	miR-21	DPV	0.1 fM–100 pM	0.05 fM	Shuai et al. (2016)
N-doped functionalized graphene (NFG)/AgNPs/polyaniline	miR-21	DPV	10 fM–10 μM	0.2 fM	Salahandish et al. (2018)
UIO-66-NH_2	let-7a	DPV	0.01–10 pM	3.6 fM	Chang et al. (2019)
	miR-21		0.02–10 pM	8.2 fM	
CoNi-MOF	miR-126	EIS	1 fM–10 nM	0.14 fM	Hu et al. (2021a)

CV cyclic voltammetry, *DPV* differential pulse voltammetry, *EIS* electrochemical impedance spectroscopy, *MOFs* metal–organic frameworks, *SWV* square wave voltammetry, *LSV* linear sweep voltammetry, *SSWV* stripping square-wave voltammetry

utilized the UIO-66-NH_2 consisting of Zr^{4+} and 2-aminoterephthalic acid as nanocarriers to encapsulate MB or TMB and linked carboxylated ssDNA onto the UIO-66-NH_2 through the amidation reaction (Fig. 3c). The ssDNA/UIO-66-NH_2 encapsulated the MB or TMB and another ssDNA, which was partially complementary to the modified ssDNA and target miRNA, hybridized with modified ssDNA, a gatekeeper to form double-stranded DNA (dsDNA)-capped MOFs. The introduced target miRNA would hybridize with complementary to release capped electroactive dyes. Moreover, these dsDNA-capped MOFs simultaneously detected the let-7a and miRNA-21 with LOD down to 3.6 fM and 8.2 fM, respectively, and they were successfully applied to detect target miRNAs spiked in serum samples.

These nanomaterials display excellent performance in amplifying signal intensity and significantly decreasing the LOD of electrochemical miRNA biosensors. The gold nanoparticles show excellent performance in sensing while are limited to high cost. The carbon nanomaterials and MOFs are low-cost but need a complex modification process. Besides, nanomaterials-based signal amplification strategies are of high background signal. Therefore, nanomaterials-based biosensors show the tendency to combine with other amplifying strategies with reasonable specificity.

