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Electrochemical-based biosensors for microRNA detection: Nanotechnology comes into view

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ABSTRACT

Nanotechnology plays an undeniable significant role in medical sciences, particularly in the field of biomedicine. Development of several diagnostic procedures in medicine has been possible through the beneficial application of nano-materials, among which electrochemical nano-biosensors can be mentioned. They can be employed to quantify various clinical biomarkers in detection, evaluation, and follow up stages of the illnesses.

MicroRNAs, a group of regulatory short RNA fragments, added a new dimension to the management and diagnosis of several diseases. Mature miRNAs are single-stranded RNA molecules approximately 22 nucleotides in length, which regulate a vast range of biological functions from cellular proliferation and death to cancer development and progression. Recently, diagnostic value of miRNAs in various diseases has been demonstrated. There are many traditional methods for detection of miRNAs including northern blotting, quantitative real time PCR (qRT-PCR), microarray technology, nanotechnology-based approaches, and molecular biology tools including miRNA biosensors. In comparison with other techniques, electrochemical nucleic acid biosensor methods exhibit many interesting features, and could play an important role in the future nucleic acid analysis. This review paper provides an overview of some different types of nanotechnology-based biosensors for detection of miRNAs.

1. Introduction

Bio-nanotechnology is one of the novel developing areas of nanotechnology, which incorporates different fields such as engineering, physics, as well as molecular engineering into otherscience areas including biology, chemistry, and biotechnology. For instance, bio-nanotechnology provides us with modern tools consisting of sensors working based on bio-techniques, nano-based medical facilities, along with bio-photonics [1]. Every biosensor can be an analytic system which is comprised of a component for recognizing biological issues when straight spatial connection is established with the conduction component to guarantee fast and exact transformation of biologic phenomena into quantifiable signals [2].

Yet, detection of valuable Nano-materials has brought about more possibilities to carry out studies in the area of bio-sensing, while it is also possible to provide considerable benefits such as being more

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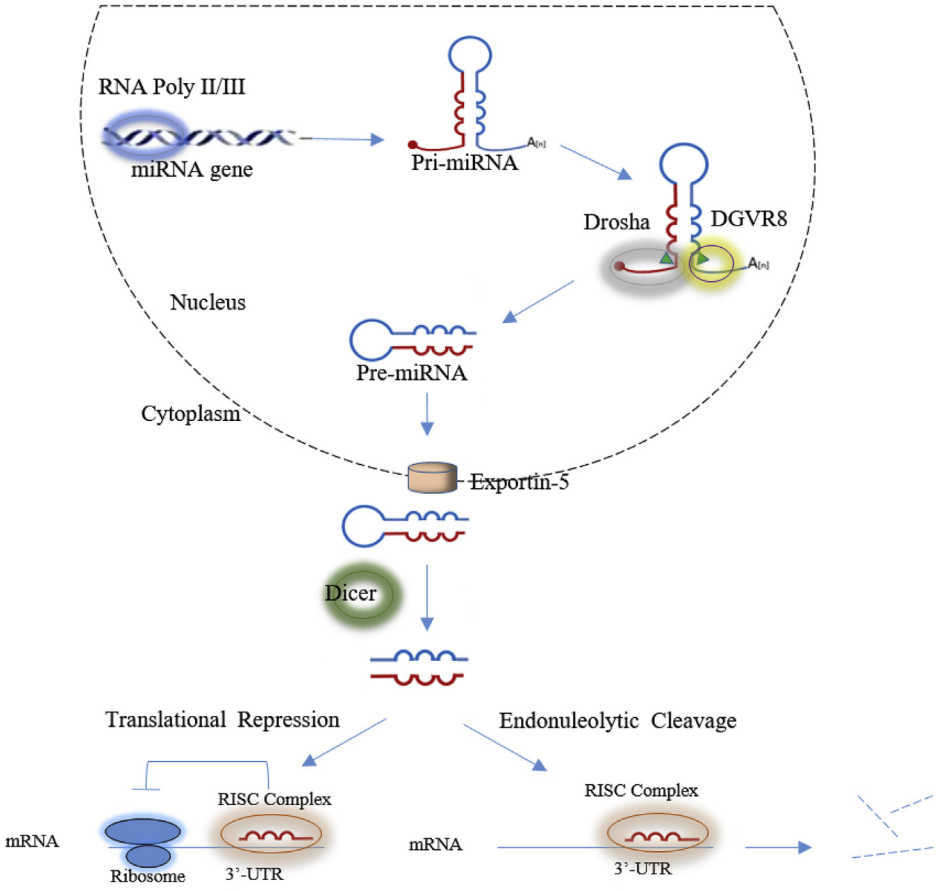

Fig. 1. Biogenesis of microRNAs. miRNA genes are transcribed in the nucleus and processed by DGCR8/Pasha and the RNAse III family enzyme, Drosha. Next, the pre-miRNA is then transported into the cytoplasm where it is processed by Dicer to generate a ~22 nt miRNA:miRNA* duplex. After unwinding, the miRNA forms part of the RISC assembly and causes translational repression or mRNA degradation.

sensitive and specific than the previous bio-diagnosis [3].

Since, carbon nano-materials possess extraordinary electrical, thermal, chemical, as well as mechanical characteristics, researchers have been largely interested in them. Consequently, they have been applied in various fields including composites, energy saving and converting, sensors, administration of medicine, field emission systems, and electronic elements at nano-scale [4,5]. Furthermore, synthesis customization is possible through connected practical elements, while their assembly in the form of 3D arrays would help the scholars devise catalysts with more surface area along with materials possessing high levels of photo-chemical as well as electrochemical functions. They are broadly applied in designing catalysts in hydrogenation, biosensors, and fuel cells due to their extraordinary features [4].

MicroRNAs (miRNAs) are a group of small noncoding RNAs that play an important regulatory role in gene expression at posttranscriptional levels by inhibiting translation or affect on RNAs degradation [6–8]. This macromolecules are potential biomarker candidates for diagnosis and prognosis of different diseases [9]. However, highly sensitive and selective methods for miRNA detection is still a challenge because of their unique characteristics such as low abundance, small size and high sequence homology among the family members [10–12].

Currently, the common conventional assays for miRNA detection include northern blotting, microarray analysis and RT-PCR [13,14]. The disadvantages of these methods are complicated handling, high costs and unstable results [15]. However, determination of miRNAs clearly requires the development of highly sensitive techniques in order to assess very low levels of sample in the bloodstream, and also needs to be very selective and comply with minimum sampling volume requirement, cost-effectiveness, and multiplexing capabilities of a diagnostic test [12,16].

