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Short Review

Strategies for Designing of Electrochemical MicroRNA Genesensors Based on the Difference in the Structure of RNA and DNA

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Electrochemical genosensors have held great promise as devices suitable for point-of-care diagnostics and multiplexed platforms for fast, simple and inexpensive nucleic acid analysis. MicroRNA (miRNA), regulating gene expression by translational repression or degradation of messenger RNA, is believed to be important for cancer diagnosis and prognosis serving as a reliable molecular biomarker. In recent years, there have been many attempts for miRNA detection using electrochemical genesensors. In this work, we first addressed the progress in designing of miRNA detection strategies based on the difference in the structure of RNA and DNA. This work should be valuable for the design of new types of miRNA genesensors.

Keywords: MicroRNA; electrochemistry; genesensors

1. INTRODUCTION

RNA is one of the three major macromolecules (along with DNA and protein) in the body that are essential to all known forms of life. Like DNA, RNA is made up of a chain of components called nucleotides. The chemical structure of RNA is very similar to that of DNA, but differs in three main ways: (1) unlike double-stranded DNA, RNA is a single-stranded molecule in many of its biological roles and has a much shorter chain of nucleotides, (2) RNA contains ribose (in deoxyribose there is no hydroxyl group attached to the pentose ring in the 2' position) while DNA contains deoxyribose, and

(3) the complementary base to adenine is not thymine, as it is in DNA, but rather uracil. There are several kinds of RNA in the body: messenger RNA (mRNA), transfer RNA (tRNA), ribosomal RNA (rRNA), small nuclear RNA (snRNA) and other non-coding RNAs (ncRNA). A ncRNA is a RNA molecule that is not translated into a protein. The number of ncRNAs encoded within the human genome is unknown; however recent transcriptomic and bioinformatics studies suggest the existence of thousands of ncRNAs [1]. Many ncRNAs show abnormal expression patterns in cancerous tissues. MicroRNA (miRNA) is one of categories of ncRNAs with 19–25 nucleotides long. It plays important roles in developmental and cell biology, including developmental regulation, stress responses, cell differentiation, cardiogenesis and epigenetic inheritance. Recently, the aberrant expression of miRNA has been correlated with cancer (prostate, breast, colon, lung, etc.) and other diseases (diabetes, heart diseases, etc.), making miRNA clinically important biomarkers and drug discovery targets [2-4]. The methods currently used for miRNA detection, such as Northern blotting, microarrays and polymerase chain reaction (PCR), are usually time-consuming, less sensitivity and/or require fluorescent-or radio-labeling and complicated instrumentation [5-7]. Therefore, it is critical to develop robust detection methods for miRNAs with high sensitivity, selectivity and simplicity. In recent years, electrochemical genosensors have held great promise as devices suitable for point-of-care diagnostics and multiplexed platforms for fast, simple and inexpensive nucleic acid analysis [8]. There have been many attempts for miRNA detection using electrochemical genesensors [8,9]. In this work, we, for the first time, reviewed the strategies for designing of electrochemical miRNA genesensors based on the difference in the structure of RNA and DNA.

2. METHODS FOR DESIGNING OF ELECTROCHEMICAL MIRNA GENESENSORS

2.1 Labeling with osmium complexes

The electroactive label of nucleic acids based on osmium (Os) complexes with nitrogenous ligands (e.g. pyridine (py), N,N,N’,N’-tetramethylethylenediamine (temed) or 2,2’-bipyridine (bipy), denoted as L) have been reported since 1981 [10-13]. Trough addition of electroactive eight-valent Os(VIII)L to the 5,6 double bond in pyrimidine bases (Fig. 1), nucleic acids including DNA and PNA were labeled covalently, which lead to the development of hybridization genesensors using carbon, gold and mercury electrodes. With this principle, Labeling of miRNA with Os(VIII)L is also feasible, but modification of bases of uracil and cytosine would interfere with the miRNA hybridization necessary for determination of specific miRNA. Deferring from Os(VIII)L, six-valent osmium complexes with nitrogenous ligands (Os(VI)L) react specifically with 1,2 diols groups in sugar residues in oligo- and polysaccharides as well as in ribosides to form osmate esters (Fig. 1) [14-16], but not react with nucleic acid bases of pyrimidine, uracil and cytosine. High specificity of Os(VI)bipy for ribose in nucleic acids and high sensitivity of the determination at mercury and solid amalgam electrodes give promise for new efficient methods of microRNA determination [17-19]. For example, ribose at the 3’-end of oligonucleotides (oligos) selectively modified by Os(VI)2,2’-bipyridine (bipy) produced two CV redox couples at pyrolytic graphite electrode [18]. As a result, 22-mer oligos can be detected down to 250 nM with square wave voltammetry (SWV). At mercury electrodes, the