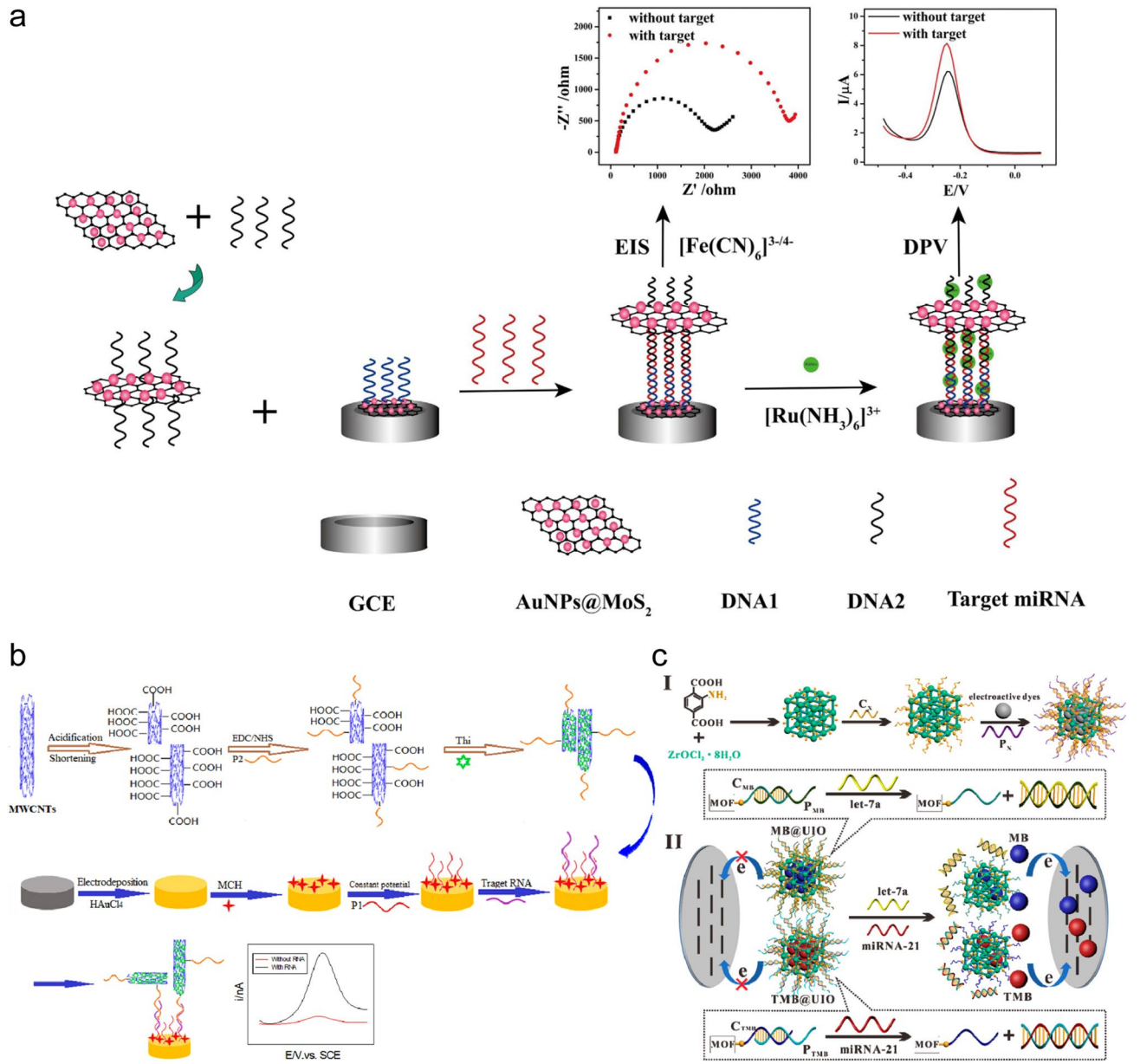

Fig. 3 Nanomaterials-based signal amplification strategies. **a** Schematic diagram of label-free and dual-mode electrochemical detection for miRNA-21 based on gold nanoparticle-decorated MoS2 nanosheet (Su et al. 2017) (Reprinted with permission from Elsevier). **b** Schematic illustration of miRNA-21 determination with the short-ened multi-walled carbon nanotubes (Deng et al. 2018) (Reprinted with permission from Elsevier). **c** Schematic representation of steps of the dsDNA-capped MOFs (I) and principles of miRNA detections (II) (Chang et al. 2019) (Reprinted with permission from ACS)

Enzyme-Based Signal Amplification Strategy

Various enzymes have been involved in amplifying the signal of electrochemical DNA biosensors for miRNA detection. Duplex-specific nuclease (DSN) can cleave DNA–DNA or DNA–RNA duplexes and disintegrate the duplex DNA long chains into short fragments. DSN is inactive towards ssDNA or RNA. DSN-assisted target recycling is widely used for signal amplification in constructing DNA biosensors (Zhu et al. 2020; Yang et al. 2020; Wu et al. 2020). Yang et al. (2014) developed a multiplexed biosensor for dual detection of miRNA-141 and miRNA-21 based on the redox labels with distinct potential positions, and DSN-assisted target recycling was employed for signal amplification. As shown in Fig. 4a, the stem-loop DNA probe hybridized with target miRNAs to form DNA–RNA duplexes. DSN shows a preference for cleavage of DNA–RNA duplexes, resulting in the release of target miRNAs, which could hybridize with the

