Recent Advances in Ultrasensitive miRNA Biomarkers Detection

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Abstract Recently, microRNAs gain a great interest in the bio-molecular field due to their fundamental role in clinical diagnostics. In this chapter, we generally discussed the biogenesis of microRNAs and the progress made for their detection. Researchers have widely tried to investigate their effort to build a sensitive, selective, and accurate platform for microRNA detection. To date, multiple techniques have been developed, ranging from the old conventional method (northern blot, RT-PCR, microarrays) to the newly established ones (biosensors, nanopores). However, given the various challenges related to miRNA detection, such as low abundance, small size, and high level of sequence similarity, different enzymatic and non-enzymatic amplification approaches were successfully exploited to improve such devices' sensitivity. Among these strategies, HCR, RCA, nanomaterials, and the use of enzyme-based target recycling like DSN enzyme. On the other hand, the combination of different methods

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has emerged as an ideal option for further enhancement of the sensitivity. In the end, knowing that the expression of a single miRNA is not enough to identify one specific disease, it is usually necessary to implant a simultaneous and multiplexed technique for more sophisticated and efficient diagnostic tools.

Keywords MicroRNAs · Optical biosensing · Electrochemical biosensors · Field-effect transistor (FET) · Nanopores assay · Nanomaterials · Amplification signal · Enzymes

1 MicroRNAs Overview

Around 98% of the human genome stand for non-proteins coding DNA. These specific regions are transcribed to generate functional non-coding RNAs (ncRNAs). Among them, microRNAs one of the highly reviewed forms of ncRNAs. MicroRNAs (miRNAs) are defined as a class of small endogenous RNAs including about 18–24 nucleotides. The Ruvkun and Ambros are the first groups to discover microRNA (line-4) in 1993. Over since, this significant finding has revolutionized the molecular biology field (Feinbaum et al. 2004; Wightman and Ruvkun 1993). MicroRNAs are key features for RNA splicing and post-transcriptional regulation of gene expression. To date, tremendous effort has been made in this field to comprehend the biogenesis and the mechanism of miRNAs action.

1.1 MiRNA Biogenesis and Functions

MiRNA biogenesis process goes through different steps. In the nucleus, RNA polymerase II generally binds to a promoter of the genome sequence transcribes miRNA genes, and so it generates the primiRNA with a length range from 1 to 3 kb. The resulting transcript is then split with the microprocessor complex Drosha DGGR8 (DiGeorge Syndrome Critical Region 8), releasing a stem-loop of about 70–100 nucleotides named pre-miRNA. The latter is carried via the aid of Exportin-5 to the cytoplasm, where the RNase-III enzyme Dicer will further cut it to obtain the 18–24 dsmiRNA. Afterward, the subsequent miRNA is hydrolyzed into single strand oligos. It yields the mature miRNA, which plays an essential and functional role in the RNA-induced silencing complex (RISC) with the argonaute (AGO) protein family. While the second miRNA strand termed "passenger" is frequently disintegrated or incorporated in downstream regulation effect along with the regulation of miRNA homeostasis. Generally, the 3'-untranslated region (3' UTR) represents the miRNA/mRNA binding sites using complementary Watson-Crick base pairings. Yet, miRNAs can interact with other regions, counting the 5' UTR, gene promoters, and coding sequences (Broughton et al. 2016). The RISC complex provokes whether degradation or translational repression or sequestration/ destabilization via

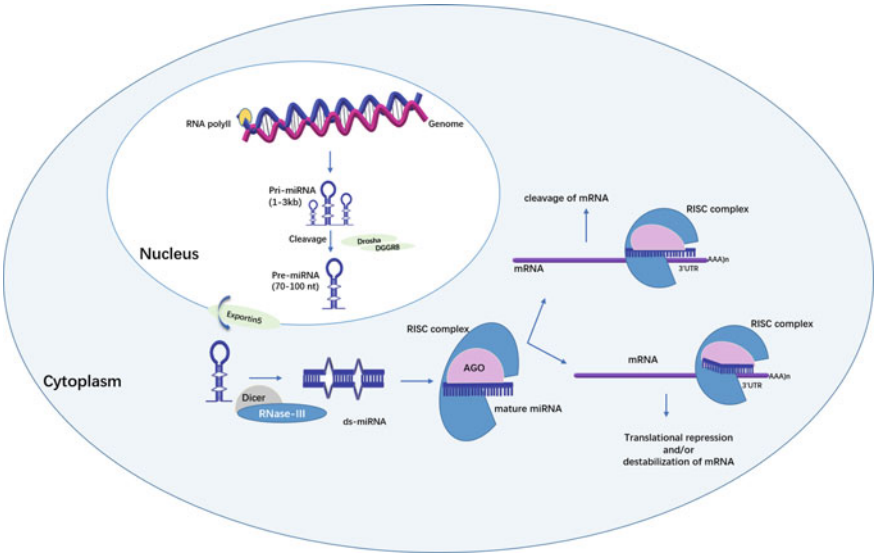


Fig. 1 Overview of miRNA biogenesis and regulation

a shortage of mRNA's polyA tail from translational machinery. At the same time, the microRNA hybridizes with its complementary mRNA target. Definite evidence has pointed out that one unique miRNA might have multiple targets, and similarly, a single gene can be regulated by numerous miRNAs. The degree of complementarity between miRNA and mRNA denotes a vital feature of the regulatory mechanism. A superior level of complementarity facilitates the mRNA degradation due to its cleavage mechanism process. On the other hand, a mismatch induces the translational repression mechanism (Fig. 1). It is ascertained that the human genome encodes for more than 1000 miRNAs, which regulate in turn approximately 60% of gene expression in different biological activities (Yu and Pan 2012). The various processes in which miRNAs are involved include cell proliferation, apoptosis, immunity, neuronal patterning, hematopoietic differentiation, and fat metabolism,

1.2 MicroRNAs in Different Pathological Processes

MicroRNAs give insight into the etiology and the progression of several pathologies. The expression profile of miRNA represents a core characteristic of all human diseases. It is a double-edged sword. It can provoke the disease or suppress it since some of these microRNAs are overexpressed in cells, tissues, VOC (volatile organic compounds), vesicles like exosomes, and biological fluids (blood, saliva, cerebrospinal fluid), while others are expressed at lower levels. For example, miRNA-21 is considered to be overexpressed in almost 80% of the tumor samples, while miRNA-143,

Table 1 Some miRNA involved in various diseases

Disease	Type of disease	microRNAs	Refs.
Cancer	Lung cancer	miR-155, miR-21, miR-126, Let7, miR-210	Wu et al. (2019)
	Breast cancer	miR-21, miR-16, miR-17, miR-106a, miR-126	Singh and Mo (2013)
	Colorectal cancer	miR-17-3p, miR-141, miR-486-3p, miR-601	Schetter et al. (2012)
	Ovarian cancer	miR-200 family, Let7 family, miR-141	Chen et al. (2019)
Neurological disease	Alzheimer's disease	miR-127-3p, miR-34a-5p, miR-93, miR-342-3p	Angelucci et al. (2019)
	Parkinson disease	miR-485, miR-29, miR-30, miR-Let7	Goh et al. (2019)
Infectious disease	HIV	miR-21,miR -122, miR-223, miR-3162-3p	Tribolet et al. (2020)
	VSV	miR-197, miR-629, miR-363, miR-132, miR-122	
	Malaria	miR-15a-3p, miR-30c-5p, miR-30e-5p	
Diabetes	Insulin resistance	miR-195, miR-135a, miR-7, miR-499, miR-96	Feng et al. (2016)
	Type 2 diabetes	miR-1249, miR-320b, miR-572, miR-126	
Vascular disease	Angiogenesis	miR-221, miR-222, miR-210, miR-130a, Let7	Qin and Zhang (2011)
	Atherosclerosis and restenosis	miR-125a,b, Let7, miR214, miR-146, miR-145	