Os(VI)bipy-oligo adducts produced an electrocatalytic peak at −1.2 V, allowing for the determination of the oligos down to picomolar concentrations. To improve the detection sensitivity, very recently, Bartosik et al. developed an electrochemical miRNA genesensor relying on the magnetic bead-based DNA/miRNA hybridization and the labeling of electroactive Os(VI)L (Fig. 2) [19]. In this work, miRNA was labeled with Os(VI)bipy and hybridized with biotinylated DNA capture probe attached to the streptavidin magnetic beads.

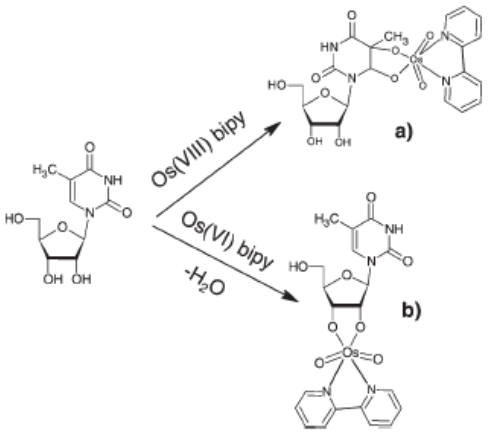

Figure 1. Different reactions of Os(VIII)bipy and Os(VI)bipy with thymine riboside (Reprinted with permission from ref. [14]. Copyright 2007 John Wiley and Sons).

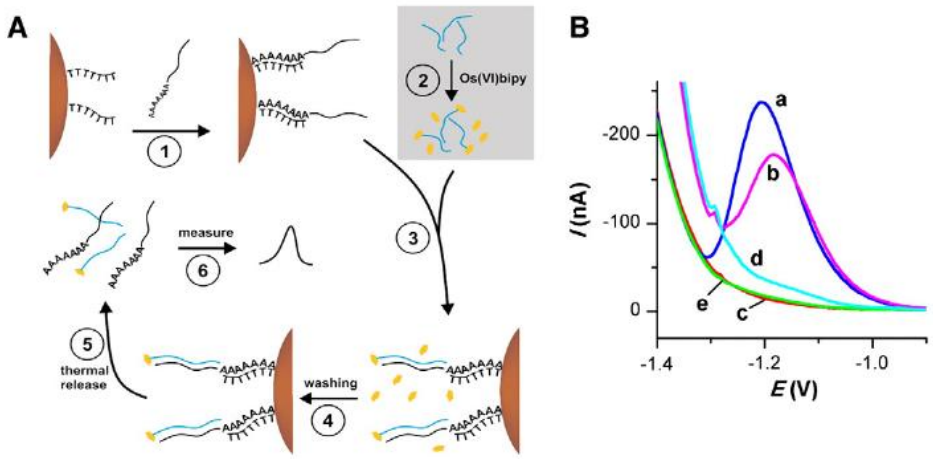

Figure 2. Detection of miRNA with specific sequence using magnetic beads. (A) Scheme of the experiment, consisting of (1) hybridization of a DNA probe with magnetic beads, (2) end-labeling of miRNA with Os(VI)bipy, (3) secondary hybridization of DNA probe with labeled miRNA, (4) thorough washing, (5) thermal release of DNA probe and labeled miRNA from the magnetic beads and (6) electrochemical detection of labeled miRNA. (B) DPV of 100 nM (a) miRNA-522 with complementary cODN-522 and (b) miRNA-522 with complementary cODN-522 hybridized in a complex mixture of other cellular miRNAs. Negative controls included (c) 100 nM miRNA-522 with noncomplementary ncODN, (d) mixture of noncomplementary miRNAs and (e) free 1 μM Os(VI)bipy reagent without any miRNA. Other conditions as in (A). (Reprinted with permission from ref. [19]. Copyright 2014 Elsevier Science B. V.)