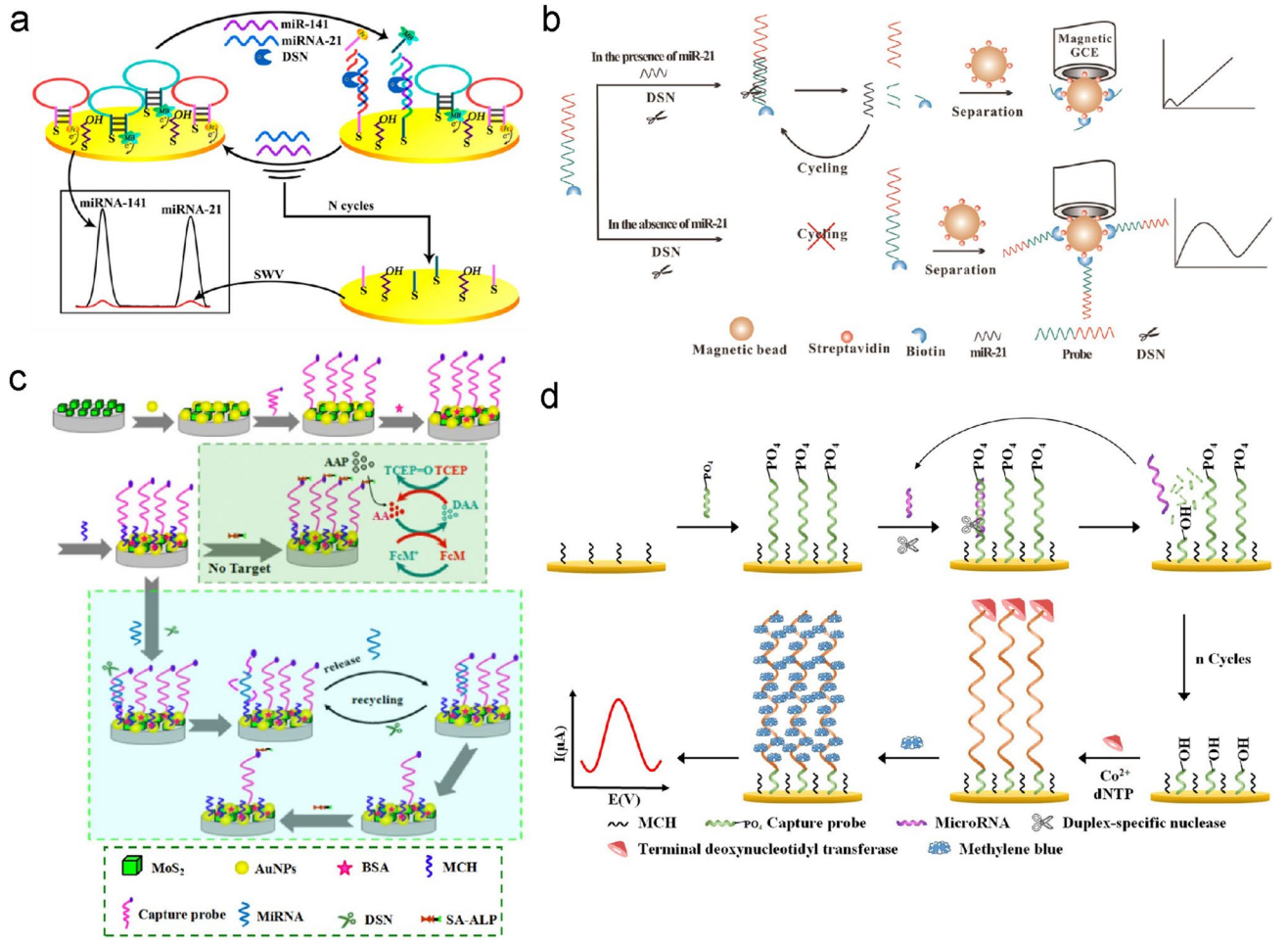

Fig. 4 Enzyme-based signal amplification strategy. **a** Schematic of DSN-assisted target recycling signal amplification via DSN for multiplexed detection of miRNA-141 and miRNA-21 by coupling different redox labels (Yang et al. 2014) (Reprinted with permission from ACS). **b** Experimental principle of biosensor includes recognizing target miRNA-21 and magnetic collection of beads without capture probes removed by DSN (Zhang et al. 2016) (Reprinted with permission from Elsevier). **c** miRNA-21 detection strategy based on AuNPs/MoS_2 modified electrode and DSN signal adding ECC redox cycling amplification strategy (Shuai et al. 2017a) (Reprinted with permission from Elsevier). **d** Schematic of miRNA-196a detection based on CESA and template-free DNA extension amplification (Guo et al. 2018) (Reprinted with permission from Elsevier)

DNA probe again to initiate the cyclic cleavage of DNA strands. The LOD of miRNA-141 and miRNA-21 in the developed biosensor was 4.2 fM and 3.0 fM, respectively.

