Electrochemical detection of miRNAs

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Abstract

The recent progress made in the development of electrochemical methods for microRNA (miRNA) detection is presented. This progress is conceived to be largely due to the invention of novel assay methodologies and the use of various bioreagents and nanostructures. These enable a rigorous control over the sensing interface, provide enormous signal amplifications and single-base mismatch specificity. Femtomolar or even subfemtomolar detection limits were shown to be feasible by electrochemical assays. Thus electrochemical detection methodologies are of perspective for diagnostic miRNA detection.

Keywords: miRNA, electrochemical detection, voltammetry, impedance, resistive pulse sensing, concatamer, peptide nucleic acid

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1. Introduction

MicroRNAs (miRNAs) are typically 21-25-nucleotide long, natural, non-coding RNAs first described in 1993 by Lee et al. [1], with the term microRNA coined much later [2]. It was discovered that miRNAs are playing important role in gene regulation [3] and their level can significantly deviate in various disease states [4-6], most notably, various types of cancers (Fig. 1) [7], cardiovascular diseases, and inflammatory diseases. Therefore, lately the use of miRNAs as tissue specific biomarkers in molecular diagnostics and prognosis generated a remarkable interest, i.e. the number of papers involving miRNA in 2012 exceeded 5500 (Fig. 2).

The prerequisite of a biomarker is to be stable on the time scale of the analysis and although RNA molecules are known to be rapidly degraded by nucleases, circulating miRNAs are in fact highly stable in blood [8]. Digestion with Ribonuclease A, repeated freeze-thaw cycles, boiling, long term storage, as well as extreme pHs were shown to have hardly any effect on the microRNA levels [9]. Since the high stability refers only to endogenous microRNAs, as exogenous microRNAs are rapidly degraded in the blood serum, indicates that microRNAs are somehow protected [10].

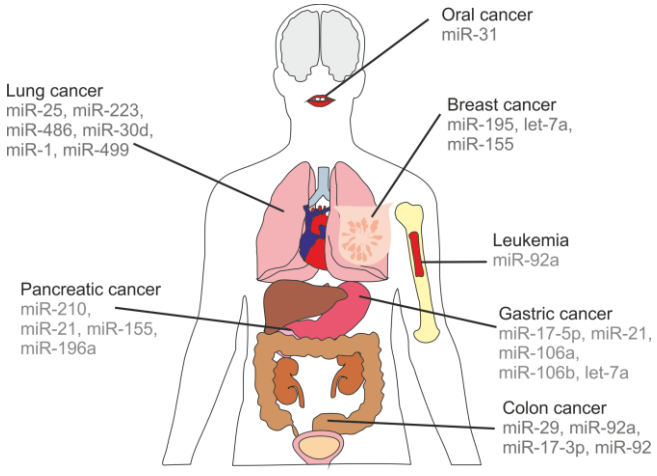

Figure 1. miRNAs associated with different type of cancers [11, 12]

Indeed, evidences were provided that miRNAs are encapsulated in particles, e.g., exosomes (Ø 50–100 nm) [13], microvesicles (Ø 0.1–1 μm), or apoptotic bodies (0.5–2 μm) [14], or in complex with lipoproteins [15] or RNA binding proteins like Nucleophosmin1 [16] and Argonaut proteins [17]. Typical levels of circulating miRNAs in serum were estimated to be within 200 aM to

20 pM [18]. As miRNAs are rarely specific to one kind of disease, employing a miRNA expression panel instead of an individual miRNA as a biomarker is more appropriate [16] and in certain cases the use of race-specific circulating miRNA based biomarkers might be additionally necessary [19].

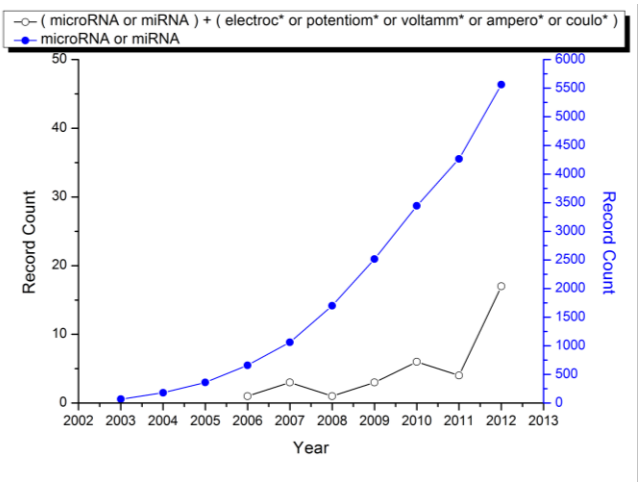

Figure 2. Number of articles published on miRNA and electrochemical detection of miRNA in the past ten years (source *Web of Science* ™)

Nowadays, common methods for miRNA detection are based on traditional molecular biology techniques, such as cloning, Northern blotting, microarray or RT-PCR [20], as well as next-generation sequencing [21]. Many of these methods are either low throughput, low sensitivity or they involve laborious sample handling and detection protocols. However, the determination of miRNAs clearly requires highly sensitive methods to assess the extremely low levels in the bloodstream, that additionally need to be very selective and to comply with the requirements for minute sample volume, cost-effectiveness, and multiplexing capabilities of a diagnostic assay. Finally, for point-of-care applications the detection method should ideally enable the direct assessment of miRNAs without prior amplification or labelling. Despite of the relatively limited number of studies on electrochemical detection of miRNAs, electrochemical methods owing to their proven compatibility with point-of-care devices, cost-effectiveness and very high sensitivities seem to have good perspectives and a clear niche for miRNA detection [22]. Therefore, owing to the rapidly expanding field of miRNA diagnostics, the purpose of this review is to introduce miRNAs to the electroanalytical community with emphasis on the latest progress in electrochemical detection strategies uncovered by previous reviews [22, 23].