The labeled miRNA was then detected at hanging mercury drop electrode at femtomole level due to an electrocatalytic nature of the peak from the Os(VI)bipy label. The assay shows good selectivity for better discrimination of perfect duplex from single and double mismatches by elevating hybridization temperature. Furthermore, the feasibility for target miRNA-522 in real total RNA samples isolated from human cancer cells was demonstrated.

2.2 Formation of boronate ester covalent bond

Boronic acids can covalently react with *cis*-diols to form five- or six-membered cyclic esters. This unique chemistry makes boronic acids attractive ligands for the many application of sensing, separation and self-assembly [20-24]. To date, many ligands with boronic acid groups immobilized on monoliths [25-27], magnetic particles [28, 29], polymer [30-36] and gold substrates [37-39] have also been shown for the immobilization and solid-phase extraction of nucleotide and RNA. For this view, Liu's group developed two label-free electrochemical miRNA genesensors [40,41]. In their first work, miRNA was captured by the pre-immobilized DNA probes on a gold electrode [41]. The cis-diol group of ribose sugar at the end of the miRNA chain allowed 4-mercaptophenylboronic acid (MBA)-capped AuNPs (MBA-AuNPs) to be attached through the formation of a boronate ester covalent bond, which facilitated the capture of electroactive dopamine (DA)-capped AuNPs (DA-AuNPs) via the interaction of boronic acids with catechol moieties on DA-AuNPs. As a result, a detection limit of 50 fM was achieved. The label-free biosensor outperforms those with the redox- or nanoparticles-labeled oligonucleotide probes in terms of its regeneration, reproducibility, long-term storage stability and/or sensitivity. However, the miRNA content is at the attomolar to femtomolar level in biological samples. To improve the detection sensitivity, they reported a highly sensitive electrochemical genesensor for miRNA detection in their second work based on the triple signal amplification of 3-aminophenylboronic acid (APBA)/biotin-modified multifunctional AuNPs (denoted as APBA–biotin–AuNPs), alkaline phosphatase (ALP) and p-aminophenol (p-AP) redox cycling (Fig. 3) [40]. Specifically, miRNA captured by the pre-immobilized DNA probes were recognized by the APBA–biotin–AuNPs through the formation of the boronate ester covalent bond. Streptavidin-conjugated alkaline phosphatase (SA–ALP) was then attached on the electrode surface via the biotin–streptavidin interaction. After the addition of the 4-aminophenyl phosphate (p-APP) substrate, the enzymatic conversion from p-APP to p-AP occurred. The resulting p-AP could be cycled by a chemical reducing reagent after its electro-oxidization on the electrode (known as p-AP redox cycling) [42,43], thus enabling an increase in the anodic current of p-AP. The results indicated that the detection limit was 3 fM and the current increased linearly with the miRNA concentration over a range of 10 fM – 5 pM. In these two works, the specific binding of phenylboronic acid to ribose sugar in ribonucleotides was also characterized by mass spectrometry. Besides the formation of boronate ester covalent bond at the 3'-end of miRNA, Zhang et al. suggested that boronic acids can bind to the 2'-hydroxyl group of all riboses in RNA, affording a novel binding mode towards miRNA [44,45].