In recent years, various electrochemical biosensors have been developed for the sensitive detection of miRNAs [12,17]. Electrochemical genosensors hold great promise to serve as devices appropriate for point-of-care diagnostic and multiplex platforms for fast, simple and low-cost nucleic acids analysis as well [18]. Thus, different strategies have been proposed to obtain a favorable miRNA biosensor. According to some reports, application of various enzymes which engage in the oxidation-reduction reactions can be beneficial in electrochemical miRNA biosensors, in which generation of the final electrochemical signal is done by the enzymes [19,20]. It should be noted that, these biosensors which work based on enzymes require completely ideal circumstances to maintain the stability as well as the activity of the enzymes when biosensor fabrication and electrochemical evaluation have been done [21].

Applying conductive polymers to modify electrodes is another novel idea, according to which the polymers and their derived forms, significantly accelerate the transmission of the electrons on the electrode. Accordingly, this results in promotion of electrochemical function of the electrode, while signaling identification is also amplified [22]. In addition, the current involvement of nano-materials in bio-sensing has been accompanied by novel horizons toward miniaturization of the electrochemical bio-sensors along with improving their analytic potential [23]. The significant influence of nanotechnology on the provision of electrochemical miRNA bio-sensing is considered in the following section.

In this review, we described miR-141, miR-155, miR-21, and Let-7 functions in human diseases especially in cancers. Afterward, we illustrated some sensitive and specific electrochemical biosensors for these miRNAs.

2. MicroRNA: insights into its biogenesis

MicroRNAs (miRNAs) are 18–27 nucleotide-long and are categorized as non-coding RNAs. ncRNAs are RNA molecules that are not translated into a protein [24]. Recent evidence suggests that the majority of the human genomes are transcribed into ncRNAs while the number of them is unknown. As the wide proportion of ncRNAs, miRNAs are important parts of “dark matter” in the human genome. Most of miRNAs are located in introns and/or exons, and also approximately 30% of miRNA genes are found in intergenic regions or in the anti-sense orientation of genes and contain their own promoter and regulatory units [25].

miRNA genes are transcribed by RNA polymerase II (pol II) or RNA polymerase III (pol III) [26]. miRNA biogenesis consists of three major steps, the first step ocurres in the nucleus, an ~80 nt primary or pri-miRNA is transcribed from the genome and is cleaved by a nuclear RNase III enzyme termed Drosha [27]. That results in the formation of a 60 to 70 nucleotide stem-loop intermediate termed a pre-miRNA which actively transported to the cytoplasm by Ran-GTP and Exportin-5 [28]. In the cytoplasm, pre-miRNA is processed by RNase III endonuclease Dicer complex, to 20-22 nt double-stranded fragment which is incorporated into RNA-induced silencing complex (RISC) (Fig. 1) [29,30].

The 5′ region of miRNAs binds to the 3′ untranslated region (3’ UTR) on target messenger RNA (mRNA) transcripts and often, they result in translational repression or mRNA degradation [31,32]. The expression patterns of miRNAs vary from ubiquitous up to tissue or cell-specific and their function depends on the cell contents and target availability within a cell [33].

3. The role of miRNAs in pathological conditions

Numerous studies have reported that certain miRNAs got highly changed during development and also disease, such as some types of cancers, heart diseases, diabetes, nervous system, kidney and liver diseases etc. [34–37]. Therefore, microRNAs which are deregulated during disease may be one of the particular research interests. its noteworthy that miRNA expression is a dynamical process and measurement of Cell-free nucleic acid has potential to be a cost-effective and non-invasive tool for disease screening and prognosis assessment. Unit now, it has been demonstrated that, some miRNAs like miRNA-141, miRNA-155, Let-7, and miRNA-21, are either increased (up regulated) or decreased (down regulated) in cancer cells [38–41]. It reveals their potential as a class of highly sensitive and specific biomarkers for tumor classification and prognosis (Table 1). New diagnostic tools including discovery and early detection of new biomarkers in addition of using new drugs and therapies have improved diagnosis and therapy outcomes.

3.1. miRNA-141

A growing body of studies have shown that tumor-derived miRNA-141 deregulation in human plasma could serve as important biomarkers for the blood-based detection of human cancers, including prostate [42,43], colon [44] and ovarian cancers [45].

A study by de Souza et al. [42] investigated the functional role of miRNA-141 in modulating tumorigenesis in prostate cancer. They analyzed miRNA-141 expression in plasma samples collected from 102 untreated prostate cancer patients and of 50 healthy controls. Early results showed that expression of the miRNA-141 was significantly up regulated in the metastatic patient's plasma specimens. Also, another study of patients with prostate cancer, reported that miRNA-141 levels in plasma can be used to screen metastatic prostate cancer with high sensitivity [43].

Moreover, Cheng et al. reported that in 102 patients with stage IV disease, elevated plasma miR-141 levels were also associated with distant metastasis and poor prognosis [44]. Their data indicated that miRNA-141 could readily discriminate distant metastasis cases from normal controls and patients with other stages.

In addition, analysis of miRNA arrays demonstrated that miRNA-141 was overexpressed in the endometrioid histotype compared to normal tissue [45]. According literatures, this miRNA is an onco-suppressor of BAP1 (as a BRCA1-associated protein) [46].

Furthermore, Bing Zhou et al. [47] indicated that miRNA-141 plays an essential role during myocardial fibrosis in the diabetic mice. They found that circRNA_010567 is markedly up-regulated in diabetic mice myocardium; sponge miRNA-141 and miRNA-141 directly target TGF-β1, caused suppressing fibrosis-associated protein resection.

Multiple studies have identified that miRNA-155 plays a significant role in various human cancers, including breast, lung, colon, pancreatic and thyroid tumors [48–52].

3.2. miRNA-155

Cheng Fang and colleagues revealed that serum miRNA-155 appeared to be potentially useful tools as a novel noninvasive biomarker for diagnosis of diffuse large B cell lymphoma (DLBCL). Data showed that miRNA-155 was significantly elevated in DLBCL serum samples compared with normal controls [53]. In addition, Costinea et al. reported that miRNA-155 induces B-cell malignancies by targeting Ship and C/EBPβ, which are two important inhibitors of the IL-6 signaling pathway, in a transgenic mouse model [54].