2. Electrochemical detection of miRNAs

A large variety of electrochemical detection strategies were proposed that are classified in this review based on the electrical readout methodology. The selective recognition of miRNAs is achieved in all cases by using complementary nucleic acid probes, either of natural or synthetic origin, within a hybridization assay. Since such probes are a prerequisite of all detection strategies a separate introductory chapter is dedicated to their description.

2.1. Selective probes for miRNA detection

A particularity of miRNAs is the high degree of sequence homology between family members that may require in certain cases discrimination between single base mismatched oligonucleotides. miRNAs represent only a very small fraction (ca. 0.01%) of total RNA mass [24]. Furthermore, pri-miRNA and pre-miRNA [25] - the longer intermediates of miRNA biogenesis - possess the sequence motifs of mature miRNA, thus they may interfere with the detection of mature miRNA. One ingenious way to overcome this problem has been reported through the use of p19 RNA binding protein. This protein binds to small, 21–23 base pair double stranded RNAs (dsRNA) with nanomolar affinity in a size dependent, but sequence independent manner [26]. Thus beside ruling out other nucleotides including single stranded RNA (ssRNA), ssDNA, and dsDNA, a size selectivity for dsRNA in the size range of miRNAs is achieved [27]. As generally the selective recognition of miRNAs is achieved through hybridization assays, the choice of the probe is essential to discriminate between single base mismatched RNAs. To address this problem the use of synthetic oligonucleotide analogues were proposed, most importantly, locked-nucleic acids (LNAs) and peptide nucleic acids (PNAs) (Fig. 3) [28].

LNAs contain a methylene linking the 2′-O and 4′-C on the furanose ring resulting in an increased rigidity of the double-stranded nucleic acid structure. For each LNA monomer incorporated in the oligonucleotide probe the melting temperature of the corresponding duplex with complementary RNA increases with 3-8°C [29]. As a consequence LNAs manifest very high hybridization affinity [30] toward complementary ssRNAs and also excellent single-base-pair mismatch discrimination.

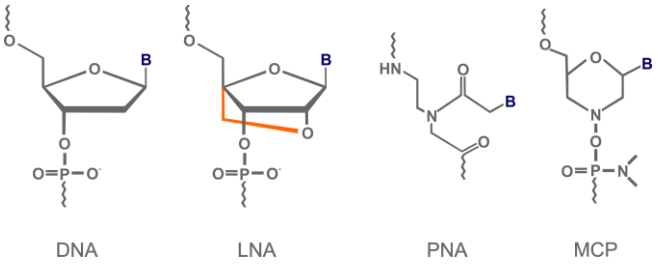

Figure 3. Natural (DNA) and synthetic units (LNA - locked nucleic acid, PNA – peptide nucleic acid, MCP –morpholino capture probe) of oligonucleotide probes for selective recognition of miRNAs

PNAs have the sugar phosphate backbone replaced by a neutral peptide backbone composed of N-(2-aminoethyl)-glycine units. Therefore, the PNA:RNA duplex lacks the electrostatic repulsion present in duplexes formed by their negatively charged natural counterparts, which results in higher affinity binding of complementary RNAs. Additionally, PNA probes have excellent chemical, biochemical and thermal stability. Their electroneutrality makes them ideal probes for electrochemical transducers based on detection of surface charge variations upon oligonucleotide hybridization, e.g., ion channel sensors [31], field effect transistors (FET) [31], and nanopore sensors [32, 33]. As a low cost alternative of PNA, antisense morpholino oligos (MCPs), which have higher affinity to complementary RNA than natural nucleic acid probes, were also reported [34]. Despite of the well-documented advantages of using synthetic nucleic acid analogues as selective miRNA probes [21] their implementation in electrochemical detection methodologies is still scarce [35, 36]. It must be mentioned that miRNA detection, beside the availability of high affinity and selectivity hybridization probes, benefits also from the implementation of new signaling probes with superior properties. One relevant example is the use of the thermostable reporter enzyme esterase 2 (ETS2) from *Alicyclobacillus acidocaldarius*.[37] EST2 offers the possibility of site specific modification with oligonucleotide probes and was found to be superior to common enzyme labels such as alkaline phosphatase and horseradish peroxidase (HRP) [38].

2.2 Nanopore-based resistive pulse sensing of microRNAs

Resistive pulse sensing with nanopores implies monitoring changes in the applied voltage driven ion flow across a nanopore that separates two electrolyte chambers as various molecular species are passing through or residing within the nanopore sensing zone [39]. Thus, nanopore-based sensors have single molecule detection capabilities, but one should bear in mind that the achievable detection limit is ultimately determined by the translocation throughput [40] of the detected species and not by the single-molecule sensitivity of the detection [41]. The nanopore-based miRNA detection methods are among the most sensitive nanopore-based sensing strategies. They benefit from the inherent size selectivity of nanopores, as different size species give different current signatures. That is, the amplitude and duration of current pulses caused by translocation events is size dependent. Thus, solid-state nanopores made in a 7 nm thick SiN membrane with a smallest constriction of 3 nm (Fig. 4) were shown to enable in ideal conditions discrimination of dsRNA even from dsDNA and transfer RNA (tRNA) [42].