miRNA-126, and miRNA-145 are downregulated in approximately 80% of the tumor samples. The first malady known to be allied with miRNA degradation was chronic lymphocytic leukemia, and the identified two microRNAs were miRNA-15a and miRNA-16-1 within locus 13q14 (Calin et al. 2002). Since then, many researchers have focused on microRNAs' role in producing genetic disorders through microRNA mediated chromatin reorganization or their implication in different human diseases and their utility as drugs. In Table 1, we listed multiple diseases associated with microRNAs based on the previous works, for instance, upon a variety of inflam-

matory stimuli, the level of miRNA-155, miRNA-147, miRNA146, and miRNA-9 increases markedly in macrophages. In cancer, two major microRNAs groups are identified: one with putative -suppressive, including the microRNA Let-7 family that plays a crucial role in stopping cancer cells' growth and it is known as a "post-transcriptional-gatekeeper" (Madison et al. 2015). The second group has oncogenic proprieties such as miR-21, which has been discovered as an inhibitor of the tumor suppressor PDCD4, promoting cancer's invasion and metastasis (Asangani et al. 2008). Various microRNAs are reported to be related to neurodegenerative maladies as Parkinson disease (increase level of miR-7 and miR-153) (Leggio et al. 2017) and Alzheimer's disease. For example, a high level of miR-125b stimulated Tau phosphorylation and aggregation by targeting phosphatases PPP1CA and DUSP6, whereas its low level induced a reduction in Tau phosphorylation (Banzhaf-Strathmann et al. 2014). MicroRNAs are also implicated in cell development and proliferation and other diverse pathological processes such as cardiovascular and autoimmune diseases, diabetes, and so more.

This chapter aims to summarize, compare, and analyze various biosensing technologies that have been developed recently towards microRNAs detection and comprehensively highlight recent advances in improving the performance of miRNAs biosensors with signal amplification using functional nanomaterials, nucleic acid circuitry, and/or enzyme.

2 MicroRNA Detection

2.1 MicroRNA Detection Challenges

Recently, multiple studies have shed light on microRNAs as biomarkers for the diagnosis of numerous diseases. Hence, the development of reliable and efficient biosensors with -cost strategies for detecting microRNA is required. Due to their unique characteristics, the analysis of microRNAs expression profile exhibits many challenges. Among the latter, the low abundance of microRNAs in real samples since it occupies a tiny fraction compared to the total RNA (around 0.01%) might affect the sensitivity, the duration of the assay and appeal for further enrichment and amplification steps. Add to that their sequence similarity among the same family and their stability to prevent accurate and selective detection. Another challenge associated with microRNAs detection is their small size. This propriety causes difficulties for a selective pairing of miRNAs and improves the cross-hybridization. Finally, the in situ detection may engender a false positive signal if researchers use a mixture of pre-miRNA and miRNA (Cissell et al. 2007). Based on these challenges, there is an ultimate need to develop a sensitive, selective, accurate, and rapid miRNA detection method.

2.2 Conventional Techniques Used for miRNA Detection

Northern blotting is the first and most used microRNA detection method and is still considered the gold standard for analyzing microRNA expression profiles. This technique is based on the target miRNA's hybridization captured on a nitrocellulose membrane's surface to a labeled probe. However, the major shortcomings of northern blotting are the high amount of sample loading (~10–30 μg), the low sensitivity with a detection limit in the range of nanomolar, and radioactive tags probes (Cissell et al. 2007; Válóczi et al. 2004; Ouyang et al. 2019). To omit these drawbacks, researchers tried to improve the detection of miRNAs via the use of locked nucleic acids (LNA) as a detection probe instead of standard DNA probes. As a result, they found that the sensitivity increases by 10-folds (Válóczi et al. 2004). Moreover, they employed digoxigenin (DIG)—labeling probe as an alternative for the previous radioactive probes. It has since proven that DIG has a short exposure time, high sensitivity, longer shelf life, and increased safety than 32P-labeled probes (Kim et al. 2010; Ramkissoon et al. 2006).

Quantitative PCR preceded by reverse transcriptome of miRNA to cDNA is also classified as a standard strategy for short ncRNAs detection. The major problem associated with this technique is the length of microRNAs that necessitate short primers. The latter may affect the efficiency of the method since it influences the melting temperature (Ouyang et al. 2019). Thus, researchers have made a great effort to solve this problem by amalgamating LNA to the microRNA primer (Pritchard et al. 2015), and combining DNA probe with the ribonucleotides via exploiting miRNA in the role of the template and T4 ligase. With the latter method, Zhang et al. were able to detect as low as 0.2 fM of miRNA (Zhang et al. 2011). They also used digital droplet PCR (ddPCR) since it is more accurate and sensitive in detecting miRNAs (Zhao et al. 2018b). Even though this method has several advantages, it is cumbersome and often used to validate or supplement other techniques (Ouyang et al. 2019). Biochip technology or microarray is another method that was frequently used for microRNA detection. A particular signal is generated to characterize the hybridization event when the labeled DNA probe attached to a solid surface binds to the target miRNA (Ouyang et al. 2019). This technique is designed for rapid and simultaneous detection of different microRNAs. However, it is more expensive, low sensitive, and selective. To ameliorate the detection results, scientists exploit nanomaterials and enzymes as signal amplification techniques. On the other hand, they used LNA instead of DNA probes for Tm normalization and better mismatch discrimination (Castoldi et al. 2006).

2.3 Emerging Techniques for microRNA Detection

Over the past decade, substantial scientific investments have been made to improve conventional methods and build up a suitable selective, sensitive, and high-throughput

platform to detect miRNAs such as biosensors. Recently biosensing gains great interest for the detection of microRNAs. Biosensor is a bioanalytical device that permits converting a biological reaction into a measurable signal in which there are different forms of transducers, including optical, electrochemical, and electrical (field-effect transistor and nanopore sensing). In this part, we will deal with these different strategies for the detection of microRNA.

2.3.1 Optical Biosensing

Optical assays have been considered an emergent tool for miRNA detection due to multiple advantages, including high sensitivity, convenience, and uncomplicatedness. Four types of assays are mainly involved in the optical sensing, fluorescent, colorimetric, surface-enhanced Raman scattering (SERS), and surface plasmon resonance (SPR). This section will include different optical biosensor for micoRNAs detection.

(1) Different probes used in optical sensing for microRNAs detection

Linear single-strand DNA/LNA/PNA represents the most used biological recognition unit for microRNA detection (Dave et al. 2019). The LNA probe possesses a high affinity towards small and highly similar targets and high thermal stability compared to traditional DNA probes. Therefore, it allows for better discriminatory function and better sensitivity (Silahtaroglu et al. 2004). PNA probe also gained great interest by scientists due to its advantages, including its faster hybridization, stability, and resistance to enzyme degradation (Vilaivan 2018). Molecular beacons (MBs) is another different form of microRNA probe characterized by a hairpin shape. It is also widely used for microRNAs detection. This probe is generally labeled from both ends, one modified with a fluorophore and the second end tagged with a quencher. Once this probe is hybridized with the target microRNA, the short double-strand will be dissociated to generate a linear form, thus restoring its fluorescence (Tyagi and Kramer 1996; Mittal et al. 2019). MB is an excellent alternative for multiplexed microRNAs detection (Lee et al. 2016) though; its major limitation is associated with the depleted quenching effectiveness (Shu Zhu et al. 2019). A Y-shaped DNA probe can also be investigated for microRNAs detection. It is formed when three oligonucleotides; two probes, and a target DNA complementary binds to each other. This kind of probe is sensitive to single base mismatch (Zhang et al. 2018; He et al. 2016).

On the other hand, one of the most used DNA nanostructures is a tetrahedron with six double-helical edges, assembled by annealing four DNA strands with partial complementarity. DNA tetrahedron have been used widely in microRNA detection in recent years (Xu et al. 2016). This DNA shape has various characteristics, such as good rigidity, enzyme resistance, and excellent cellular permeability (Goodman et al. 2005). The choice of the right probe is based on the application itself and the craved results.