Furthermore, Mattiske et al. reviewed the role of miRNA-155 in breast cancer progression [55]. They suggested new avenues of research for this oncogenic miRNA. Yang et al. had also the same results [56]. They performed a meta-analysis of two specific miRNAs (miRNA-155 and miRNA-21) as important prognostic classifiers for non-small cell lung cancer (NSCLC). Gao et al. also introduced miRNA-155 as a diagnostic marker for the early detection of lung adenocarcinoma. They demonstrated that expression of miRNA-155 was significantly higher in serum of lung adenocarcinoma patients than that in normal controls [57].

Another study showed the relative expressions of miRNA-155 in

Table 1
Circulating miRNAs as diagnostic markers for different cancer.

	miRNAs	alteration	Type of tissue	Diseases	Refs.
1	MiR-141	Up	Plasma	Prostate cancer	[43]
2	MiR-21, MiR-141	Up	Plasma	Ovarian cancer	[123]
3	MiR-155	UP	Serum	Breast cancer	[124]
4	MiR-155, MiR-21	Up	Plasma	Lung cancer	[125]
5	MiR-155, MiR-21	Up	Plasma	Pancreatic cancer	[126]
6	MiR-155, MiR-21	Up	Serum	DLBCL(diffuse large B-cell lymphoma)	[127]
7	MiR-155	Down	Serum	Ovarian cancer	[128]
8	MiR-21	Up	Serum	Ovarian cancer	[128]
9	let-7a	Down	Serum	Gastric Cancer	[129]
10	let-7a	Down	Serum	Breast cancer	[130]

esophageal tissue significantly associated with increased risk for esophageal cancer [58]. Zhong-chuan et al. also performed independent validation experiments using a large cohort of 146 patients with colorectal cancer (CRC) and 60 control subjects. They revealed that the detection of miRNA-155 levels in the serum might serve as a useful tumor biomarker in the assessment of prognosis and diagnosis of colorectal cancer [59].

3.3. miRNA-21

Many articles have published promising results for miRNA-21. This miRNA not only to be used in early diagnosis and drug development in cancer biology but also in cardiovascular disease [60–63]. In addition, Iorio et al. showed that miRNA-21 is one of the most consistently up-regulated miRNAs in breast cancer. Indeed, miRNA-21 is progressively up regulated with increasing tumor stages and from normal breast to cancer [50].

Moreover, Nam et al. demonstrated that miRNA-21 was the most frequently up regulated miRNA in the serous ovarian carcinoma biopsies compared to normal control ovarian tissue. They suggested relevance of miRNA-21 to ovarian cancer and it may be a universal fluid biomarker for cancers [64]. In another study, northern blot results revealed that the expression of miRNA-21 was significantly increased in 21 out of 29 tumor samples [65].

Significant up-regulation of miRNA-21 was also observed in CRC samples that explain why high tumor-specific miRNA-21 expression levels positively is correlated with the cancer stage and development of distant metastases [66,67].

In another study, analysis of miRNA expression profiling in hepatocellular carcinoma (HCC) tumor and normal liver tissues showed a highly increased miRNA-21 expression in tumor compared to the normal samples [68].

Moreover, miRNA-21 appears to be strongly deregulated in cardiovascular disease. Tatsuguchi et al. showed that miR-21 expression is induced during hypertrophy and, most importantly, their data suggest miR-21 as a negative regulator in cardiac hypertrophy [62].

3.4. Let-7 family

Let-7 family has multiple isoforms and composed of nine mature miRNAs in human body (Table 2). These isoforms encoded by 13 distinct genomic loci, some of which are clustered together [69]. Let-7 has also been implicated in various diseases, most notably cancer [70]. In higher organisms, reduced expression of multiple let-7 members has been found to be associated with cancers while the levels of let-7 rise during embryogenesis [71,72].

In many cancers, activation of non-coding genes, LIN28A and LIN28B, two highly related RNA binding proteins (RBPs) and proto-oncogenes, are responsible for the global post-transcriptional down regulation of the let-7 miRNA family [73]. For example, miRNA-203 and miRNA-146a enhance let-7 biogenesis by targeting LIN28B in order to suppress tumor growth in lung and breast cancer, respectively [74,75].

Table 2
Sequence of the let-7 family miRNAs. The red nucleotides are the positions that are identical among all members of the let-7 family.

1	Let-7a	5′UGAGGUAGUAGGUUGUAUAGUU 3′
2	Let-7b	5′UGAGGUAGUAGGUUGUGUGGUU 3′
3	Let-7c	5′UGAGGUAGUAGGUUGUAUGGUU 3′
4	Let-7d	5′AGAGGUAGUAGGUUGCAUAGUU 3′
5	Let-7e	5′UGAGGUAGGAGGUUGUAUAGUU 3′
6	Let-7f	5′UGAGGUAGUAGAUUGUAUAGUU 3′
7	Let-7g	5′UGAGGUAGUAGUUUGUACAGUU 3′
8	Let-7i	5′UGAGGUAGUAGUUUGUGCUGUU 3′
9	has-miR-98	5′UGAGGUAGUAAGUUGUAUUGUU 3′

In addition ofcancer, Let-7 family appears to be strongly deregulated in cardiovascular biology and diseases. Brennan et al. reported that let-7 levels are decreased in diabetic human carotid plaques and suggested the restoration of let-7 expression could provide a new target for an anti-inflammatory approach in diabetes-associated atherosclerosis [76]. Also, Faccini's study introduced let-7c, miRNA-145 and miRNA-155 as diagnostic biomarkers in coronary artery disease [77].

4. Electrochemical-based biosensors

According to IUPAC, a biosensor is an integrated receptor transducer device, which can provide selective quantitative or semi-quantitative analysis of an analyte using bio-recognition elements [78]. A biosensor gives us rapid, real-time, precise and authentic information about the interrogation analyte, and can detect pathogenic and physiological relevant molecules with desirable sensitivity and specificity [79,80]. This analytical device converts a biological response into a detectable electrical signal, and functions by a biological sensing (bio-recognition) element with a detector system using a transducer (Fig. 2) [81]. The biosensors are mainly consist of three sections: (i) Bio-recognition element can be a biological component including enzyme, antibody, DNA, aptamer, or even a whole cell, etc.,(ii) Transducer that is detector element and functions in a physicochemical; optical, calorimetrical, piezoelectrical, electrochemical approaches and etc. The signal results from analyte and bio-recognition element interactions will convert by transducer in order to display signals to understandable manner, (iii) signal processing system is the associated electronic section, which comprises of signal amplifier, processor and a display unit [82].