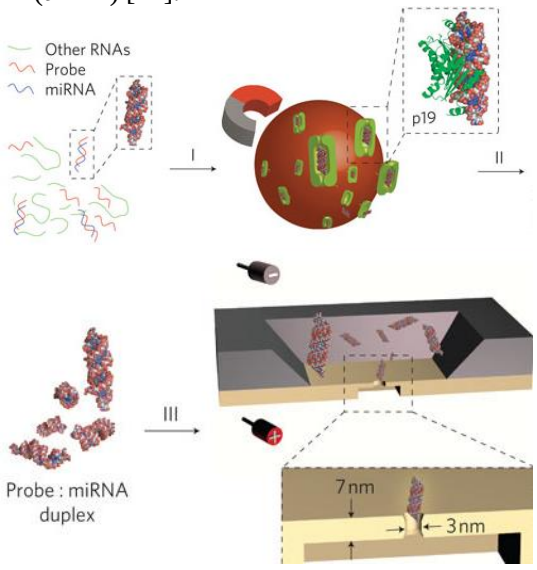

Figure 4. Selective detection of miRNA by resistive pulse sensing using solid state nanopores. A separation and pre-concentration step based on magnetic beads modified with dsRNA-selective p19 protein was used to separate the target miRNAs from cellular RNA.[42] Reprinted by permission from Macmillan Publishers Ltd: Nature Nanotechnology, Ref. [42], copyright (2010).

However, to make the method operational for detecting miRNAs (i.e., liver-specific miR122a) in cellular RNA mixtures, a separation and concentration step needed to be employed. This was based on adding a complementary probe, to generate the probe:miRNA duplex, and viral protein p19 coated magnetic beads that bind selectively the formed RNA duplex. The probe:miRNA duplex is separated under magnetic field from the sample matrix with concurrent, ca. 100,000-fold enrichment. Following their elution from the magnetic beads, the probe:miRNA duplex was quantified down to 1 fmol level based on the frequency of the translocation events.

Wang et al. [43] proposed another nanopore detection approach, using α-hemolysin protein pore, which was claimed to enable selective detection of miRNA in blood with a detection limit of ca. 100 fM. For selective detection, first an RNA extraction was performed and then an oligonucleotide probe with a miRNA capture zone flanked by 2 poly$(dC)_{30}$ tags was added to the plasma RNA. After hybridization the duplexes were electrically driven into the nanopore, where they resided longer than single stranded oligos owing to the time required to unzip the duplex before translocation. Thus the longer duration pulses produced by miRNA duplexes as compared to other RNAs and the non-hybridized probe, enabled the selective detection of target miRNA in the presence of high plasma RNA background [44]. It was shown that the method can distinguish the relative levels of miR-155 (indicative of lung cancer) of healthy and lung cancer patients in close agreement with RT-PCR based measurements [43].

2.3 miRNA detection by nanogap sensors

While nanopore sensors are generally based on modulation of the ionic current through a nanopore, nanogap sensors are based on measuring the ionic current between two closely placed electrodes (<1 μm) that define an inter-electrode space, i.e., nanogap. Changes in the conductivity of the nanogap environment is detected through changes in the ionic current (e.g., provided by a reversible redox mediator cycled between the two electrodes) [45] or electron conduction (e.g., formation of an interconnected network of conductive material) [46]. Thus, if microelectrodes are flanking a receptor modified surface, the specific binding of a target can be monitored through conductivity changes it causes in the gap. Fan et al. [35] reported a biosensor array consisting of 100 pairs of interdigitated microelectrodes with a gap of 300 nm between adjacent electrodes that features immobilized PNA probes. The selective binding of the target miRNA generated negative charges on the surface, which facilitated in subsequent steps the peroxidase catalyzed deposition of conducting polyaniline nanowires. The conductance of the deposited nanowires was indicative of the amount of miRNA bound to PNA within 10 fM to 20 pM with a detection limit of 5.0 fM.

2.4 Detection of miRNAs by nanowire-based field effect transistors

Nanowire-based FETs are known to enable sensitive label-free detection of nucleic acids [47]. This principle was applied also for the detection of miRNAs [48] by using PNA-modified n-type silicon nanowires (SiNWs) that were 50 nm in diameter and 100 μm long. Upon miRNA hybridization negative charge is generated on the nanowire surface, which causes an increase in the resistance of the SiNWs due to depletion of charge carriers in its "bulk". The sensor was applied for the detection of let-7b miRNA in total RNA extracted from HeLa cells. This label-free method had an appealing detection limit of ca. 1 fM, however, the proposed FET construction does not allow the regeneration of the surface for repeated measurements.