(2) Colorimetric sensor

Attributable to its advantages, including low cost and quick response, colorimetric biosensor has become an appealing microRNA detection technique. It is based on organic chromogenic substrates, such as ABTS and TMB to form colored products via enzymatic catalysis (Zhu and Gao 2018). However, this type of biosensor lacks sensitivity and selectivity compared to others. Hence, noble metal-based localized surface plasmon resonance (LSPR) represents a better alternative for the traditional colorimetric sensor for microRNAs detection since its first use by Joshi et al. (2014), Bellassai et al. (2019). In most cases, researchers profited from AuNPs for the LSPR based colorimetric sensor since they can control their colloidal aggregation and dispersion phases by utilizing the target analytes. The color of the solution and the aggregation of nanoparticles are mainly influenced by the balance between particle attraction and repulsion forces. In the case of aggregation, the reason can be attributed to the "interparticle-crosslinking" or the "no crosslinking-aggregation" mechanism. In this context, Huang et al. designed a colorimetric experiment for the detection of miRNA-21 using AuNPs and DSN enzyme for better signal amplification. After the different amplification cycles, the aggregation of AuNPs is derived by the use of divalent cations (Mg_2^+). The detection limit was about 50 pM with a linear range from m 50 pM to 1 nM. This method shows great discrimination results even for a single mismatch and it reveals high performance for biomedical application since it was tested for cell lysates (Fig. 4) (Huang et al. 2019).

(3) Surface plasmon resonance (SPR) sensor

Surface plasmon resonance (SPR) represents the fact where the electrons of the surface metal layer are excited via the light photons with a certain incidence angle and then spread parallel to the metal layer (Fig. 2c). A slight variation in the reflective index will occur in the analyte presence, making it possible for microRNAs detection (Zeng et al. 2017). Nanomaterials represent an ultimate strategy for signal amplification in SPR based biosensors. Mouzavi and his co-workers succeeded in developing a dual hybridization biosensor based on the SPR on capped gold nanoslits using magnetic nanoparticles as a target enrichment tool and signal amplification strategy to detect the urinary miRNA-16-5p. They were able to reach 17 fM as a limit of detection. This technique allocates the detection of the target miRNA in the urine of acute kidney injury patients (Mousavi et al. 2015). Li and his co-workers developed an SPR biosensor composed of two layers of graphene oxide-gold nanoparticles (GO-AuNPs) composites (Fig. 2b). The first layer of the nanocomposite functionalized with the capture probe was immobilized onto the surface of Au film modified with thiocyanuric acid In contrast, the upper layer (used for amplification) is modified with an assistant probe. In the presence of the target miR-141, both layers are joined (Fig. 2a). The detection limit reached 0.1 fM, and the selectivity was tested in the presence of different miRNA-200 family members (Li et al. 2017). Another study by Wang's group wherein they resorted to an SPR platform employing gold film on which is immobilized the capture probe for miRNA-141 detection and gold nanoparticles-decorated molybdenum sulfide where the assisted DNA that binds to

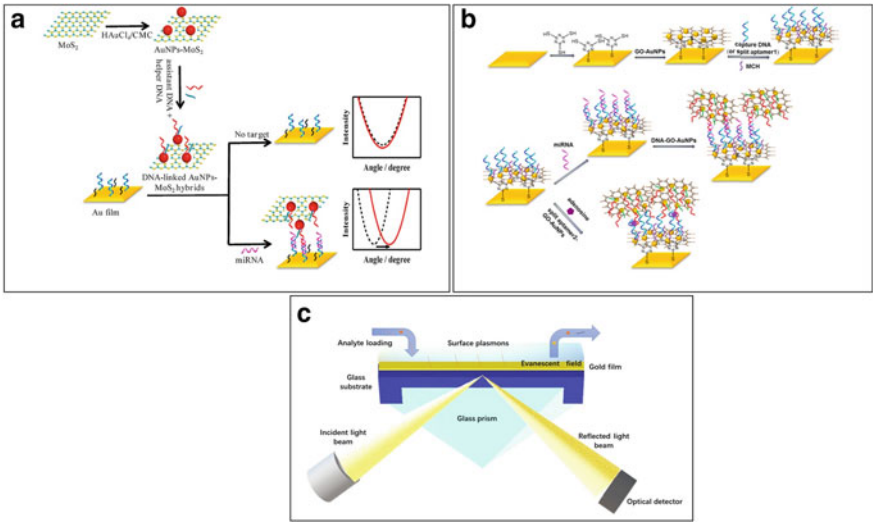


Fig. 2 **a** SPR biosensor for microRNA-141 detection using AuNPs-MOS_2 hybrids for signal enhancement **b** SPR biosensor for microRNA-141 detection using double layers of graphene oxide-AuNPs for signal enhancement **c** SPR detection principle. Reproduced with permission (Nie et al. 2017; Li et al. 2017)

a segment of miRNA-141 is linked. This enzyme-free method displays high sensitivity with a limit of detection equal to 0.5 fM and high selectivity. Moreover, this method has an excellent capacity for complex samples, such as human serum (Nie et al. 2017).

(4) Surface-enhanced Raman scattering

SERS is a surface-sensitive method based on Raman scattering enhancement via the use of irregular metal shells or nanostructures. Considering that the signal boost may attain 1011-fold, SERS has become a captivating tool for disease-related microRNAs detection. For SERS, two significant theories exist; the electromagnetic theory relies on the excitation of localized surface plasmons and the chemical concept that aims to create charge-transfer complexes. Guo et al. designed a sandwich-type method for simultaneous detection of three different microRNAs (miR-21, Let-7d, and miR-141). In this study, they fastened thiol-containing Raman dyes on Au@Ag nanosnowmen nanoprobes. They employed the gold substrate in SERS-active substrates' role, wherein they fixed the different capture probes. The limit of detection was 1.089 fM, 0.839 fM, and 1.019 fM, respectively (Guo et al. 2018). Some groups took advantage of combining the use of nanomaterials with the enzyme-based signal amplification such as DSN and exonuclease to improve the sensitivity. For instance, Yang and his colleagues developed a sensitive platform for miR-155 detection based on TB@$CaCO_3$ loading DNA microcapsule with the SERS active substrate Si@Nafion@Ag. In the presence of the target miRNA and via the aid

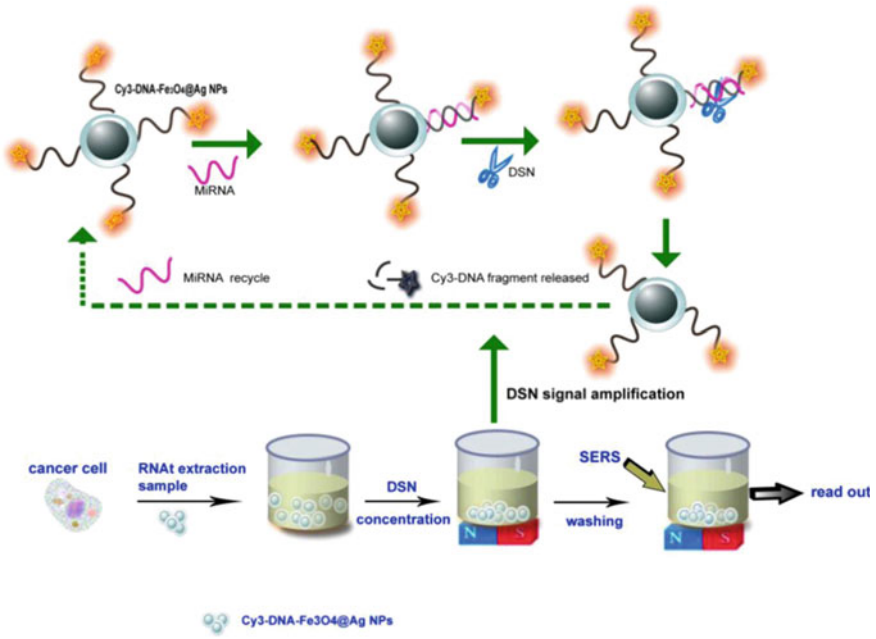


Fig. 3 SERS detection of the miRNA Let-7b using Fe_3O_4@Ag nanocomposites and DSN enzyme for signal amplification. Reproduced with permission (Pang et al. 2016)