Hence, a new era of advancement in technology and science has emerged by development of biosensors. Thus, due to validated results, biosensor has been widely applied in different scientific issues. In medicine, they can be utilized for accurate and reliable detection of tumors, toxins, pathogens and physiological molecules, offer a powerful opportunity in the early diagnosis and treatment of disease [83]. In this field, several type of biosensors based on calorimetry, piezoelectricity, optic and as well as electrochemistry have been develop [82,84].

5. Electrochemical-based biosensors and nanotechnology

Integrating the recognition and electronic components had been the center of emphasis through the beneficial features including convenient handling, economic feasibility, sensitivity, portability, and easy construction in order to produce electrochemical sensors as well as biosensors. A variety of electrochemical tools consisting of amperometrics, sensors for electrochemical impedance, along with electrochemical luminescence and photo-electrochemical sensors make a broad range of usages regarding identification of chemical as well as biological targets [85].

Given the considerable accomplishments in nanotechnology and the related sciences, augmentation of electrochemical signals based on nano-materials has the capability to improve sensitive as well as selective electrochemical and bio-sensors. Initially, the material of electrodes plays an obviously essential role for establishment of highly efficient electrochemical sensing systems which are able to recognize the target molecules following different analytic procedures [86].

Moreover, besides the material of the electrodes, these practical nano-materials can provide synergetic effects of the catalytic functions, conduction, and bio-adaptability. This synergism enhances signal conduction, while it also reinforces bio-realization of the incidents through the special signal tags which result in high sensitivity in bio-sensors. Comprehensive studies regarding the provision of practical materials for electrodes, along with a wide range of electrochemical procedures have increasingly advanced the utilization of electrochemical systems [85].

For instance, Walcarius et al. have referred to the current

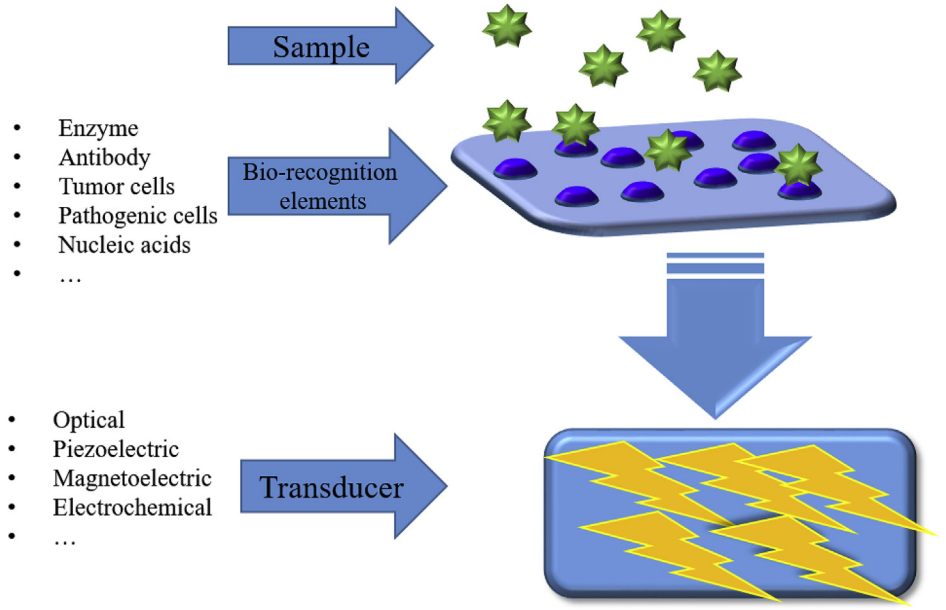

Fig. 2. Schematic diagram of a biosensor. A biosensor consists of a bio-recognition element, for the specific detection of the analyte in contact to a transducer for converting the signal into an electrically manageable format and a signal processing unit.

developments in nano-objects as well as nano-engineered/structured materials to provide logical bio-functional electrodes along with associated bio-sensors [87]. These nano-materials are interesting due to their capability of performing efficient immobilization indices and their internal as well as incomparable characteristics which were discussed before. These characteristics along with the function of biomolecules can influence the promotion of bio-electrode application. Hence, regarding the qualities of being sensitive and specific, taking nanomaterial-enabled paper-based biosensors such as lateral-flow test-strips and paper microfluidic systems into account [88].

6. Electrochemical-based biosensors in detection of miRNAs

Electrochemical biosensors hold great promises to serve as devices that are suitable for point-of-care diagnostics and multiplex platforms for impressive sensitive, simple and affordable nucleic acid and miRNA detection [89]. In a typical electrochemical miRNA biosensor, the transduction element is a solid electrode, (i.e. most often gold or graphite), is a selective short single-strand single nucleotide probe, which immobilizes on electrodes. DNA probe and the target miRNA influence the electrode or interfacial performance. Often, an electrochemically active reporter species transduces changes in electrode properties caused by miRNA hybridization to signal. Horse radish peroxidase (HRP), hydrogen peroxide (H2O2), ferrocyanide (i.e., $[Fe(CN)_6]^{-3}$, $[Fe(CN)_6]^{-4}$) glucose oxidase, alkaline phosphatase, methylene blue and hydroquinone are some examples of electrochemically active reporters used in miRNA detection biosensors [90–93]. Furthermore, amperometric [92,94], resistive [95], potentiometric [94,96] as well as impedance-based [94,97] approaches have been used to transduction.

Unique characteristics of miRNAs such as small size, extremely low abundance in total RNA samples, and high sequence homology among family members have made their quantitative analysis to a great challenge. In recent years, however, convenient and sensitive approaches including rolling circle amplification (RCA) [98], sequencing [99], surface enhanced Raman spectroscopy (SERS) [100], bioluminescence [101], surface plasmon resonance (SPR) spectroscopy [102], fluorescence [103], as well as electrochemistry [104] have been developed. Despite these approaches, given that advances of electrochemistry, there is an impressive interesting in electrochemical methods due to simplicity, sensitivity and low cost [19].