2.5 Impedimetric detection of miRNAs

Ren et al. proposed an impedimetric method for the detection of miRNA, which is based on an ingenious signal amplification mechanism that uses a duplex-specific nuclease (DSN) (Fig. 5) [49]. A gold electrode was modified with a mixed monolayer of thioglycolic acid and thiol-labeled DNA probe that captures the complementary miRNA by hybridization. Concurrently, the DNA:miRNA duplexes are cleaved off from the surface by DSN enzyme added into the sample solution, i.e., the capture probes hybridized with the target are removed from the surface of the gold electrode. Thus the hybridized miRNA strands are released back to the sample solution and become available for further hybridization with the remaining capture probes, i.e., recycled. This results in an isothermal signal amplification through which one target miRNA strand induces the removal of thousands of capture probes during repeated cycles of: (i) selective hybridization, (ii) duplex removal, (iii) miRNA release and rebinding. Upon removing the capture probe and exposing the bare gold surface the charge transfer resistance of a negatively charged redox mediator system ($K_3Fe(CN)_6/K_4Fe(CN)_6$) decreases as detected by electrochemical impedance spectroscopy (EIS). Thus, there is an inverse relationship between the charge transfer resistance and the miRNA concentration of the sample. Excellent selectivity and detection limit as low as 1 fM were reported with this approach.

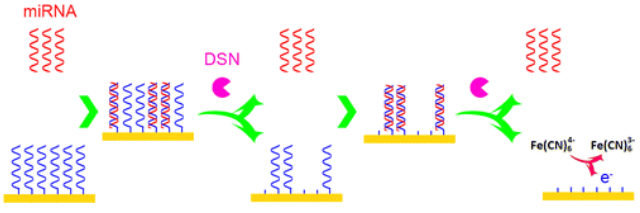

Figure 5. Schematic illustration of the working principle of the impedimetric detection method for miRNA based on isothermal amplification conveyed by the use of a duplex–specific

nuclease. [49] Reprinted with permission from Ref. [49]. Copyright (2013) American Chemical Society.

A further amplification method for label-free impedimetric detection of miRNAs is based on DNAzyme catalyzed deposition of an electrically insulating polymer [50]. A monolayer of immobilized capture probes was used for the hybridization-based binding of target miRNAs. This was followed by enzymatic cleaving of the unreacted capture probes with exonucleases and the hybridization of an oligonucleotide labeled DNAzyme to the bottom part of the capture probes (Fig. 6). The G-quadruplex-hemin DNAzyme exhibits peroxidase-like activity and effectively catalyzes the polymerization of 3,3′-dimethoxybenzidine (DB) to form an insulating poly(3,3′-dimethoxybenzidine) (PDB) film on the electrode surface, which increases the charge transfer resistance of a redox mediator. As the exonuclease-based removal of unreacted capture probes ensures that the DNAzyme enzymes bind only to the miRNA hybridized probes, the enzyme activity will be proportionally to the bound miRNA. This method was shown to have a detection limit of ca. 2.0 fM [50].

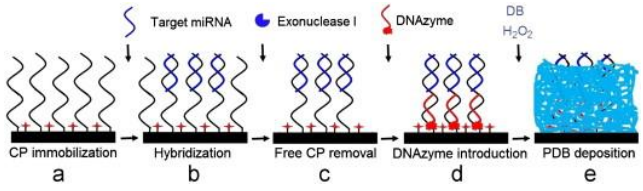

Figure 6. Schematic illustration of the impedimetric miRNA assay based on using DNAzyme with peroxidase-like activity as label to generate an electrically insulating polymer film. The assay included the following succession (a) capture probe (CP) immobilization onto the electrode surface, (b) hybridization with target miRNA, (c) removal of unreacted CPs by Exonuclease I digestion, (d) introduction of DNAzyme, which catalyzes the (e) PDB deposition.[50] Reprinted from Ref. [50], Copyright (2013), with permission from Elsevier.

To reduce the complexity of the assay, Gao et al. [36] proposed a variation of this method, which eliminates the need for enzymatic cleavage of the unreacted CP while achieving the same detection limit of ca. 2 fM. Uncharged MCPs were immobilized on an indium-tin oxide (ITO) electrode surface to which target miRNA was hybridized. The miRNA binding results in the formation of a negative surface charge, which effectively adsorbs the DB monomer (Fig. 7). In a subsequent step, the high surface concentration of DB facilitates the surface confined polymerization of DB in the presence of horseradish peroxidase (HRP) and H_2O_2. This results in the generation of a thin electrically insulating PDB film on the sensor surface which increases the charge transfer resistance.[36]

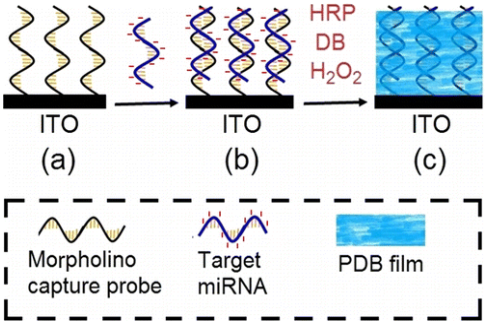

Figure 7. Schematic of the three main steps of the miRNA assay with the MCP-based impedimetric biosensor: (a) formation of a monolayer of uncharged MCPs on silane activated ITO electrode; (b) capture of the target miRNA resulting in an increase of the negative surface charge density; and (c) electrostatic adsorption of DB on the negatively charged surface and its surface confined polymerization in the presence of HRP and H_2O_2.[36] Reprinted with permission from Ref. [36] Copyright (2013) American Chemical Society.

2.6 Voltammetric detection of miRNAs

Lusi et al.[51] reported a direct, label-free and reagentless method based on the oxidation of miRNA guanines following the hybridization of the target microRNA to a DNA capture probe with inosine substituted guanines. The oxidation of guanine is performed by using differential pulse voltammetry (Fig. 8) [51].