of enzymatic amplification strategy using duplex-specific nuclease, the Raman dye (TB) was discharged from TB@$CaCO_3$ composite. The achieved detection limit was about 0.67 fM (Yang et al. 2018). Zhu's group created a "signal-off" platform for miRNA-21 detection by incorporating the T7-Exo enzyme as a recycling amplification approach along with gold decorated silicon nanowire arrays. To ensure further enhance the sensitivity of their SERS sensor; they employed Au nanostar probes to provide a stronger electromagnetic field. Via this technique, they were able to detect as low as 0.34 fM (Wen et al. 2019). Another study conducted by Xiao's group for Let-7b detection relies on the immobilization of DNA probe onto the surface of Fe_3O_4@Ag NPs. When the hybridization with the target miRNA takes place, the DSN initiates the target recycling cycles. The nanoparticles were collected using a permanent magnet, and the SERS signal was measured (Fig. 3). This technique possesses a fabulous limit of detection (0.3 fM) and an excellent recovery while checking it for different cell lines (Pang et al. 2016).

(5) Fluorescence-based sensor

Fluorescence assay is the most predominantly method used for microRNA biosensing (Ye et al. 2019). It depicts the fact that a fluorescent dye or molecule absorbs and emits light. In the absorption phase, the fluorescent tag is excited by light with high-energy (short wavelength), stimulating electrons' transition within the fundamental

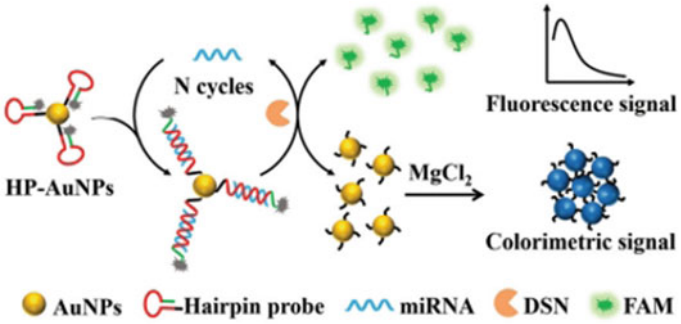


Fig. 4 Principle of the fluorescent and colorimetric biosensor using AuNPs designed for microRNA-21 detection based on DSN assisted target recycling. Reproduced with permission (Huang et al. 2019)

state's molecule to the excited state. Once in this state, and within nanoseconds (the fluorescence lifetime), the electrons will release their stored energy and rest back to the ground state, generating an emitted photon with lower energy (longer wavelength) compared to the absorbed light (Zhu and Gao 2018). Recently, a new fluorescence approach called fluorescence resonance energy transfer or Förster resonance energy transfer (FRET) has gained great interest in miRNAs detection. This strategy relies on energy transmission from a fluorescent donor to an acceptor. Two main conditions are needed for FRET: (i) the donor's emission wavelength corresponds to the fluorescent acceptor's excitation wavelength, and (ii) the distance between them does not exceed 10 nm (Sapsford et al. 2006).

Attributable to the effect of the considerable limitations, many nanomaterials can directly engender fluorescent emissions with the exalted significant harvest, excellent photo-stability, and long fluorescence lifetime, including quantum dots (QDs). Jie et al. reported a multiplexed fluorescent sensor for detecting miRNA-141 and miRNA-21 using two different kinds of quantum dot (CdSe@ZnS and CdTe) probes and target recycling amplification strategy. The thiolated hairpin DNA probe was immobilized onto the magnetic bead's surface decorated with Au nanoparticles (MB@Au). During hybridization with the target miRNAs, Exo III-based target recycling is unthreaded, generating short ssDNA sequences attached to the surface of MB@Au. Afterward, the latter is hybridized with QDs fluorescent probes that will activate the cleavage by adding Nb.BbvCI enzyme followed by magnetic separation and underwent for fluorescence measurements. The achieved detection limit for miR-21 and miR-141 was 1.5 pM (Jie et al. 2017).

Nanomaterials can also serve as effective fluorescence quenchers (Fig. 5). For instance, some of these nanomaterials, such as gold nanoparticles (AuNPs), (Huang et al. 2019) graphene, (Tan et al. 2019) WS_2, (Xi et al. 2014) were investigated to detect miR-21 using DSN based signal amplification. The calculated limit of detection was 50 pM, 1.5 pM, and 300 fM, respectively. Moreover, the same approach for miRNA Let-7a detection was used employing metal nanomaterials such as a

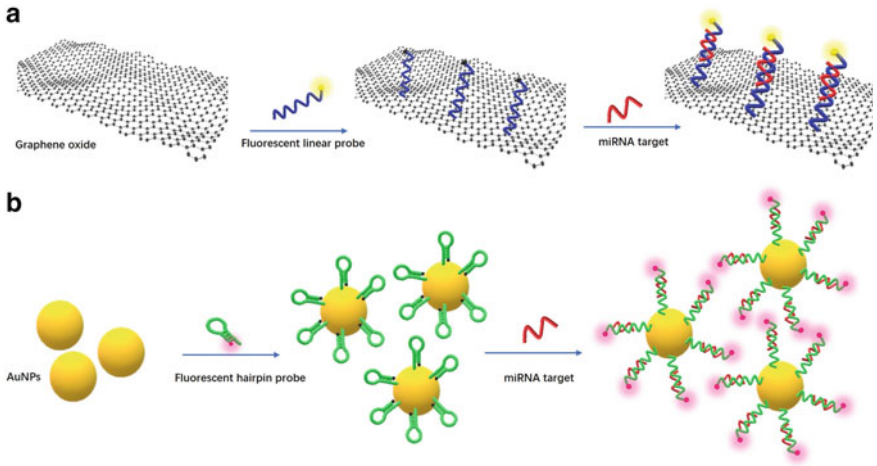


Fig. 5 Principle of the fluorescence quenching: **a** using graphene oxide, **b** using gold nanoparticles

magnetic bead, and they were able to detect as low as 300 fM (Shen et al. 2015). Xie group reported a fluorescent-based biosensor to detect miRNA-141 using graphene oxide and rolling circle amplification along with Exo III. This method exhibits a good selectivity and sensitivity along with high performance when testing the spiked miRNA in the human serum, and the LOD was about 0.1 aM (Li et al. 2019). All these methods show tremendous results for the selectivity test towards mismatches and different miRNAs families. The exploitation of nanomaterials for fluorescent sensors not only expands the selectivity, the sensitivity, and the performance of the detection of clinical biomarkers (miRNA), but also it opens the door to plenty of novel approaches to conceive fluorescent sensors.

2.3.2 Electrochemical Biosensor for miRNA Detection

Electrochemical methods exhibit superior benefits own to their capability of miniaturization, selectivity, and capability compared to other analytical techniques, which uplift it to have widespread use in agriculture, food, environment fields, and medical application (Bahri et al. 2019). Also, electrochemical biosensors demonstrated pleasant for serving simple, fast, and point of care analysis. The transduction element for such a genosensor can be in multiple materials: glassy carbon, gold, indium tin oxide, graphite, or the modification of these materials using multiples nanoparticle, nanotubes and/or nanowires. The fundamental principle in the electrochemical detection of miRNAs is measuring the variations either in the electrode properties (resistance or capacitance) or in the electrochemical tag's signal against the target miRNA hybridization with the complementary probe.