Similarly, an immobilization-free electrochemical biosensor was developed for amplified detection of miRNA-21 (Zhang et al. 2016). The capture probe was modified with the biotin group at the end and partly complementary with the target miRNA, while the rest sequences were used for signal output (Fig. 4b). When the target miRNA-21 existed in the detection system, capture probes partially hybridized with the target miRNA-21 to form DNA–RNA duplexes. DSN selectively cleaved the duplexes and released the target miRNA-21 for further hybridization. Due to the lack of biotin-labeled at the end, capture probes could not attach to the streptavidin-modified magnetic beads, resulting in a small charge-transfer resistance. An ultralow detection limit of 60 aM was obtained through electrochemical impedance spectroscopy. As shown in Fig. 4c, a miRNA sensing platform was constructed based on DSN and the electrochemical–chemical–chemical (ECC) redox cycling (Shuai et al. 2017a). DSN cleaved the DNA–RNAs duplexes that miRNAs could recycle in the detection system. Besides, FcM, TCEP, and ascorbic acid 2-phosphate (AAP) were used as the redox mediator, reducing reagent and enzyme–substrate, respectively. Ascorbic acid (AA) produced from alkaline phosphatase enzyme triggered the ECC redox cycling that generates a tremendous electrochemical response. The LOD of the developed sensing platform was reported to be 86 aM.

Polymerase extension is another amplification strategy that usually uses in electrochemical DNA biosensor

fabrication. Template-independent DNA polymerase (TdT) is a polymerase that can catalyze the sequential addition of deoxyribonucleoside 5′-triphosphates (dNTP) at 3′-OH group of DNA to produce a long ssDNA structure directly. Guo et al. (2018) developed a sensitive biosensor by immobilizing 3′-PO_4 terminated capture probes on the gold electrode blocked with MCH. The fabrication process of the developed biosensor was detailed in Fig. 3d. DSN was used to cleave the DNA–RNAs duplexes after hybridization with the target miRNAs of which 3′-OH groups were exposed. Simultaneously, the recycling of target miRNAs generated large numbers of cleaved capture probe fragments with 3′-OH terminals. In TdT and dNTP, the 3′-OH groups on the gold electrode surface triggered sequential DNA extension. Positively charged methylene blue used as an electroactive redox indicator was specifically absorbed on the DNA extension, generating amplified electrochemical signals with a LOD of 15 aM. Poly (U) polymerase can catalyze the template-independent addition of uridine monophosphate (UMP) from uridine triphosphate (UTP) to the 3′-terminal of RNA (Zhou et al. 2016). Zhou et al. (2016) adopted a Poly (U) polymerase mediated isothermal signal amplification strategy to detect miRNA. Biotin conjugated UTP (biotin-UTP) was selected as a UMP donor to form poly (U)-biotin tail at the 3′-terminal of hybridized miRNA. Avidin conjugated alkaline phosphatase (avidin-ALP) was captured to catalyze the hydrolysis reaction of *p*-nitrophenyl phosphate disodium salt hexahydrate (PNPP) via biotin–avidin interaction. A detection limit of 1.7 fM was achieved.

Hybridization Chain Reaction (HCR)

Hybridization chain reaction (HCR) has drawn attractive attention in fabricating electrochemical biosensors for miRNA detection since Dirks and Pierce (2004) proposed it in 2004. HCR is a kinetics-controlled reaction and can act as an amplification signal transducer without any external inputs to realize sensitive miRNAs detection (Zhang et al. 2020; Zeng et al. 2020). For a basic HCR amplification strategy, two stable species of DNA hairpins coexist in the solution until the introduction of initiator miRNA, which can open the first kind of DNA hairpins and expose a new single-stranded region to open the other species of hairpins (Dirks and Pierce 2004). In turn, the initiator miRNA for the first hairpins will form and start another round of hybridization events. HCR has a specific advantage in biosensor fabrication since it can fulfill both recognition and amplification roles.

The target-triggered HCR strategy was introduced for miRNA detection that simultaneous detection of miRNA-141 and miRNA-21 was realized (Yuan et al. 2017). Yuan et al. (2017) synthesized two kinds of magnetic nanoprobes by hydrothermal treatment, and a target-triggered HCR strategy was adopted to increase the binding sites of nanoprobes (Fig. 5a). The two distinguishable nanoprobes carry extensive redox signals, which can realize the dual signal amplification when target miRNAs exist. Besides, DSN has added in the detection system that miRNA-initiated cleavage of DNA in RNA–DNA duplexes lead to the release of target miRNA and another cleaves cycle (Yuan et al. 2018). An ultralow detection limit of 11 aM was obtained by utilizing this combination strategy. Tian et al. (2018b) developed an electrochemical biosensor for miRNA detection based on the novel planar intercalated copper (II) complex molecule coupled with a target-triggered HCR strategy. The planar copper (II) complex could intercalate well into the long-range DNA structures formed by HCR, resulting in an intense electrochemical response. The detection limit of miRNA was down to 33 aM. DNA tetrahedron structure was introduced to combine with HCR amplification strategy shown in Fig. 5b. Ge et al. (2014) used the synergetic effects of DNA tetrahedron probes and HCR amplification to detect miRNAs. The avidin-modified HRP attached to the HCR products produced an electrocatalytic signal, and the detection sensitivity was significantly improved to 10 aM for miRNA detection.