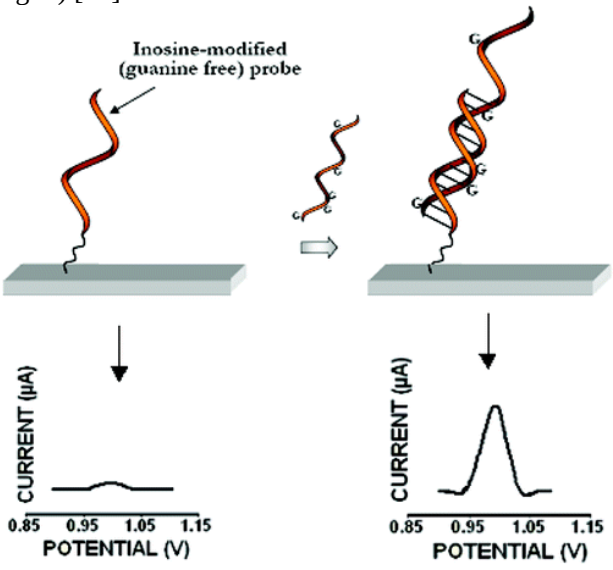

Figure 8. Electrochemical detection of the hybridization between the inosine-modified capture probe and the miR-122 target. The oxidation signal of guanine is indicative of the duplex formation, i.e., of the presence of the miRNA target.[51]

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A comparable high detection limit, ca. 2 nM, was obtained by using a voltammetric method based on labeling the target miRNA with an electroactive complex composed of six-valent osmium and 2,2′-bipyridine (Os(VI)bipy) in combination with a miRNA specific DNA-magnetic bead conjugate to separate the target miRNA [52]. The miRNA amount was determined through the electrocatalytic peak of the Os(VI)bipy-miRNA at a hanging mercury drop electrode [52].

Tran et al. described a direct, reagentless, and label-free detection strategy of microRNAs, based on a DNA probe grafted conjugated copolymer, poly(5-hydroxy-1,4-naphthoquinoneco-5-hydroxy-2-carboxyethyl-1,4-naphthoquinone), acting as hybridization transducer (Fig. 9). The electroactivity of the quinone redox system detected by square-wave voltammetry (SWV) was found to be influenced by the conformation change of the grafted DNA probes upon hybridization with complementary miRNAs, i.e., an increase in the peak current was found upon hybridization. It was suggested that the closely packed layer of coiled probes on the electrode surface decreases the apparent diffusion coefficient of charge compensating counter-ions and consequently the peak current. Contrariwise, the formation of straight double strands upon hybridization with miRNA creates a more permeable layer and induces a significant current increase. The method was shown to be applicable in diluted human serum and enabled the direct electrochemical detection of the target miR-141 with a detection limit of 8 fM [53, 54].

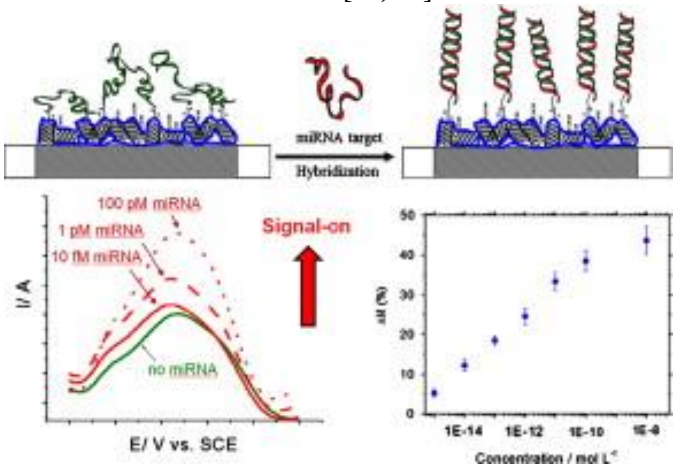

Figure 9. Voltammetric method for direct, reagentless, and label-free detection of microRNAs based on detecting permeability changes of a DNA probe layer upon hybridization with target miRNAs.[54] Reprinted from Ref. [54], Copyright (2013), with permission from Elsevier.

The use of sandwich-type assays for miRNA determination, while common in DNA analysis, are rendered difficult by the small size of miRNAs. Wen et al. [55] overcame this problem by adapting a base stacking-based strategy to stabilize the sandwich complex. A tetrahedral DNA nanostructure was designed and applied for utmost spatial control of the DNA probe immobilization, which ensured their optimal accessibility. Three vertices ending in thiol groups anchored the DNA tetrahedron to the electrode surface, keeping the 10-mer DNA probe appended to the fourth vertex of the tetrahedron in an upright position, i.e., perpendicular to the surface (Fig. 10).

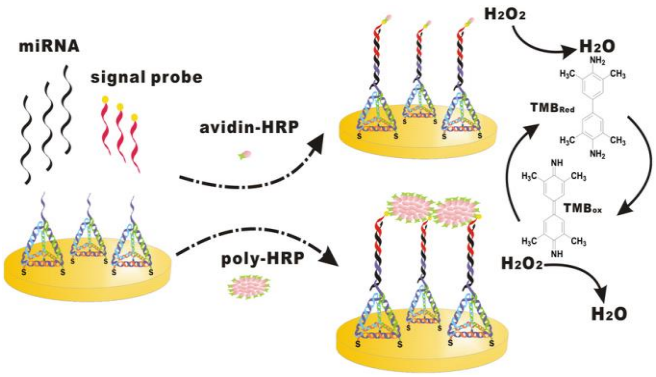

Figure 10. miRNA detection with the tetrahedron-based electrochemical miRNA sensor using enzyme-based signal transduction (either avidin-HRP or high-activity poly-HRP).[55] Reprinted by permission from Macmillan Publishers Ltd: Scientific Reports Ref. [55], copyright (2012).