Generally, employing nano-materials in the layout and provision of biosensors will reduce the size of the system and bring about additional beneficial features for efficient advancements in precision and replicability of the results. These features are considered important for nanomaterials, because they usually indicate new and various optic, electric, electrochemical, as well as magnetic traits (in a size range of 1–100 nm) in comparison with bulk forms [105]. A considerable proportion of the surface to the volume as well as a size range like biomolecules would account for the effective reactions between nano-materials and biomarkers without subsequent destructions [106]. Then, electrochemical biosensors have been fabricated to determine miRNA using a broad scope of nano-particles, rods, wires, clusters, composites, and so on. All of them would primarily engage in amplifying electrochemical signaling, bio-separating, and being a platform to immobilize biomolecules [23]. All of electrochemistry based miRNA detection methods, which introduce in this literature, rely on hybridization (Table 3).

6.1. miR-141

Tran et al. reported an electrochemical immunosensor for detection of miR-141 in which gold electrodes modified with reduced graphene oxide (GO) and carbon nanotubes (CNT) has been applied. Amino modified oligonucleotide capture probe was immobilized on electrodes and complementary miRNAs added. Following hybridization, an antibody directed to the RNA. Then, DNA hybrids bound selectively to the hybrids. After that, secondary antibody conjugated to horseradish peroxidase (HRP). They used hydroquinone chromophore which is well known to be oxidized into benzoquinone by HRP/H2O2 catalytic system [90]. Detection limit of this method was down to 10 fM. Schematic configuration of this biosensor is illustrated in Fig. 3.

In another research, a label-free miR-141 sensor based on a network of CNT and electro-active polymer was described. This one is a probe of miR-141 that bound to Poly (JUG-co-JUGA)/o-multiwall carbon nanotube (MWCNT)-modified electrodes (Fig. 4). Due to quinone group embedded in polymer backbone, polymer film presented very well electro-active property in neutral aqueous medium. Addition of target miR-141 increased current because of enhancement of the polymer electro-activity, but non-complementary miRNAs could not cause any significant current changes [107].

Table 3
Electrochemical biosensor for detection of Let-7, miR-21, miR-141 and miR-155.

N	Sensing platform	Reporter	Target miRNA	LOD	Linear range	Ref
1	capture probe tagged with OsO2 nanoparticle	hydrazine	Let-7b	80 fM	0.30–200 pM	[131]
2	miRNAs were ligated with Os(dmpy)2(IN)Cl +	ascorbic acid	Let-7b	800 fM	1.0–300 nM	[132]
3	Ru(PD)2Cl2 was tagged onto miRNA	hydrazine	Let-7b	0.20 pM	0.50–400 pM	[133]
4	long oligonucleotide capture probes comprising miRNA and glucose oxidase detection probe capturing segments	glucose	Let-7c	4.0 fM	8.0 fM to 10 pM	[134]
5	detection probe labeled with glucose oxidase	glucose	Let-7b	10 fM	20 fM to 10 pM	[92]
6	target-guided formation of conducting polymer nanowires in nanogaps	H_2O_2	let-7b	5.0 fM	10 fM to 20 pM	[135]
7	off–on sensing platform based on LNA capture probe beacon	H_2O_2	miR-21	6 fM	0.01–700 pM	[136]
8	LNA hairpin probe-biotin and streptavidin-HRP	H_2O_2	miR-21	0.4 pM	1–5000 pM	[137]
9	Stem loop capture probe hybridized with target miRNA and detection probe	hemin	miR-21	3.96 pM	5–5000 pM	[138]
10	detection based on hybridization of capture probe and target DNA, p19 binding protein and protein displacement	K3[Fe(CN)6] And [Ru(NH3)6]Cl	miR-21,miR-141	5 aM	10 aM -1 μM	[139]
11	capture probe immobilized on Pd-Nps-modified electrode	H_2O_2	miR-155	1.87 pM	5.6 pM -5.6×10^5 pM	[140]
12	target miRNA, cyt C and alcohol oxidase bound to hairpin capture probe	H_2O_2	miR-155	0.35 fM	0.8 fM -1 nM	[141]
13	anti-miR oligonucleotide immobilized on electrode	Fe(CN)6	miR-155	5.7 aM	10 aM- 1.0 nM	[142]
14	methylene blue-probes were released by target hybridization	methylene blue and hemin	miR-155	5.2 pM	No data	[143]
15	target miRNA hybridized with capture probe and made double strand DNA	oracet blue	miR-155	13.5 pM	50 pM -15 nM	[144]
16	capture probe incorporated on tetrahedral DNA nanostructures	H_2O_2	miR141,miR21	10 aM	No data	[145]
17	molecular beacon capture probe immobilized on electrode and tetrahedral DNA	H_2O_2	miR141	1 fM	No data	[146]
18	hairpin capture probe of target miRNAs labeled with electrochemically active agents	Methylene blue, ferrocene	miR141,miR21	4.2 fM, 3 fM	5.0 fM to 50 pM	[147]

6.2. miR-155

Azimizade et al. presented an oligo-hybridization-based electrochemical biosensor for plasma miR-155 detection [108]. This biosensor uses gold nanorods (GNRs) decorated on GO sheets on the glassy carbon electrode (GCE). After that, thiolated probe was immobilized on the gold nanorods (GNRs). Following hybridization of probe and miR-155, Oracet blue (OB) intercalated into the hybrid and reduction signals were measured by differential pulse voltammetry (DPV) method (Fig. 5). A linear relationship was found with the concentration of miR-155 ranging from 2.0 fM to 8.0pM. This nano-biosensor had a great specificity, and could discriminate sharply between complementary target miRNAs, three-base mismatch, and non-complementary miRNAs. Furthermore, sensitivity and selectivity of biosensor was evaluated in plasma samples and demonstrated high functionality for direct detection of miR-155 in plasma without any sample preparation, extraction and amplification.

Hu et al. also developed an electrochemical biosensor for quantitative detection of miR-155 [20]. Thiol-tethered oligonucleotide probes (capture DNA) assembled on the gold electrode and then complemented with target miRNA and aminated indicator probe (NH2-DNA). After construction of the double strand structure, carboxyl groups of graphene quantum dots (GQDs) bound to NH2-DNA and then HRP immobilized on GQDs, noncovalently. HRP modified biosensor effectively catalyzed H2O2 mediated oxidation of 3, 3',5,5′-tetramethylbenzidine (TMB) and caused a change from colorless to blue in solution as well as an increment in electrochemical current signal (Fig. 6). This high performance presented biosensor could detect 1 fM to 100 pM of miR-155 with a detection limit of 0.14 fM.