(1) Impedimetric miRNA biosensors

Electrochemical impedance spectroscopy (EIS) is a useful detection technique that delivers crucial information while modification processes like; hybridization, nanostructure formation, and bio-functionalization. Furthermore, EIS is a powerful tool for surface adsorption explore and electrochemical reaction mechanisms investigation (Tlili et al. 2005, 2003) . When it comes to miRNA detection, EIS has been employed because it is label-free and practical. Some works detail EIS as a detection strategy for miRNA (Erdem et al. 2017) while others describe it as a characterization method (Erdem et al. 2015). Peng and Gao (2011) suggested an electrochemical impedance-based biosensor using a novel sensing protocol via the amplification of miRNA's electrochemical detection and greatly enhanced sensitivity and specificity. The developed biosensor was based on a direct ligation procedure through a direct tag of miRNAs with ruthenium oxide nanoparticles (RuO_2 NPs). The RuO_2 NPs-initiated polymerization of 3, 3'-dimethoxybenzidine (DB), and the hybridized miRNA strands and free capture probe (CP) strands guided the deposition of poly(3, 3-dimethoxybenzidine) (PDB). Whereas the biosensor surface was made of a mixed monolayer of 4-mercaptoaniline and oligonucleotide capture probes on a gold electrode. Following this protocol, and after 60 min of incubation into DB/H_2O_2 mixture, the developed biosensor showed a linear charge transfer resistance-concentration relationship from 6 fM to 2 pM and a detection limit (LOD) of 3 fM (Fig. 6a).

Similarly, using PDB polymerization strategy, Gao and co-workers suggested another label-free detection of circulating miRNAs in blood along with miRNAs extracted from cultured cells with a detection limit of 2 fM (Gao et al. 2013a) (Fig. 6b). Graphene oxide modified pencil graphite electrode (PGE) was also utilized for electrochemical detection of miRNA-34a and showed appealing discrimination of the target miRNA against miRNA-155, miRNA-660, and miRNA-15a in buffer medium and in the fetal bovine serum (Congur et al. 2015) (Fig. 6c). In another work, the graphene-modified disposable pencil graphite electrode (GME) was utilized by Ozsoz team (Kilic et al. 2015), wherein they used voltammetric and impedimetric detection techniques for the detection of microRNA-21. They reported a high performance of the suggested technique toward miRNA-21 in both buffer and cell lysates samples with a LOD of 2.09 μg/mL. Zhang et al. (2016) developed an electrochemical impedance biosensor with immobilization-free for amplified detection of miRNA-21. In their work, they have used a strategy based on a duplex-specific nuclease (DSN) assisted target recycling and capture probe enriched from the solvent to the magnetic glass carbon electrode surface (as a working electrode) using magnetic beads. Due to the low activity of duplex-specific nuclease (DSN) to ssDNA, capture probes cannot be hydrolyzed in the absence of miRNA-21. However, in the presence of the target miRNA-21 forming DNA-RNA heteroduplex through the hybridization with the capture probes, the DSN hydrolyzed the target-binding portion of the capture probe while liberating the intact miRNA-21 to be hybridized with another capture probe and a second hydrolization cycle is starting. Finally, all capture probes were digested. Moreover, real sample measurements were made using human serum samples from breast cancer patients selective attempts toward miRNA-21 detection. By

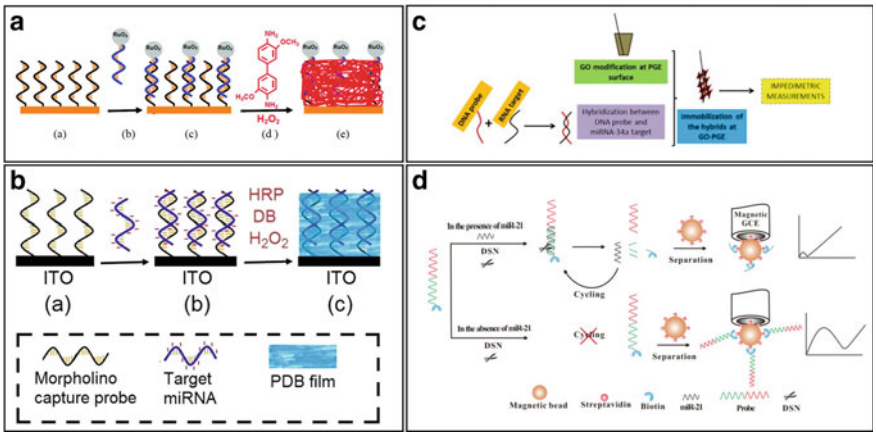


Fig. 6 Examples of impedimetric miRNA biosensors strategies. **a** Experimental principle of the amplified detection of miRNA based on RuO_2 NP. **b** Schematic illustration of the label-free biosensor for electrochemical detection of femtomolar microRNAs. **c** The working strategy of the impedimetric Detection of microRNA at Graphene Oxide Modified Sensors. **d** Experimental principle of immobilization-free electrochemical impedance biosensor based on DSN assisted target recycling for amplified microRNA detection. **a** Reproduced with permission (Peng and Gao 2011), **b** Reproduced with permission (Gao et al. 2013a), **c** Reproduced with permission (Congur et al. 2015), **d** Reproduced with permission (Zhang et al. 2016)

employing such a strategy the developed biosensor displayed an ultrahigh sensitivity for miRNA-21 with a LOD of 60 aM (Fig. 6d).

(2) Amperometric miRNA biosensors

Amperometry is an electroanalytical method based on the current resulting from the application of a constant oxidizing or reduction potential and respect to time to a working electrode (indicator). Usually, the magnitude of the obtained current depends on the concentration of the oxidized or reduced substance. Thus this method can be used for various analytical applications.

(a) Chronoamperometry

Chronoamperometry is a time-dependent method where the square-wave potential is applied to the working electrode. The current to time measurement of the electrode fluctuates depending on the diffusion of the solution immersed in the bulk analyte toward the sensor surface. Therefore, chronoamperometry can be applied to detect current-time dependence for the diffusion-controlled process occurring at an electrode, which varies with analyte concentration. For example, Liu et al. (2014) developed a label-free and highly-sensitive strategy using triple signal amplification via AuNps, AP and p-aminophenol (p-AP) redox cycling for miRNA detection into a range of 10 fM–5 pM (Fig. 7a), with a limit of detection (LOD) of 3 fM. The followed strategy was based on the difference between RNA and DNA structures. The DNA probes were first immobilized onto the Au electrode, then it hybridized with

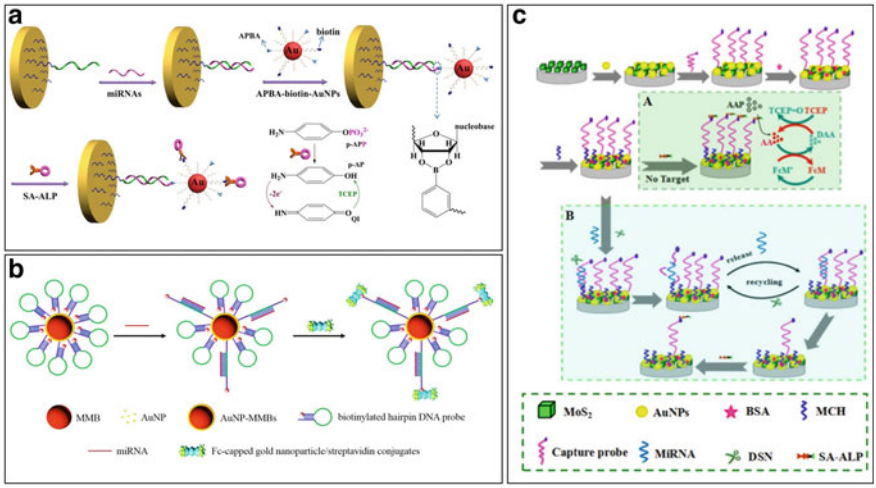


Fig. 7 Examples of electrochemical detection of miRNA strategies. **a** Schematic illustration of the label-free detection of miRNA using triple signal amplification of APBA-biotin-AuNPs, SA-ALP, and the p-AP redox-cycling reaction. **b** Amplified voltammetric detection of miRNA via the emergence of conducting magnetic microbeads and ferrocene-capped gold nanoparticle/streptavidin conjugates. **c** miRNA-21 detection using signal amplification of DSN and ECC redox cycling via AuNPs/MOS_2 modified electrode. **a** reproduced with permission (Liu et al. 2014), **b** reproduced with permission (Lu et al. 2016), **c** reproduced with permission (Shuai et al. 2017)