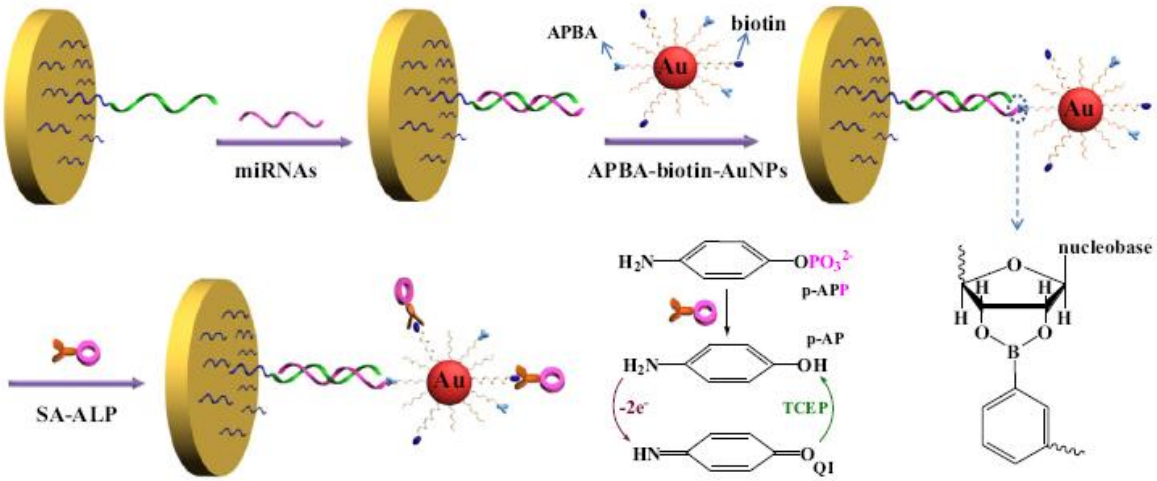

Figure 3. Schematic representation of the label-free detection of miRNA based on the triple signal amplification of APBA–biotin–AuNPs,SA–ALP and the p-AP redox-cycling reaction. (From ref. [40], with permission. Copyright E 2014 Elsevier Science B. V.)

2.3 Oxidation of 2', 3'-diol into dialdehyde

The 2', 3'-diol on the ribose ring at the 3'-terminus of the RNA molecules can be oxidized to a dialdehyde by sodium periodate. Based on this reaction, Weiler *et al*. developed an approach for labeling mRNA with dye molecules by two-step reaction [46,47]. A condensation reaction with a hydrazide derivative was performed to covalently couple a single label onto the 3' end of each miRNA. Since this approach requires an intact 2', 3'-diol, possible background signal contributions from contaminant RNA bearing 3'-phosphate groups or DNA can be minimized. In addition to dye labels, the oxidized miRNA can also be labeled with biotinylate hydrazide prior to microarray analysis, which facilitates the detection of miRNA with fluorescence measurements of streptavidin-quantum dot (QD) conjugates that bind to the miRNA via the streptavidin-biotin interaction (Fig. 4) [48]. The reported detection limit of 40 pM (0.4 fmol) was similar to a comparable approach by the same authors based on colorimetric measurements using streptavidin-modified gold nanoparticles. Based on the reaction, Gao *et al.* reported the first electrochemical miRNA genesensor in 2006 using electrocatalytic OsO_2 nanoparticles [49]. In the work, the OsO_2 nanoparticles was modified with isoniazid and then used to capture the oxidized miRNA through the isoniazid-dialdehyde interaction. Amperometric measurements involving the OsO_2 electrocatalyzed oxidation of hydrazine could be applied to detect 80 fM concentrations. Moreover, they also reported the label of miRNA with with an isoniazid-substituted osmium complex, Os(dmpy)2(IN)Cl+ (dmpy = 4,4'-dimethyl-2,2'-bipyridine, IN = isoniazid). Following hybridization with pre-immobilized complementary capture probes, the resulting sensor shows excellent electrocatalytic activity towards the oxidation of ascorbic acid, allowing for the detection of miRNA with a detection limit of 800 fM and a linear current–concentration relationship up to 300 pM are obtained.