6.3. miR-21

Kilic et al. reported a novel electrochemical biosensor for detection of miR-21 in breast cancer cell lines based on enzyme amplified method [93]. The pencil graphite electrode (PGE) was modified by capture probes and/or cell lysates. N-(dimethylamino) propyl-N′-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysulfosuccinimide (NHS) intermediated covalently attachment of probes and/or cell lysates on surface of electrode. Hybridization was then accomplished with a biotinylated complementary target. The oxidation signal of alpha naphtol, alkaline phosphatase reaction product was detected in the presence of the hybrid. Fabrication steps of the biosensor are demonstrated in Fig. 7. For the first time, they designed an enzyme based miRNA biosensor for detection of miRNA from cell lysates without any modification. Compared to conventional guanine oxidation based method, the proposed biosensor seems to provide more reproducible results for cell lysates.

Cheng et al. also reported a novel, simple, sensitive, and specific electrochemical biosensor based on a metal ion functionalized titanium phosphate nanospheres for detection of miR-21 [109]. In this design they used Cd^{+2} modified titanium phosphate (TiP-Cd^{+2}) as signal unit and two DNA capture probes. Incorporation of large amounts of Cd^{+2} ions into TiP nanosphere improved electrochemical signals more than 5 times. Ru $(NH3)_6{}^{+3}$ played an electron transfer mediator role that interacts with DNA-base-pairs. Steps of this method have been illustrated in Fig. 8. This method indicated a wide linear range from 1.0 aM to 10.0 pM and a substantial improvement in sensitivity which is 0.76 aM. The proposed biosensor discriminated miR-21 target from homologous miRNA, and detected the miRNA in human serum as well, rapidly.

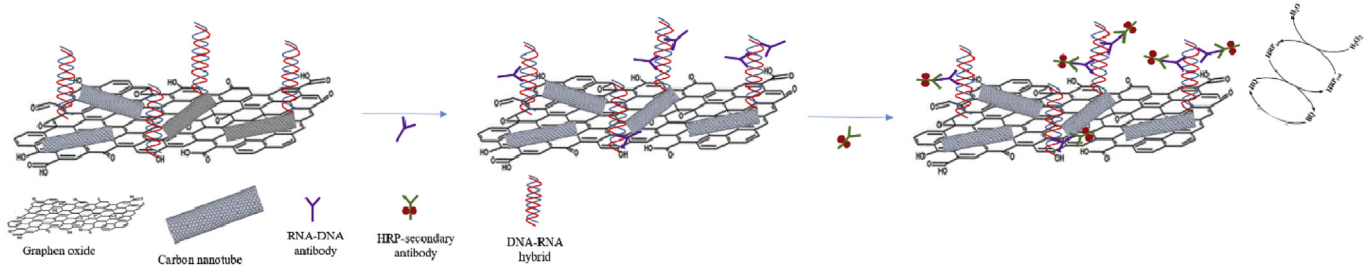

Fig. 3. Schematic representation of ELISA-like immunosensor on electrodes modified with GO and CNT.

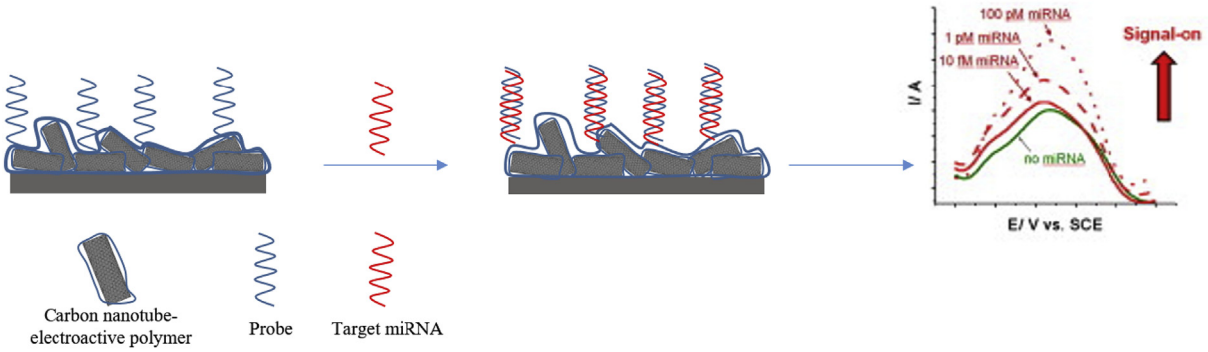

Fig. 4. label and reagent-free biosensor using a conducting polymer nanostructured by CNT.

Locked nucleic acid (LNA) is another form of RNA in which the ribose ring is constrained by a methylene bind between the 2′-oxygen and the 4′-carbon. This unique binding and conformation restrictions increase binding affinity for complementary sequences and decrease non-specific binding and mismatches in double strand hybrids [110]. A research described a highly sensitive biosensor for sequence specific miRNA-21 detection without any labeling or enrichment [111]. This biosensor was based on graphene, LNA integrated molecular beacon, AuNPs and hourse radish peroxidase (HRP). LNA integrated reporter DNA, biotin functionalized signal DNA were attached on AuNPs surface. Beside, glassy carbon electrode (GCE) was functionalized with graphene and dendritic gold nanostructures (DenAu). Substantially, DenAu/graphene/GCE was obtained. Locked nucleic acid (LNA) integrated hairpin molecular beacon (MB) capture probes against miRNA-21 were well aligned probe on functionalized electrode. After hybridization of target miRNA-21, the stem loop structure of MB probe was unfold and 3′-end was far away from electrode surface to hybridize with LNA integrated DNA. So, the biotin functionalized signal DNA loaded on AuNPs could bind with target miRNA-probe hybrids. After that, Streptavidin-HRP interacted with biotinylated signal DNA to catalyze the chemical oxidation of hydroquinone by H2O2 to form benzoquinone. The electrochemical reduction of benzoquinone on amperometric signal was monitored the miRNA hybridization (Fig. 9). Due to specific confirmation of probe and tri-amplification effects of DenAu/graphene/GCE, multi-functionalized Au-NPs and HRP, the biosensor was selective and sensitive with low detection limit of 0.06pM. miRNA-21 expression in two human cell lines (i.e. BEL-7402 as a hepatocarcinoma cell line and L-02, anormal human hepatic cell line), has been analyzed with this proposed biosensor, successfully.