the target miRNA. The cis-diol ribose sugar group at the end of the miRNAs chain interacted with the 3-aminophenylboronic acid (APBA)/biotin-modified AuNPs via a boronate ester covalent bond. The obtained complex was then consented to react with the streptavidin-conjugated alkaline phosphatase through the biotin-streptavidin interaction. After accumulating the 4-aminophenylphosphate (p-APP) substrate, the enzymatic conversion from p-APP to p-AP developed. The resulting p-AP possibly cycled via a chemical reducing reagent after its electro-oxidization on the electrode, thus increasing the anodic current. In another work, Castaneda et al. (2017) combined the electrocatalytic amplification (ECA), and duplex-specific nuclease (DSN) catalyzed amplification for the detection of miRNA. In their work, they have used Pt nanoparticles to enhance the catalytic electrochemical reaction on inert Au ultramicroelectrodes (UMEs) after miRNA-capture probe hybridization followed by DSN enzyme reaction on DNA.

(b) Chronocoulometry

Compared to chronoamperometry, Chronocoulometry is also similar, except that the readout is a charge variation in function of time. Chronocoulometry exhibits the advantages of effective integration in terms of reducing the noise signal, where it is easy to distinguish and separate the capacitive charge and the faradic charge. Masud et al. (2017) developed a nonenzymatic, amplification-free, and highly sensitive miRNA based on gold-loaded nanoporous iron-oxide nanocubes

(Au@NPFe$_2$O$_3$NC). The assay displayed a remarkable selectivity toward miRNA21 with a detection limit down to 100 fM in cell lines and tissue samples from oesophageal squamous-cell carcinoma patients. Whereas, Miao et al. (2016) demonstrated an electrochemical miRNA biosensor based on DSN enzyme, which relied on the cleavage of DNA-RNA hybrid duplex cleavage by DSN and the miRNA target association with $[Ru(NH_3)_6]^{3+}$ upon introduction of AuNPs due to DNA1 and DNA2 probes hybridization. The used method exhibited a LOD as low as 50 aM into a dynamic range from 0.1 fM to 100 pM. This direct detection method without the requirement of miRNA conversion to cDNA also showed excellent discrimination of miRNA family in real complex sample formed of throat swabs H1N1 influenza-infected patients diluted with sterile saline.

(3) Voltammetric miRNA biosensors

Voltammetry method is classified as an electroanalytical technique, where the current measurement is achieved at a potential ramp. The wide variety of methods in which the applied potential may be varied leads to numerous type of voltammetric techniques, including Cyclic voltammetry (CV), Square wave voltammetry (SWV), and Difference pulse voltammetry (DPV) (Rezaei and Irannejad 2019).

(a) Cyclic voltammetry (CV)

CV is widely used to get useful information about redox potentials and explore the mechanisms and kinetics parameters involved in electroactive analyte solutions' reactions. When it comes to miRNA detection CV technique can be used for label-free and labeled detection. Label-free detection of miRNAs is generally a simple method where no additional labeling steps are required, making it less time consumption, more practical, and usually low cost compared to label-based methods. The plurality of the label-free miRNA biosensors approaches is based on identifying electroactive nucleic acid base signals before and after hybridization. AuNPs have been widely used and so popular for nucleic acid-based detection strategies. Following the same strategy of dsDNA and ssDNA's adsorption ability on AuNPs, Li et al. (2016) developed a let-7a miRNA biosensor with a LOD of 16 fM in human breast adenocarcinoma cells. Lu et al. (2016) reached a lower LOD of 0.14 fM using AuNPs based method and the higher loading density of biotinylated hairpin-structured DNA probes that opened after hybridization and further interacted with ferrocene-capped streptavidin-conjugated (Fig. 7b).

(b) Square wave voltammetry (SWV)

SWV technique is one of the most sensitive and fastest pulse voltammetry techniques. The obtained detection limits are comparable with those obtained using spectroscopic and chromatographic techniques (Simões and Xavier 2017). In one study, Tran et al. (2013) have used the SWV to detect miRNA-141 by taking the advantage of the quinone group existing inside the nanostructured polymer film containing an electroactive polymer and carbon nanotube. The presence of the target microRNA-141 generated a signal-on response resulting from the enhancement of the polymer. The detection of prostate cancer biomarker mir-141 was achieved using square wave

voltammograms leading to a LOD of 8 fM. In a similar context, Labib et al. (2013) designed a three-mode experience electrochemical biosensor based on hybridization, protein displacement, and P19 protein binding for the detection and quantification of miRNAs with a LOD of 5 aM without the requirement of PCR amplification. MiRNA biosensors-based hybrid nanomaterials also showed a greatly enhancement of sensitivity and limit of detection. For example, an interesting hierarchical flower-like Au nanostructure that exhibits a large surface area and thus enhances the sensitivity up to 1 fM also enables discrimination of the target miRNA from cervical cancer cells (HeLa) and lung cancer cells (A549) (Su et al. 2016). Besides the AuNPs, ruthenium nanoparticle (RuO_2) was also used for miRNA detection along with high conductivity and a robust catalytic activity (Peng et al. 2010).

(c) Difference pulse voltammetry (DPV)

DPV is a technique that implicates applying amplitude potential pulses on a linear ramp potential. Using DPV, a base potential value is selected at which there is no faradaic reaction and is applied to the electrode. The bias potential is raised between pulses with equal increments. The current is instantly measured before the pulse application and at the end of the pulse. Consequently, the difference between them is registered. Label-free detection of miRNA based on DPV measurements was also widely used, leading to high discrimination of the target biomolecules along with high sensitivity and a low limit of detection (LOD). Kilic et al. (2013) suggested an alternative procedure in which they used the P19 viral protein as a bio-recognition element for label-free electrochemical detection of miRNA-21 due to its particular binding ability RNA duplexes. The developed mir-21 biosensor was based on the oxidation signal of tryptophan in p19 protein afore and after interaction of the protein with miRNA hybrid and displayed a picomolar detection limit in real samples measurement. The selectivity test was proven with two control experiments, firstly via a non-complementary miRNA (miRNA-192), which leads to a non-hybridization or p19 sequencing. Whereas the second test was based on glucose oxidase enzyme (GOX) instead of p19, confirming the specificity of protein toward the double-stranded RNA (dsRNA).

Yang et al. (2009) also developed a label-free electrochemically inactive inosine-substituted probe sequence that was utilized to get an assay with a yes/no signal when the target miRNA was detected using the DPV technique. Isin et al. (2017) modified the graphene oxide onto the surface of a pencil graphite electrode (PGEs) and the modified electrode was used for the first time for voltammetric monitoring of miRNAs-34a biomarker related to Alzheimer disease. The CA/GO/PGE characterization was investigated via CV, EIS, and scanning electron microscopy (SEM). The GO concentration, DNA probe concentration, and miRNA-34a were optimized. Thus, under the optimum conditions, the developed biosensor exhibited a LOD of 7.52 μg/mL into a linear concentration range from 5 to 35 μg/mL.