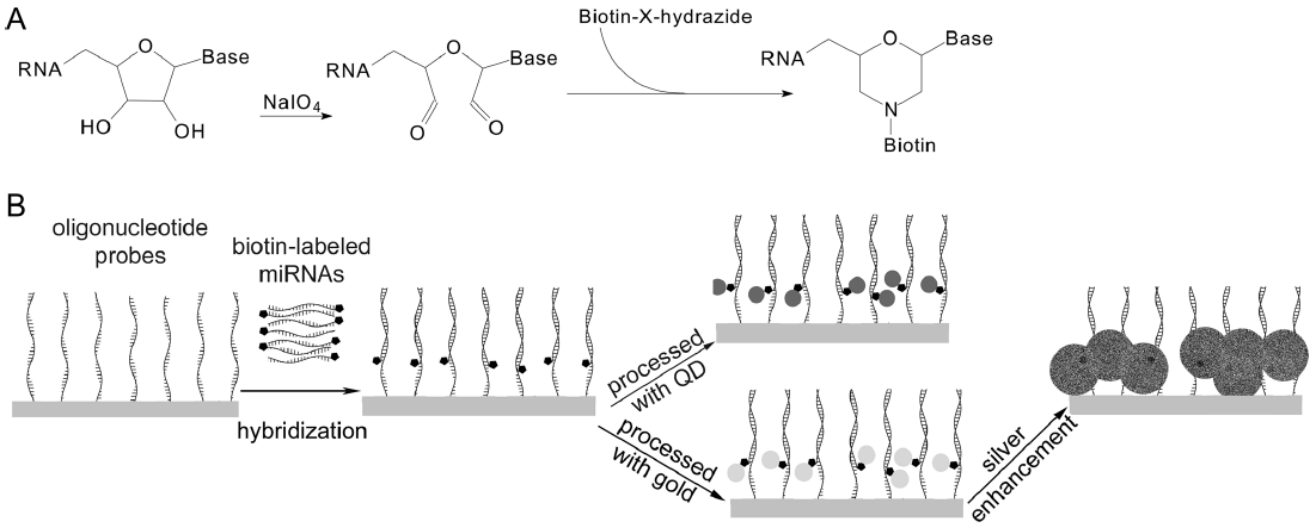

Figure 4. Schematic principles of the miRNA profiling microarray. (A) Principle of labeling miRNA at the 30 terminus with biotin. (B) Principle of the miRNA profiling microarray detected with QD or colorimetric method. (From ref. [48], with permission. Copyright E 2005 Oxford University Press)

Nucleic acid can guide the deposition of polymer where the phosphate groups serve as templates. Recently, Gao's group developed a simple and sensitive electrical miRNA genesensor based on the miRNA-guided deposition of insulating poly(3,3′-dimethoxybenzidine) (PDB) polymer film with ruthenium oxide nanoparticles (RuO_2 NPs) polymerization method on gold electrodes covered with the mixed monolayers of DNA capture probes (CPs) and 4-mercaptoaniline (MAn) [50]. In the work, RuO_2 NPs were coated with 4-(2-aminoethyl) pyridine (AEP), which allows for the label of the oxidized miRNA through the formation of an imine bond between dialdehyde and AEP. In the system, the captured miRNA and the tagged RuO_2 NPs act as the templates and catalysts, respectively. Consequently, hybridization with RuO_2-tagged miRNA and incubation in a mixture of 3,3′-dimethoxybenzidine (DB)/H_2O_2 led to the formation of an insulating PDB film and the increase in the electrochemical impedance. The amount and insulating capability of the deposited PDB correlated to the miRNA concentration in the range of 6 fM to 2 pM. After incubating the sensing electrode in the mixed DB/H_2O_2 solution for 60 min, a detection limit of 3 fM was obtained by electrochemical impedance spectroscopy.