The DNA probe bound the 22-mer target miRNA at one of its ends, while the other end of the miRNA was reacted with a complementary biotinylated DNA probe. The resulting sandwich complex is stabilized by stacking interactions between adjacent bases of the two oligonucleotides (DNA probe and biotinylated DNA), while forming a contiguous duplex with miRNA [56]. Either avidin-HRP or poly-HRP was added to react with the biotin tag of the signal probe, followed by the addition of H_2O_2. The enzyme-catalyzed reduction of H_2O_2 was electrically coupled to the electrode surface by using 3,3′,5,5′ tetramethylbenzidine (TMB) as electron-shuttle. The amperometric current of TMB reduction was proportional with the miRNA concentration in a large concentration range [55]. This detection strategy was reported to enable attomolar detection limits for target miRNAs and discrimination single-base mismatched strands.

Further on the line of developing complex miRNA probes Cai et al. [57] reported a functional allosteric molecular beacon (aMB). The aMB consisted of four domains: a Streptavidin (SA) binding aptamer, a miRNA

binding domain, a blocking domain that internally hybridizes with a part of the SA aptamer sequence (blocking its binding activity towards SA), and an immobilization/spacer domain to anchor the aMB onto the electrode surface through Au-S bonds. The spacer domain additionally keeps the binding sites of aMB at proper distance from the surface to reduce steric hindrance (Fig. 10). In the absence of the miRNA target, the aMB forms a stable hairpin structure which blocks the binding capability of the SA aptamer. Binding of the target miRNA to the aMB probe "liberates" the SA aptamer part to bind SA-HRP conjugate added into the solution. After a washing step, TMB and H_2O_2 are added and the surface confined HRP catalyzes the oxidation of TMB that is reduced back at the electrode (Fig. 11). The aMB-based assay was applied for the detection of let-7a miRNA and showed to enable single-base mismatch selectivity and a detection limit of 13.6 amol in 4 μL sample [57].

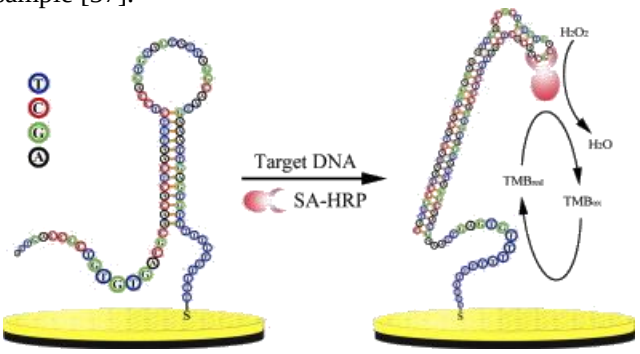

Figure 10. The detection principle of the allosteric molecular beacon (aMB) based sensor[57] Reprinted from Ref. [57], with permission from Elsevier.

Table 1. Some representative electrochemical detection methods for miRNA and their detection limit

Method (principle)	Probe	miRNA	LOD	Sample	Ref.
Conductance (miRNA-guided formation of conducting polymer nanowires in nanogaps)	immobilized PNA	let-7b	5.0 fM	total RNA extracted from HeLa cells and lung cancer cells	Fan et al. (2007)[35]
Voltammetric (Electrochemical oxidation of guanines in the target miRNA)	immobilized inosine substitute DNA capture probe	miR-122	0.1 pmol	-	Lusi et al. (2009)[51]
Conductance (PNA-modified n-type Silicon nanowire based FET for label-free miRNA detection)	immobilized PNA probe	let-7b	1 fM	RNA extract from HeLa cells	Zhang et al. [48]
Resistive pulse sensing (solid-state nanopore-based detection)	solution-based DNA and viral protein p19	miR-122a	1 fmol	cellular RNA extract	Wanunu et al. (2010) [42]
Resistive pulse sensing (α-haemolysin-based nanopore)	solution-based programmable oligonucleotide probe	miR-155	100 fM	plasma RNA	Wang et al. (2011)[43]
Voltammetric (based on multifunctional bio-barcodes and enzymatic assay)	immobilized LNA integrated molecular beacon	miRNA-21	60 fM	diluted total RNA extract from human hepatocarcinoma BEL-7402 and normal human hepatic L02 cells	Yin et al. (2012) [58]
Voltammetric (Conducting polymer nanostructured by carbon nanotubes)	DNA capture probe	miR-141, miR-29b-1, miR-103	~8 fM	diluted serum	Tran et al. (2013)[54]