To date, most HCR systems are mainly formed into linear nanostructures. Some nonlinear HCR systems for miRNA detection have been developed (Liu et al. 2015; Liang et al. 2018; Zhou et al. 2019). Liang et al. (2018) formed a nonlinear HCR system, in which the cascade HCR was employed. As shown in Fig. 5c, captured DNAs modified onto the electrode were partially complemented by the target miRNA-21, disclosing a single-stranded toehold. The disclosed toehold region as an initiator sequence (trigger 1) led to the first HCR process (HCR1), which results in polymer chains of HCR1 that are encoded with another single-stranded initiator (trigger 2) for another HCR process (HCR2) on the chains of the previous HCR product. Thus, the cascade HCR process generated a nonlinear HCR system, and it was further amplified by the interaction of redox probes. This biosensor can detect the miRNA-21 in the range from 30 pM to 7 nM, and the detection limit is estimated to be as low as 11 pM.

Catalytic Hairpin Assembly (CHA)

Catalytic hairpin assembly (CHA) is a new signal amplification strategy based on DNA structure without the assistance of enzymes (Si et al. 2020). Two designed stem-loop DNA probes were triggered by the target miRNAs, leading to the recycling of target miRNAs (Liu et al. 2019; Ning et al. 2020). Liu et al. (2018) introduced an electrochemical biosensor based on Fe_3O_4/CeO_2@Au MNPs as nanocatalyst and CHA for signal amplification. As shown in Fig. 6a, target miRNAs hybridized with stem-loop DNA probe H_2