6.4. Let-7

Ren et al. developed an ultrasensitive label-free biosensor specific for Let-7b [112]. Thiolated DNA capture probes were immobilized on the surface of a gold electrode, acting as target miRNA capturing interface, and then were hybridized to target miRNA. Capture probes in resulted duplexes were cleaved by duplex specific nuclease (DSN), simultaneously. DSN is a thermostable enzyme derived from kamchatka crab (*Paralithodes camtschaticus*) that cleaves double strand (ds) DNA and DNA in DNA-RNA hybrids with little activity toward single strand DNA and RNA-RNA hybrids, irrespective of sequence length [113,114]. The target miRNA was released and stranded back to the sample solution and then hybridized again with remaining capture probes. Fig. 10 depicts the working principles and structure of the biosensor. So, hybridization/DSN incubation formed an isothermal amplification cycle through which one target miRNA strand leaded to thousands of the capture strands cleaves. Amount of miRNA was realized through distinct difference in electrochemical impedance between a control and

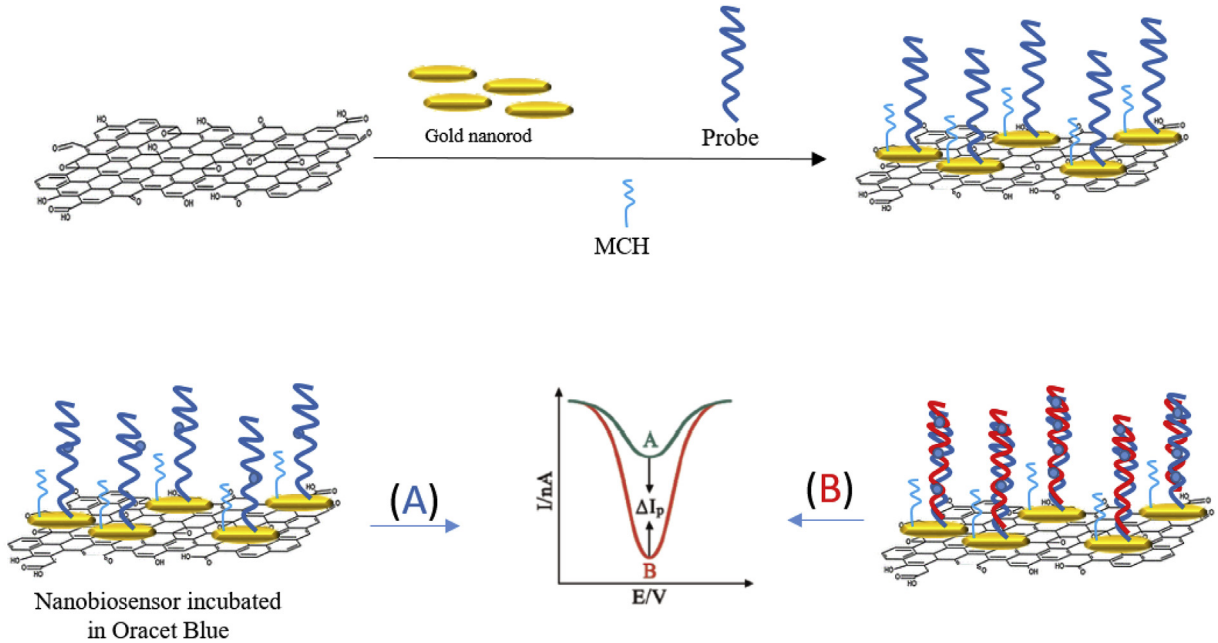

Fig. 5. An electrochemical biosensor based on GO and gold nonaorod. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