Voltammetric (sandwich assay with peroxidase labeling)	Tetrahedral DNA nanostructure-based immobilized capture probe	miR-21, mir-31, let-7a, let-7b, let-7c, let7-d, let-7e, let-7f, let-7g, let-7i, miR-98	10 fM	total RNA extract from tissues	Wen et al. (2013)[55]
Impedimetric (DSN-based *in-situ* isothermical amplification)	DNA capture probe	let-7b	10 aM	circulating miRNAs in blood and in total RNA extracts from blood and cancer cells	Ren et al. (2013)[49]
Voltammetric (Concatamer-based amplification)	DNA capture probe (requires 2 auxiliary DNA probes)	miRNA-21	100 aM	serum samples	Hong et al. (2013)[59]
Impedimetric (DNAzyme-catalyzed and microRNA-guided formation of insulating polymer film)	DNA capture probe	miR-720, let-7c, miR-1248	2.0 fM	total RNA extracts from cultured cells	Shen et al. (2013)[50]
Impedimetric (Probe:miRNA duplex templated deposition of an insulating polymer)	NH_2-terminated MCP	let-7a, let-7b, let-7c	0.10 nM	serum and total RNA extracts from cultured cells	Gao et al. (2013)[36]
Voltammetric (Os(VI)bipy label-based electrochemical assay)	immobilized DNA capture probe	miR-522	2 nM	spiked into a mixture of small RNAs	Bartosik et al. (2013)[52]
Voltammetric (3 detection strategies: based on hybridization, p19 protein binding, and protein displacement)	immobilized DNA capture probe	miR-21, miR-32, miR-122, miR-141, miR-200	0.4 fM	human serum sample with an excess of yeast tRNA added to protect endogenous miRNAs from nucleases	Labib et al. (2013)[60]
Voltammetric (conformational changes in the p19 protein upon dsRNA binding that exposes tryptophan residues for oxidation)	solution-based RNA probe and P19 protein	miR-21	1.6 pmol	-	Kilic et al. (2013)[61]

The loop opening of a molecular beacon probe upon selective miRNA binding was also used in the context of freeing the end part of the beacon for binding a bio-barcode reporter (Fig. 11) [58]. The bio-barcode consists of an Au nanoparticle modified with a reporter LNA probe that binds to the released end of the molecular beacon and a signal probe featuring biotin-modified oligos. The latter are in great excess on the Au nanoparticle surface with respect of the reporter LNA probe so that further addition of a SA-HRP conjugate results in a large number of peroxidase molecules bound to the surface. The activity of the surface confined HRP labels was detected through the electrochemical reduction of benzoquinone resulted from the HRP catalyzed oxidation of hydroquinone by H_2O_2. The method was applied for the determination of miRNA-21 with a detection limit of ca. 60 fM.

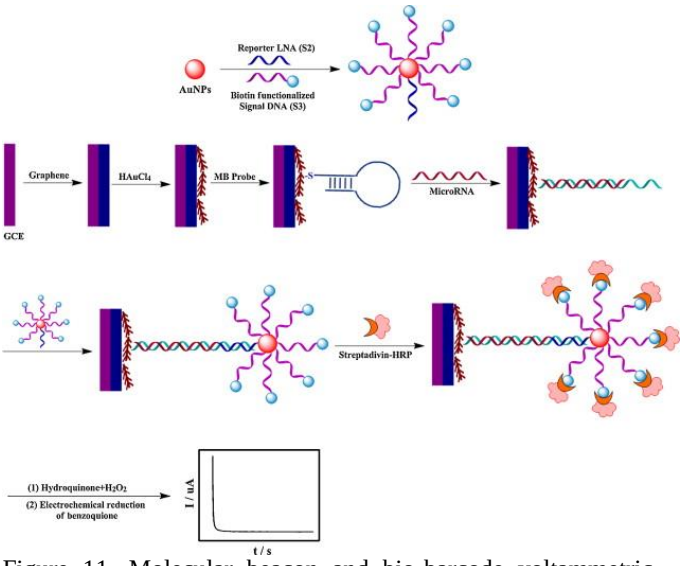

Figure 11. Molecular beacon and bio-barcode voltammetric assay of miRNAs. The miRNA binding to the molecular beacon probe releases the end of the beacon to bind a bio-barcode based on HRP reporter. The enzymatic activity is detected through the reduction of bezoquinone.[58] Reprinted from Ref. [58], with permission from Elsevier.

A concatamer based hybridization chain reaction method was developed by Hong et al. [59]. In this method a hairpin capture probe comprising the sequence complementary to the target miRNA in the loop is immobilized onto the sensor surface (Fig. 12).

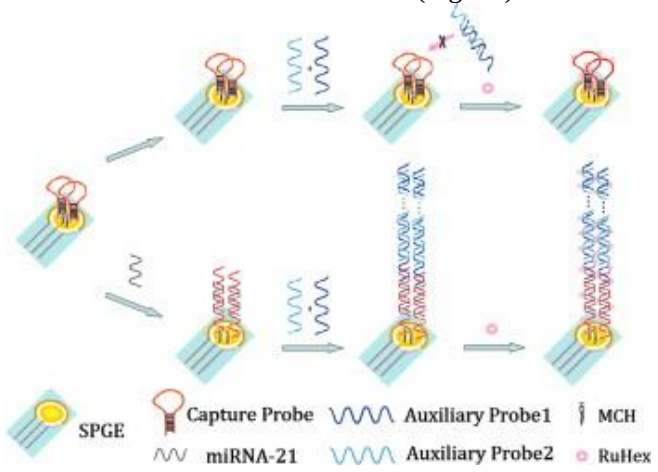

Figure 12. Flow chart of the concatamer-based ultrasensitive electrochemical assay for the detection of miRNA-21.[59] Reprinted from Ref. [59], with permission from Elsevier.