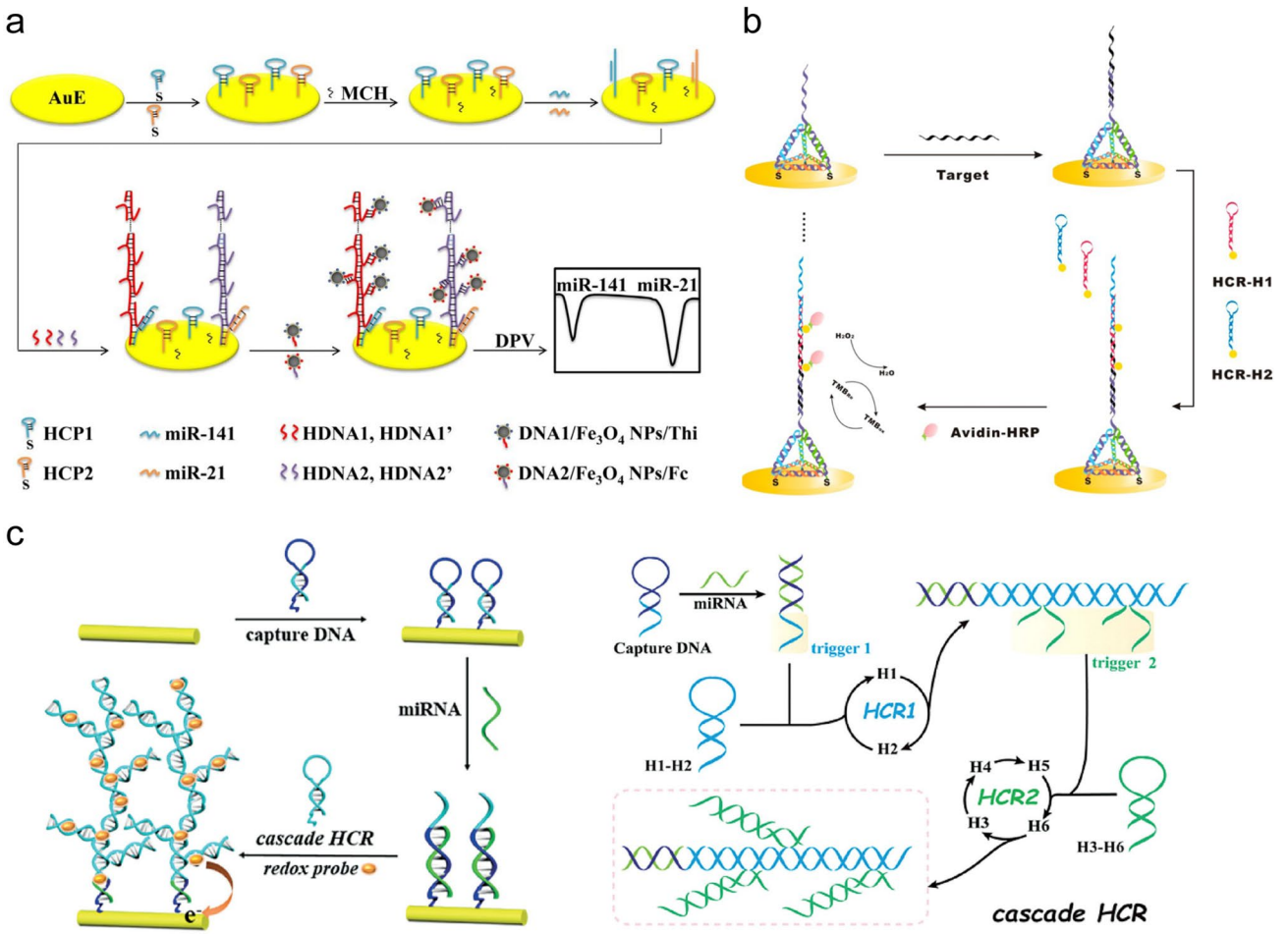

Fig. 5 Hybridization chain reaction (HCR). **a** Schematic of simultaneous detection of miRNA-141 and miRNA-21 based on multifunctional magnetic nanoparticles combining HCR (Yuan et al. 2017) (Reprinted with permission from Elsevier). **b** Design of miRNA-122b biosensors utilizing the synergetic effects of tetrahedral probes and HCR amplification strategy (Ge et al. 2014) (Reprinted with permission from ACS). **c** Design of cascade HCR for miRNA-21 detection (Liang et al. 2018) (Reprinted with permission from John Wiley and Sons)

to form a DNA duplex, which could further open the stem-loop DNA probe H_1 to form an H_1–H_2 DNA duplex. Target miRNAs were released during the process and recycled for signal amplification. A detection limit of 0.33 FM was obtained. CHA was combined with HCR in biosensor construction for signal amplification in miRNA detection (Bao et al. 2019). In Fig. 6b, Au/PPy–rGO nanocomposite was used as a substrate to enhance the efficient electron transport of biosensors. The DNA capture probes were self-assembled on the nanocomposite. Target miRNAs triggered stem-loop structures of H1 and H2 to form the double helix structure of H1-H2, leading to the recycling of target miRNAs. Under H3 and H4, the exposed sequence of H2 triggered the HCR to form a long DNA strand. MB was used as redox probes that intercalated into the DNA duplexes to achieve amplified electrochemical signals. The LOD of the fabricated biosensor was as low as 1.57 fM.

Conclusions

In this review, recent advances in miRNA detection with electrochemical DNA biosensors were summarized. The working principles and amplification methodologies tailored to miRNA of unique properties were discussed. To fulfill the various requirements of laboratory and clinical applications, especially for early disease diagnosis, numerous efforts have been devoted to developing emerging strategies of miRNA detection and signal amplification, accompanying the improvements of nanomaterials and microfabrication technology.

Electrochemical DNA biosensors, which are capable of integration and portability, have a great potential to realize miRNA detection as point-of-care devices with a significant impact on the diagnosis of diseases such as cancers and neurodegenerative diseases in practice. The advancement