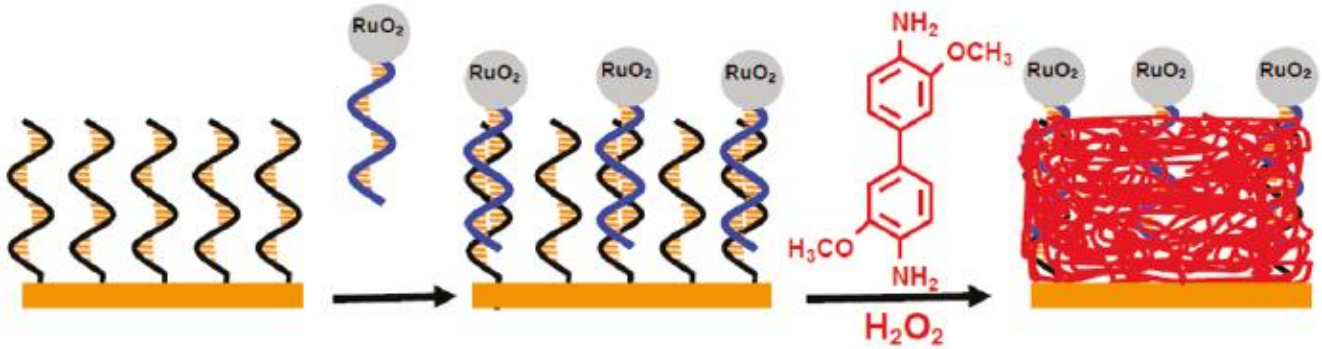

Figure 5. Schematic illustration of the miRNA biosensor based on RuO_2 NP-catalyzed miRNA-templated deposition of a thin PBD insulating film. (Reprinted with permission from ref. [50]. Copyright 2011 American Chemical Society)

With the same condensation reaction, Gao's group suggested that miRNA in total RNA could be directly ligated with an isoniazid-substituted osmium complex, $Os(dmpy)_2(IN)Cl^+$ (dmpy = 4,4'-dimethyl-2,2'-bipyridine, IN = isoniazid) [51]. After hybridization with pre-immobilized complementary capture probes, the labeled miRNA showed excellent electrocatalytic activity towards the oxidation of ascorbic acid. An increase in sensitivity of 2000-fold over direct voltammetry is obtained in electrochemically amplified amperometry, allowing ultrasensitive detection of miRNA. Under optimized experimental conditions, a detection limit of 800 fM and linear current–concentration relationship up to 300 pM are obtained. The sensor is applied to the quantitation of miRNA in total RNA extracted from HeLa cells.

2.4 MiRNA-initiated cleavage of DNA by duplex specific nuclease

Duplex specific nuclease (DSN) displays a strong preference for cleaving double-stranded DNA or DNA in DNA:RNA heteroduplexes and is practically inactive toward single-stranded DNA, or single- or double-stranded RNA [52,53]. It shows a good capability to discriminate between perfectly and nonperfectly matched (up to one mismatch) short duplexes and is widely applied in molecular biology field, including full-length cDNA library normalization, subtraction, quantitative telomeric overhang determination, and genomic single-nucleotide polymorphism detection. Based on this property, Gao's group developed a highly sensitive and selective label-free miRNA genesensor based on hybridized target miRNA strands initiated cleavage of hybridized DNA capture probes (CPs) by a DSN on a gold electrode (Fig. 6) [54]. Briefly, the hybridized CPs in the miRNA-CP duplexes are simultaneously cleaved by the DSN, releasing the target miRNA strands back to the sample solution. The released target miRNA strands again hybridize with the remaining CPs on the electrode, thus forming an isothermal amplification cycle. The distinct difference in electrochemical impedance between a control and the DSN cleaved genesensor allows label-free detection of miRNA down to femtomolar levels. The mismatch discrimination ability of the DSN permits miRNA expression to be profiled with high selectivity.