Although these methods display a more straightforward, fast solution for miRNA detection and quantification, but still required sensitivity enhancement that could not be met most of the time, modifying the surface properties or the electrode's conductivity via metal nanoparticles, electroactive materials or taking advantage of

enzymes for the signal amplification could significantly lower the limit of detection and enhance the biosensor's sensitivity. Electroactive materials such as methylene blue (MB) and meldola's blue (MDB) have been employed as electroactive redox labels for hybridization indicators in electrochemical nucleic acid as well as miRNA biosensors. Using DPV technique, Kilic et al. (2012) studied miRNAs detection based on alkaline phosphatase (AP) enzyme. In this work, kilic and coworkers described an assay usage of (PGE) for oligonucleotide capture probe immobilization via EDC/NHS chemistry to record the α-naphthol oxidation signal of AP enzyme after the hybridization with target mir-21. Similarly, Rafiee et al. (2016) developed a simple electrochemical miRNA biosensor using methylene blue (MB) as an electroactive intercalator.

The DPV technique was used to study the oxidation of MB associated with the hybridization event between the probe DNA and the target miRNA-21. This approach displays a high selectivity and sensitivity with a LOD of about 84.3 fM. In another study, Kapton et al. (2017) reported an electrochemical sensor for the detection of mir-21 extracted from breast cancer cell (MCF-7) based on Meldola's blue (MDB) reduction. Wherein, they employed the electropolymerized polypyrrole modified pencil graphite as an electrode (PPy/PGE). This method displayed a high sensitivity and a LOD of 0.17 nM.

Hybrid nanomaterials also showed superior features for miRNA detection, like molybdenum disulfide (MOS_2) microcubes (Shuai et al. 2017) and tungsten oxide-graphene composites (Shuai et al. 2016). Shai et al. (2017) designed an ultrasensitive electrochemical biosensor for miRNA detection using hollow molybdenum disulfide (MOS_2) microcubes and DSN enzyme was used for signal amplification.

Firstly, the biotinylated ssDNA probe was immobilized onto AuNPS/MOS_2 modified electrode for streptavidin-conjugated alkaline phosphatase combination (Fig. 7c). When the DSN cleaves the formative duplexes of the capture probes and miRNAs, the biotin group strips from the electrode's surface, and the streptavidin-conjugated alkaline phosphatase is not able to be attached to the electrode surface. Subsequently, ascorbic acids create the electrochemical-chemical-chemical redox cycling and making the electrochemical response in the attendance of ferrocene methanol and tris (2-carboxyethyl) phosphine. The suggested method exhibits a LOD of 0.086 fM into a concentration range from 0.1 fM to 0.1 pM. Additionally, the biosensor showed a successful capability to detect target miRNA-21 in human serum samples.

2.3.3 Field-Effect Transistor (FET)

Additionally to the electrochemical detection method, field-effect transistor (FET) is also a prominent part of the electrical detection. Recently, label-free detection based FET biosensors has shown great attention and prospects considering no electrochemical tags. However, attain high capability detection and sensitivity. Gao et al. (2013b) reported the design of an ultrasensitive, real-time, and label-free detection of miR-21 using silicon nanowire (SiNWs) field-effect transistor biosensor array. The

sensing channel of silicon nanowires was synthesized via optical lithography and anisotropic self-stop etching. Further, the obtained device was functionalized with 3-aminopropyltriethoxysilane (APTES). The terminal carboxyl group of the capture probe DNA was then conjugated to the amine group of the APTES-modified SiNWs via EDC/NHS chemistry. The described strategy leads to the detection of miR-21 with a LOD of 1 fM and high selectivity for single-nucleotide polymorphism discrimination.

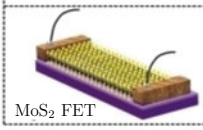
Graphene a 2D sheet of sp^2 bonded carbon atoms sparked research on 2D materials that are blooming at a tremendous rate owing to its unique properties, such as high carrier mobility, low electrical noises, ease of fabrication and functionalization, and large active detection area (Hwang et al. 2020). Song et al. (2020) developed a 3D graphene FET (GFET) biosensor, ssDNA as a probe was immobilized with 1-pyrenebutanoic acid succinimidyl ester (PBASE) as a molecule linker.

The manufactured biosensor showed a linear detection response in a concentration range of 100 pM–100 nM with high sensitivity of about 35 mV/nM and a LOD of 100 pM. Femtomolar miRNA detection was also proposed by Zhang team (Cai et al. 2015) based on GFET decorated gold nanoparticles.

The emerge of gold nanoparticles with peptide nucleic acid (PNA) employment instead of DNA leads to a higher hybridization efficiency with a LOD as low as 10 fM. What's more, the obtained assay exhibits accurate discrimination of complementary miRNA from non-complementary miRNA and one-base mismatched miRNA. Very recently, Gao et al. (2020) reported a free labeling and flexible GFET biosensor along with robust performance, specific and ultrasensitive detection of miRNA. The DNA probes were immobilized onto the graphene channel via $\pi - \pi$ stacking interaction without molecules linker or surface functionalization. The proposed biosensor finished the miRNA detection in only 20 min and displayed a detection ability down to 10 fM.

Molybdenum disulfide (MOS_2) as a transition metal dichalcogenides (TMDCs) material has also added exceptional value to the electrical measurement-based biosensors field-effect transistor. MOS_2, with its direct-to indirect tuning structure as varying its layer number from single layer to bulk, respectively (Sarkar et al. 2014). Numerous studies on MOS_2 have fully emphasized its extraordinary potential for electronic components. Also MOS_2-based biosensors for DNA or proteins detection have been reported (Sarkar et al. 2014). Furthermore, a label-free ultrasensitive biosensor platform using MOS_2 FET gadgets were developed for breast cancer biomarker (miRNA-155) in human serum and cell line samples (Majd et al. 2018). The developed biosensors displayed high mobility of $1.98 \times 103\ cm^2V^{-1}\ s^{-1}$ and a LOD of 0.03 fM under a complementary target miRNA-155 concentration range from 0.1 fM to 10 nM. Table 2 is resuming and illustrating the conventional development methods of miRNA biosensors based field-effect transistors.

Table 2 Conventional development methods of miRNA biosensors based field-effect transistors

FET structure	Sensing material	Surface functionalization with	DNA probe	miRNA	Work example	LOD
 SiNW FET	 Silicon Nanowires	APTES + NH_2 Probe			Gao et al., 2013	1 fM
 GFET	 Graphene	AuNPs + SH Probe			Cai et al., 2015	10 fM
		PBASE + NH_2 Probe			Song et al., 2020	100 fM
		Adsorption			Gao et al., 2020	10 fM
 MoS_2 FET	 TMDCs (MoS_2)	Adsorption			Majd et al., 2018	0.03 fM

2.3.4 Nanopore Technology

Nanopore technology has a strong potential to enhance the development of low coast high-performance single-molecule sensing platforms. Nanopore-based sensors have been successfully used as a detection platform for different biological targets, such as DNAs, aptamers, and MicroRNAs (Wanunu 2012; Zhao et al. 2018a; Sultan and Kanavarioti 2019). The detection principle of nanopore-based sensors is inspired from the Coulter classical counter routine; a nanopore chip is embedded inside a flow cell between two chambers (cis and trans). The two reservoirs are filled with an ionic buffer (KCL, LiCl, $CsCl_2 \cdots$). The application of an electric potential through 2 Ag/AgCl electrodes will force electrons to move from one chamber to another, establishing a current baseline called open pore current. When the target molecule is added to one side of the flow cell, the molecules will be driven to pass through the pore under the applied potential. The passage of the molecules through the pore will temporarily block the pore and prevent electrons from circulating. However, a current drop will appear in the as-established baseline current whenever a molecule passes through the pore. The main parameters to extract from a nanopore sensor are the current drop intensity and the dwell time, which are the characteristics of the target (Lee et al. 2018). Nanopore sensors are divided into two families biological and synthetical nanopores (Fig. 8). The first family is based on a protein channel embedded directly within the lipid bilayer. In contrast, the second one is founded on a nanometric pore drilled in a free-standing membrane (Shi et al. 2017).