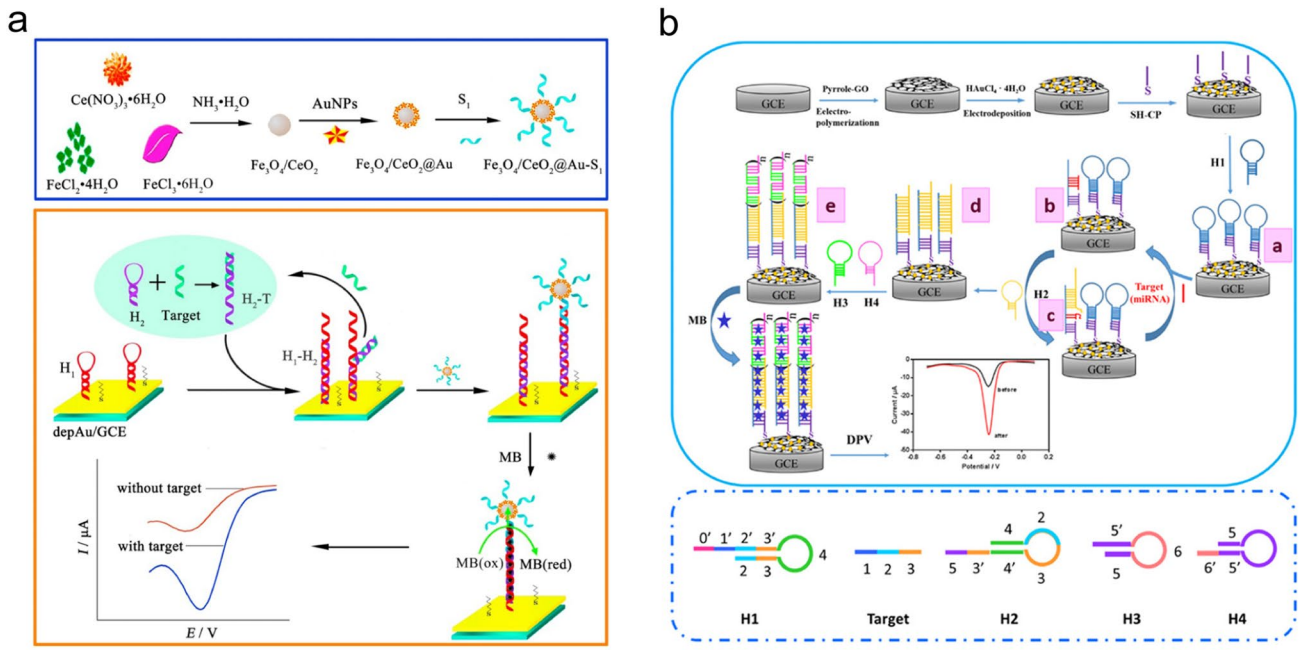

Fig. 6 Catalytic hairpin assembly (CHA). **a** Fabrication process of miRNA-21 detection biosensors including preparation of Fe_3O_4/CeO_2@Au MNPs and CHA signal amplification strategy (Liu et al. 2018) (Reprinted with permission from Elsevier). **b** Schematic illustration of miRNA-16 based on enzyme-free signal amplification of CHA and HCR (Bao et al. 2019) (Reprinted with permission from Elsevier)

of biosensors will facilitate the general data acquisition for phenomics and disease prognosis.

As summarized in Table 2, different amplification strategies have specific advantages and limitations. Nanomaterial-based amplification strategies could improve the sensitivity without long time reaction while are limited by specificity and complicated synthesis process of nanomaterials. Enzymes-based electrochemical biosensors could realize excellent amplification efficacy without complicated design while the enzyme activity and high cost still concern. HCR-based electrochemical biosensor has an excellent performance in specific recognition of target miRNAs but needs specific probe design and longtime reaction. CHA amplification strategy is advantageous for low background and good stability but also needs a long reaction time and specific DNA sequence design. This review summarized the primary amplification strategies and their recent development, which might provide information for designing electrochemical sensing platforms with excellent sensitivity and specificity, ideal stability and reproducibility, low cost, easy operation, and potential for accurate early disease diagnosis.

However, although numerous amplification-based strategies for miRNA detection were proposed, common technical challenges still exist. Compared with the well-established traditional methods, the electrochemical DNA platforms are still in the mid-to-late research stage. Technically, most miRNA electrochemical biosensors were utilized to totally extract miRNAs from cells or blood samples with pre-treatment. The stability and specificity of measurement results, especially in complicated samples, still need to be improved by developing reproducible and stable modification methods on electrodes and amplification strategies. Clinically, the detection of several miRNAs simultaneously is of great significance to identify disease-associated features. The amplification strategies which could be compatible with multiplex channels or chips are necessary. Since the electrochemical biosensors for glucose in the blood have been realized, the electrochemical biosensors are advantaged in analyzing miRNAs of actual samples. It is expected that the commercial electrochemical biosensors of reliable stability and accuracy of miRNA detection could be available with the advancement of technologies shortly.