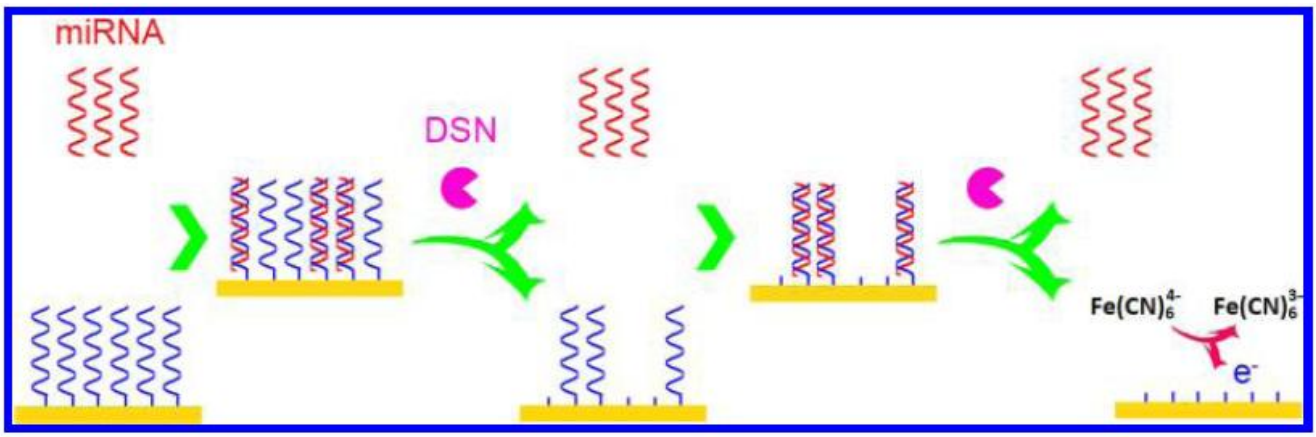

Figure 6. Schematic illustration of the working principle of the label-free electrochemical biosensor. (Reprinted with permission from ref. [54]. Copyright 2013 American Chemical Society)

2.5 Interaction with p19 binding protein

p19 RNA binding protein from carnation Italian ring spot virus (p19) can bind to small 21−23 bp dsRNA with nanomolar affinity via electrostatic and hydrogen-bonding interactions between the β-sheet formed by the p19 homodimer and the sugar−phosphate backbone of dsRNA, thereby making its binding sequence independent of the RNA substrate [55-57]. Remarkably, p19 protein does not bind to ssRNA, rRNA, mRNA, ssDNA, or dsDNA. Recently, Labib et al. developed a three-mode electrochemical sensor for analysis of five miRNA based on the self assembly of a thiolated RNA onto gold nanoparticles-modified screen-printed carbon electrode (GNPs-SPGE) [58]. The three-way analysis detects one or multiple miRNA by a hybridization-based modality (H-SENS), a protein-based modality (P-SENS), and a displacement-based modality (D-SENS). In H-SENS, the hybridization of the target miRNA to its complementary immobilized probe causes a modulation of the electrical signal (Figure 7b).