(1) Biological nanopore

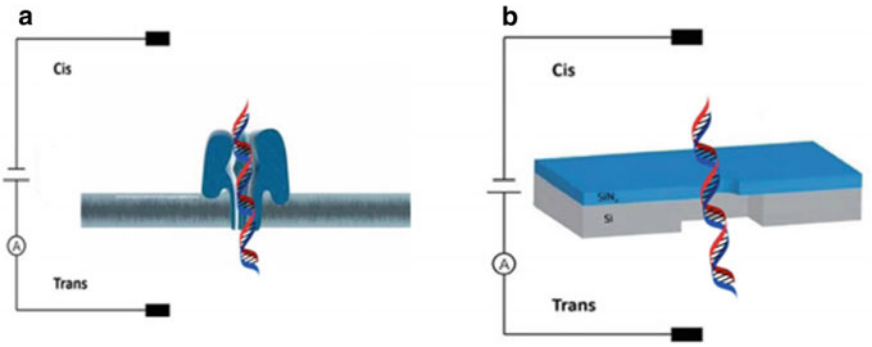


Fig. 8 **a** Biological nanopore. **b** Solid state nanopore

So far, several studies have been published based on biological nanopores to detect different microRNAs. In one study Ryuji Kawano and his team developed an alpha-hemolysin (αHL) nanopore-based miRNAs sensor that utilizes isothermal amplification and asymmetric nanopore measurement for indirect and label-free detection of miRNA-20. In their work, they have reported the detection of miR-20a with a limit of detection (LoD) of 1 fM. This ultra-low concentration was mainly achieved by designing the three-way junction structure with the catalytic enzyme reaction using miRNA-20 as the input and poly-thymines (polyT20) as outputs molecules. This approach can be applied for ultra-low concentration detection of other microRNAs biomarkers by simply changing the nucleotide sequences of the DNA template and primer (Zhang et al. 2017). Another work reported by Ivica et al. was based on α-hemolysin pore for the detection of miR155, which is considered as a lung cancer biomarker. In their work, the target MicroRNA was firstly hybridized with a specific probe. Then, the prob-miRNA duplex was unzipped before translocating through the pore. In order to improve the analytical performance of their nanopore assay, they have studied the effect of different salt gradients between the nanopore chambers and the DNA probe design effect. Based on their funding, the 8-fold KCl gradient enabled a linear relationship between pulse frequency and miRNA concentration in the range of 100 pM–100 nM with a limit of quantification of 100 pM (Ivica et al. 2017).

For the sake of selective simultaneous detection of different microRNA targets by using a biological nanopore sensor, Li-Qun Gu and his team have done innovative work and succeed in detecting four different microRNAs miR-155, miR-182-5p, miR-210, and miR21. The approach is based on designing different barcodes probes and attach each one to a target microRNA.

However, the interaction between the probe and the target could modulate the ion flow through the nanopore. Resulting in a specific signature attributed to each complex (barcode probe + target) (Zhang et al. 2014). Moreover, a novel method based on an α-hemolysin pore was proposed by Tian et, al. to selectively detect

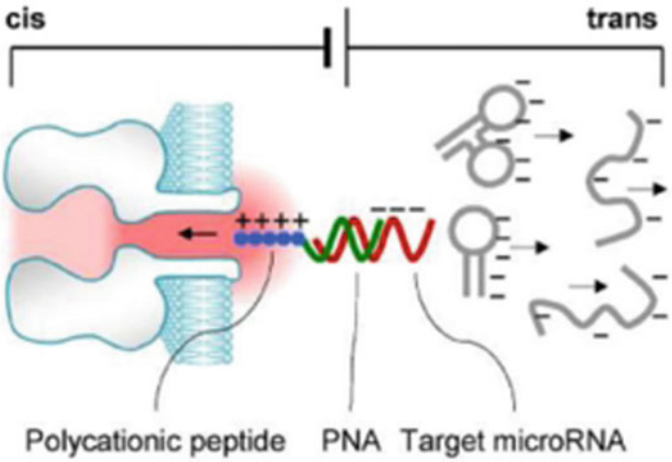


Fig. 9 Cationic peptide-PNA probe hybridized microRNA selective detection principle. Adapted with permission from (Tian et al. 2013)

microRNA in a nucleic acid mixture (Fig. 9). They have proposed to form a dipole complex by hybridizing a polycationic peptide-PNA probe with the target microRNA.

However, the application of a voltage bias, opposed to the bias needed to capture the nucleic acids, will drive the complex to pass through the pore, and the other nucleic acid will be pushed away. The selectivity test in the presence of nucleic acids and different other microRNAs proved that this approach could be used in a complex solution (Tian et al. 2013).

(2) Solid-state nanopore

Besides biological nanopore, solid-state nanopores with all their diversity have also been commonly used to detect MicroRNA. Taking the example of micropipette nanopore, Hao Wang et al. used a micropipette nanopore to detect miRNA-21 as

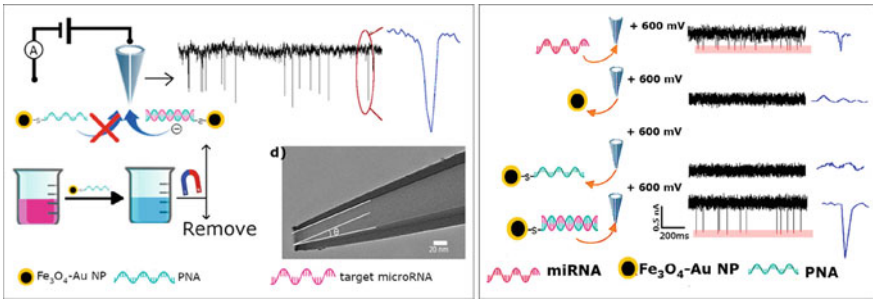


Fig. 10 Working principle of the nanopipette nanopore sensor. Adapted with permission from Wang et al. (2019)

a lung cancer biomarker (Fig. 10). The team has used an innovative strategy, and the target microRNA was hybridized with neutral peptide nucleic acid (PNA) modified Fe_3O_4—Au nanoparticles, then the complex Fe_3O_4-Au-PNA-miRNA was translocated through the nanopipette nanopore by a positive potential application and eliminating neutral Fe_3O_4-Au-PNA. The authors were able to detect the miRNA-21 in the range of 2–50 nM in PBS with a limit of detection of 2 nM. Moreover, they demonstrated that their proposed sensor can be potentially applied to detect miRNA-21 in complicated samples (Wang et al. 2019). In the same context, Wanunu et al. have succeeded in detecting miR122a using a 5 nm pore drilled on a 10 nm thick silicon nitride membrane. In their platform, the miR122a was first hybridized with a specific probe, then the DNA probe/miRNA-122 was enriched by binding to the viral proteinp19-modified magnetic beads and finally translocated through the nanopore. In this work, they have studied several microRNA probes and the effect of the membrane thickness. Under the optimal conditions, the sensor could detect concentrations at the femtomolar level (Wanunu et al. 2010).

(3) Comparison between biological and solid-state nanopore

Although the sensing principle of the solid-state and biological nanopore is the same, the choice of an appropriate platform depending on the application and the experiment's chemical environment, the key differences between the two categories are the adjustment of the pore size, which is adjustable for the solid solid-state nanopores (synthetic nanopores) and not adjustable for the biological nanopores because the proteins' size is unchangeable, and they always keep the same size and shape. The other key difference is the resistivity of harsh chemical conditions; solid-state nanopores are more immune to rough chemical conditions. While biological nanopores are more suitable for surface modification (Shi et al. 2017). However, the first commercialized nanopore platform, and so far, the most used platform is based on biological nanopores delivered in 2014 by Oxford Nanopore Technology (Plesivkova et al. 2019).

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