Table 2 Comparison of different amplification strategies in this review

Methods	Mechanism	LOD	Linear range	Advantages	Limitations	References
Nanomaterials	PdNPs@Fe-MOFs/CHA	0.003 fM	0.01 fM–10 pM	Large surface area, numerous active sites, fascinating catalytic activity, enzyme-free, good sensitivity, and selectivity	Complicated material synthesis processes	Li et al. (2018)
	Au/PPy–rGO/CHA/HCR	1.57 fM	10 fM–5 nM	Excellent conductivity, enzymes free, high selectivity, satisfactory stability, and ideal reproducibility	Complicated sequence design, long reaction time	Bao et al. (2019)
	MWCNTs	0.032 pM	0.1–12,000 pM	Enzyme-free, high selectivity, wide linear range, simple design	Low sensitivity, long reaction time	Deng et al. (2018)
Enzyme	DSN/AuNP/GO	1.5 fM	1 fM−10 pM	Easy to operate	High cost, special enzymes needed, long reaction time	Han et al. (2019)
	SDR/Ca^{2+}-dependent DNAzyme	2.3×10^{-17} M	10^{-16}–10^{-11} M	Excellent sensitivity and selectivity	High cost, special enzymes needed	Yang et al. (2018)
	ALP/MgO nanoflower	50 aM	0.1–100 fM	Good stability and reproducibility, high selectivity	Special enzymes needed, long reaction time, low sensitivity	Shuai et al. (2017b)
HCR	DNAzyme-driven 3D walker and hyperbranched HCR-DNAzyme cascade	0.02 fM	0.1 fM–100 nM	Good sensitivity and anti-interference ability	Complex design of oligonucleotides sequence, specific enzyme needed	Li et al. (2021)
	HCR	53 aM	100–1×10^{11} aM	Cheap, easy to operate, simplicity, exosomal miRNA detection, wide linear range, highly selective and reproducible	Long reaction time, specific sequence design	Guo et al. (2020)
	Y-DNA recognition/nonlinear HCR	0.3334 fM	1 fM–10 pM	High specificity, cost efficiency, excellent sensitivity	Requirement of a particular sequence design, long reaction time	Zhou et al. (2019)
CHA	CHA/copper nanoparticles	1.1 fM	10 fM–10 nM	Low background, high sensitivity, reasonable specificity, single-base mismatch discrimination capability, excellent stability, and reproducibility	Requirement of a particular sequence design, ALP needed	Cui et al. (2020)
	CHA/DNA-TWJ	3.6 fM	10 fM–10 nM	Enzyme free, high sensitivity, good specificity	Requirement of a special sequence design	Jiang et al. (2020)
	CHA/NPG film	0.17 pM	0.5–500 pM	Facile, ultrasensitive, low-cost, and high anti-interference capability	Requirement of a particular sequence design, narrow linear range	Xu et al. (2021)

ALP alkaline phosphatase, *LOD* limit of detection, *MWCNTs* multi-walled carbon nanotubes, *NPG* three-dimensional nanoporous gold, *TWJ* three-way junction, *SDR* strand displacement reaction

Authors' Contributions LZ and WS contributed equally to this work. LZ and WS performed the literature survey and wrote the manuscript. SL and CH corrected the manuscript. BG and DA modified the paper. XD provided the theme and guided the manuscript writing.

Funding We gratefully thank the financial support from Shanghai Municipal Science and Technology Project (18430760500 and 2017SHZDZX01), Shanghai Municipal Education Commission Project (ZXWF082101), Shanghai Jiao Tong University Projects (YG2021ZD19, Agri-X20200101, SL2020MS026, 19X190020154, ZH2018ZDA01, YG2016QN24, YG2016MS60, 2020 SJTU-HUJI, 2019 SJTU-Usyd, SD0820016), Shanghai Municipal Health Commission Project (2019CXJQ03), Shanghai Clinical Medical Research Center Project (19MC1910800). Thanks for the support of Advanced Electronics Materials and Devices, Shanghai Jiao Tong University.

Availability of Data and Materials Not applicable.

Code Availability Not applicable.

Declarations

Conflicts of interest The authors declare that they have no competing interests.

Ethical approval Not applicable.

Consent to participate Not applicable.

Consent to publication Not applicable.

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