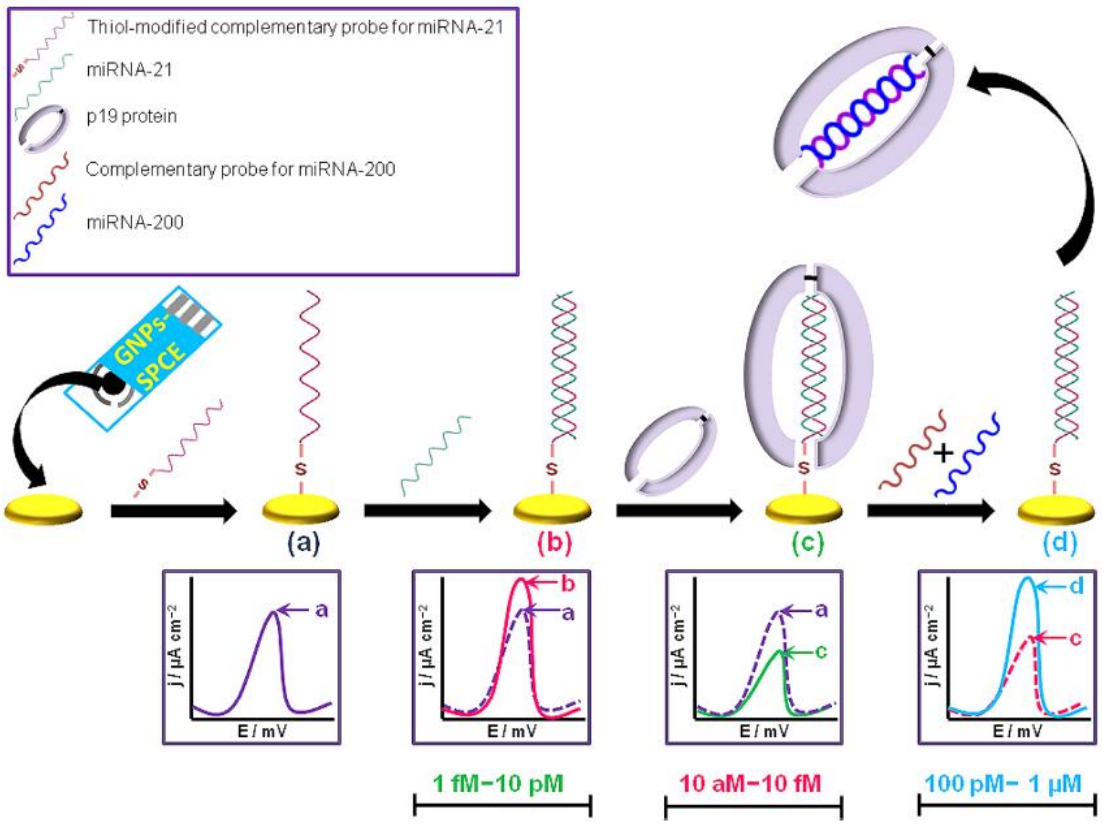

Figure 7. Schematic representation of the 3-mode electrochemical sensor (HPD-SENS) for detection of miRNA. (Reprinted with permission from ref. [58]. Copyright 2013 American Chemical Society)

Furthermore, addition of the p19 protein dimer to the formed hybrid amplifies the signal and allows an ultrasensitive detection of the target miRNA via binding of the bulky p19 protein and consequently shielding the electrode surface (Figure 7c). A universal displacement-based sensor (Figure 7d) is formed on the basis of the self-assembled thiolated RNA probe bound to the saturation concentration of a miRNA, whereas the p19 is attached to the formed hybrid. Subsequently, a mixture

of a target miRNA and a nonthiolated RNA probe is incubated with the p19-modified sensor. If the RNA probe is complementary to the miRNA sequence, a newly formed hybrid at relatively higher concentration compared to the concentration of the immobilized hybrid can force the p19 to dissociate from the immobilized hybrid on the electrode surface and to bind to the newly formed hybrid causing a shift back of the signal. Square wave voltammetry (SWV) and electrochemical impedance spectroscopy (EIS) was employed to assess the electrochemical results. The sensor can identify as low as 5 aM or 90 molecules of miRNA per 30 μL of sample. The linear detection range is from 100 pM to 1 μM.

Field-effect transistor (FET) is one of promising techniques to develop label-free, rapid and sensitive electrochemical genesensors. Ramnani et al. reported an electrochemical miRNA nanobiosensor using a p19-functionalized CNTs FET [59]. Interaction of dsRNA and p19 induced the conductance change of CNTs. The target miRNA in the concentration range from 1 aM to 10 fM could be detected readily in the presence of a million fold excess of total RNA and other miRNA.

Very recently, Campuzano et al. developed an electrochemical magnetosensor for direct determination of miRNA in RNAt raw sample on commercial screen-printed electrodes [60]. In this work, p19 protein was immobilized onto chitin-functionalized magnetic beads (chitin–magnetic beads) for the capture of the miRNA/anti-miRNA duplex. SA–HRP was anchored onto the magnetic beads through the streptavidin-biotin interaction. The resulting magnetic beads were then magnetically captured by the screen-printed carbon electrodes (SPCEs). By measuring the catalytic amperometric current upon the addition of H_2O_2 and hydroquinone, the method showed linearity between 0.14 and 10.0 nM with a detection limit of 0.04 nM.

3. CONCLUSION

MiRNA plays significant roles in numerous developmental, metabolic, and disease processes and have been regarded as the biomarker and therapeutic target in cancer treatment. The short length, low expression and high sequence similarity of miRNA arouse the creation of efficient tools for their rapid, specific and sensitive detection in complex samples. Electrochemical nucleic acid genesensors are able to provide high sensitivity at low cost with simple miniaturised redout. In this work, we, for the first time, addressed the progress in the design of electrochemcial miRNA genesensors based on the difference in the structure of RNA and DNA. We believe that the work would be valuable for the design of new types of label-free and sensitive miRNA genesensors.

ACKOWLEDGMENTS
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