In the absence of target miRNA, the capture probe exits predominantly in the hairpin form that prohibits the binding of two auxiliary probes (that are able to self-assemble to form one-dimensional DNA concatamers) complementary to the end section of the hairpin probe.

However, in the presence of target miRNA, the stem-loop structure of capture probe is unfolded and the DNA concatamers can hybridize with the terminus of DNA capture probe. The long DNA structures on the surface can electrostatically adsorb a large amount of positively charged redox indicator $[Ru(NH_3)_6]^{3+}$ added into the solution. Differential pulse voltammetry (DPV) was used to detect the adsorb redox indicator with the peak current proportional to the miRNA concentration in the sample. The proposed concatamer-based assay can detect as low as 100 aM target miRNA-21. Furthermore, it was shown that miRNA-21 can be directly detected in human serum without enrichment.[59]

Labib et al. designed a three-mode electrochemical biosensor for miRNA detection (Fig. 13). The method is based on using DNA probes immobilized on a gold screen printed electrode surface and square wave voltammetric detection of the miRNA binding to the surface confined nucleic acids in 4 mM $K_3[Fe(CN)_6]$ and 10 μM $[Ru(NH_3)_6]Cl_3$. The use of this redox mediator mixture amplifies the reduction current of the positively charged Ru^{3+} complex that electrostatically adsorbs on negatively charged oligos, as the Fe(III) in the solution reoxidizes (recycles) the Ru^{2+}. There is no direct signal from the negatively charged Fe(III) complex as it cannot access the electrode surface owing to their electrostatic repulsion by the immobilized negatively charged DNA probes.

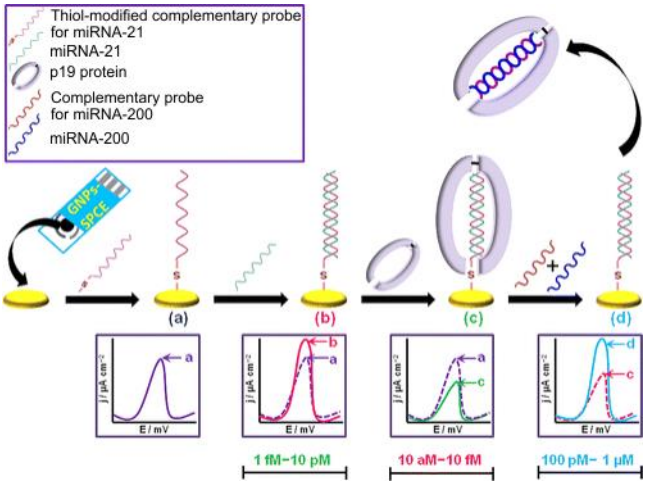

Figure 13. Schematic representation of the 3 type of assays for SWV-based detection miRNA by using modified gold screen-printed electrode and a mixture of 4 mM $K_3[Fe(CN)_6]$ and 10 μM $[Ru(NH_3)_6]Cl_3$ in solution (b) hybridization assay of miR-21 to the complementary probe modified gold electrode (c) Amplification using p19 protein which causes a large decrease in current density (d) p19-displacement-assay where the duplex formed by miR-200 and its complementary probe forces the release of p19 protein and by that a decrease in the peak current.[60] Reprinted with permission from Ref. [60]. Copyright (2013) American Chemical Society.

The interesting particularity of the sensing principle is that three successive detection steps can be employed each of them enabling miRNA assessment in a different concentration range with a different detection limit. Thus the straightforward hybridization of the miRNA to the surface immobilized probe increases the current as measured by SWV providing a dynamic range of 1 fM to 10 pM for miR-21. Further addition of p19 protein that binds to the probe:miR-21 duplex results in a decrease in the SWV peak current and provides the most sensitive mean for miRNA detection, i.e., a dynamic range from 10 aM to 10 fM. Finally, a displacement-based assay in which the addition of a miR-200 and its complementary probe duplex induces the release of the p19 protein from the sensing surface. Thus the current increase is now indicative of the miR-200 concentration in the range of 100 pM to 1 μM. [60] This latter displacement assay is fairly universal and does not needs the immobilization of a complementary DNA probe on the surface.

A more direct approach for the detection of miRNAs based on the use of p19 protein was reported recently to reach picomole detection limit for miR-21 without any preamplification step before hybridization. Hybridization of an RNA probe and its miRNA target creates dsRNA structure and this formation firmly binds p19 proteins. Using a graphite electrode changes of intrinsic p19 oxidation signals upon binding the duplex were observed at +0.80 V with DPV. The exact mechanism seem to be unclear but suggested to involve a change in the conformation of the p19 protein upon binding the duplex that exposes tryptophan residues for oxidation. The method was used to quantify miR-21 [61].

3. Conclusion

The progress made in the last few years in the development of electrochemical methods for miRNA detection is largely due to the novel assay methodologies and the use of various bioreagents and nanostructures. These enable a rigorous control over the sensing interface, provide enormous signal amplifications and single-base mismatch specificity, but still RNA extraction from body fluids seems to be required in most cases prior of the assay. The results are extremely promising as excellent selectivities and femtomolar or even subfemtomolar detection limits are clearly feasible with electrochemical detection (Table 1). Some assay formats, though very innovative, are rather complex and entail multiple steps and labeled reagents. Therefore, apparently there is room for further development and the microfluidic integration and automation is one direction that has not been given enough attention.

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