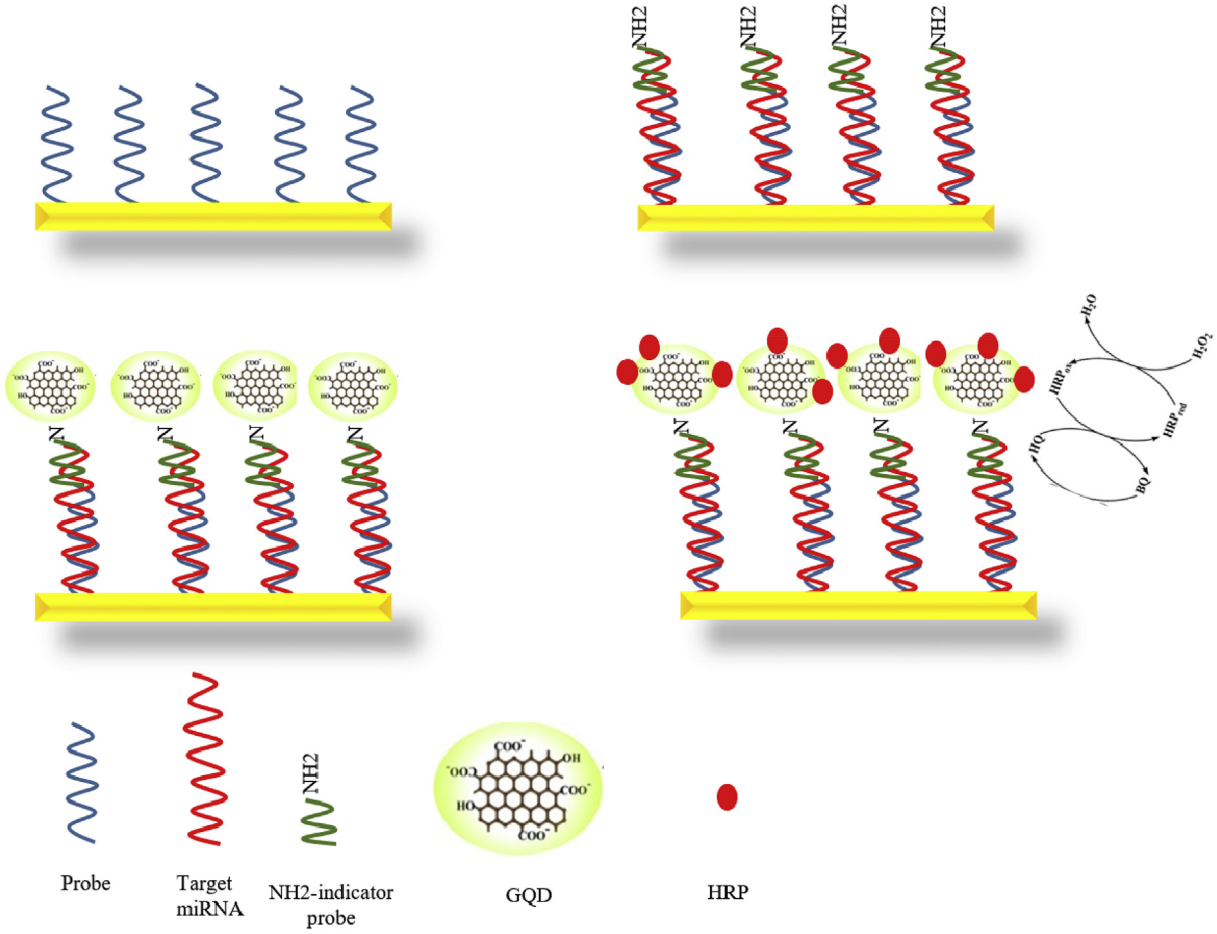

Fig. 6. An electrochemical miRNA biosensor based on GQD.

DSN-cleaved condition. The magnitude of the charge-transfer resistance (R_{ct}) of redox probes had an inverse correlation with the number of DNA-RNA hybrids or with concentration of the target miRNA. This method permitted the detection of target miRNA with 1.0 fM in concentration. Application potential of biosensors for profiling circulating miRNA in blood or blood and cancerous cell total RNAs were investigated. Magnitude of Let-7a, let-7b and let-7c in Hela cells, serum (direct analysis and extracted RNA) were measured by the proposed biosensor. These results were consistent with those obtained by qPCR, thus confirmed the practical value of the biosensor.

Another method has been reported that is based on polymerase extension [19]. A large amount of streptavidin loaded on Au-NPs to make streptavidin-AuNPs (SA-AuNPs) complex (Fig. 11). This complex and alkaline phosphatase (ALP) were responsible for signal amplification. Let-7a as target DNA bound to capture probe immobilized on the electrode, so it could be used as a primer and triggered a primer extension reaction. They used an isothermal DNA polymerase, Bst DNA polymerase, which has a helicase activity and no exonuclease activity [115,116]. During extension, polymerase incorporated biotinylated nucleotides into the synthesized strand. Through the streptavidin-biotin interaction, SA-AuNPs plays a role as a linker to bind biotinylated alkaline phosphatase (biotin-ALP) to the duplex. The ALP converted an electrochemical inactive 1-naphthyl phosphate into an electrochemical active naphthol to generate an amplified electrochemical signal which exhibited a linear correlation ranging from 100 fM to 1 nM of target miRNA, with a detection limit of 99.2 fM. This method showed high specificity to distinguish single nucleotide differences between let7-a and other members of let family.

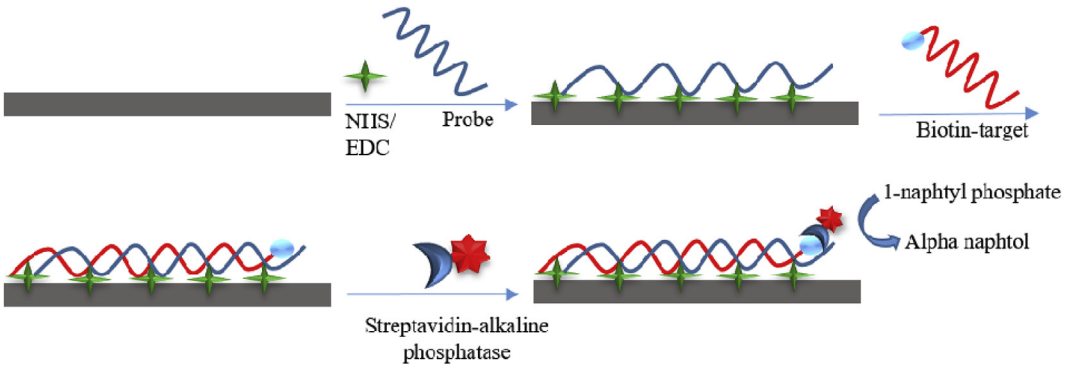

Fig. 7. Schematic representation of DNA-biosensor for detection of miRNA.

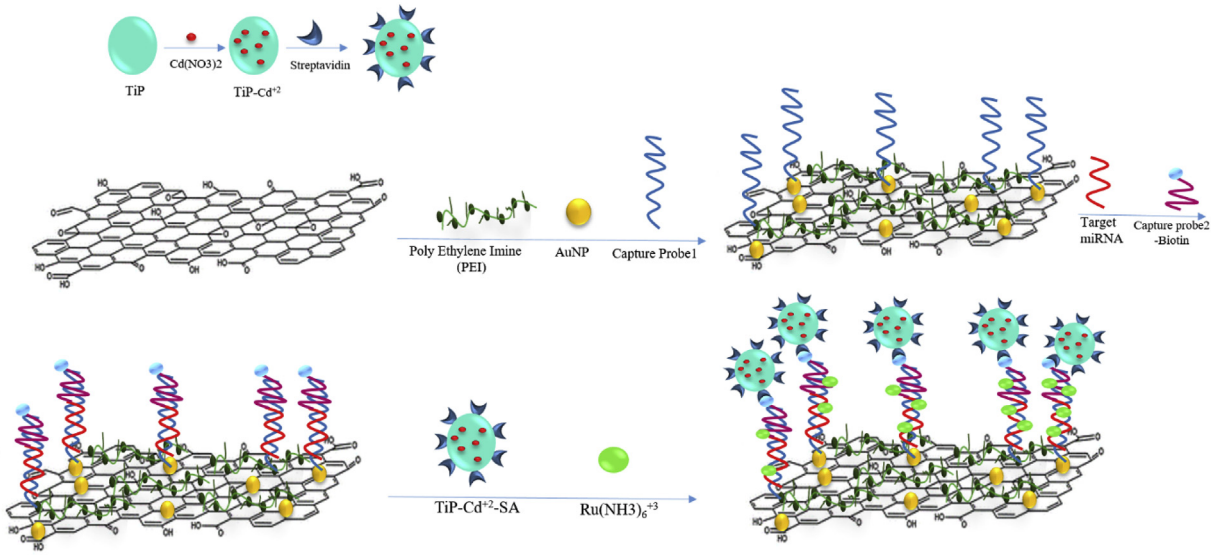

Fig. 8. Electron transfer electrochemical biosensor based on metal ion functionalized Titanium phosphate nanospheres.

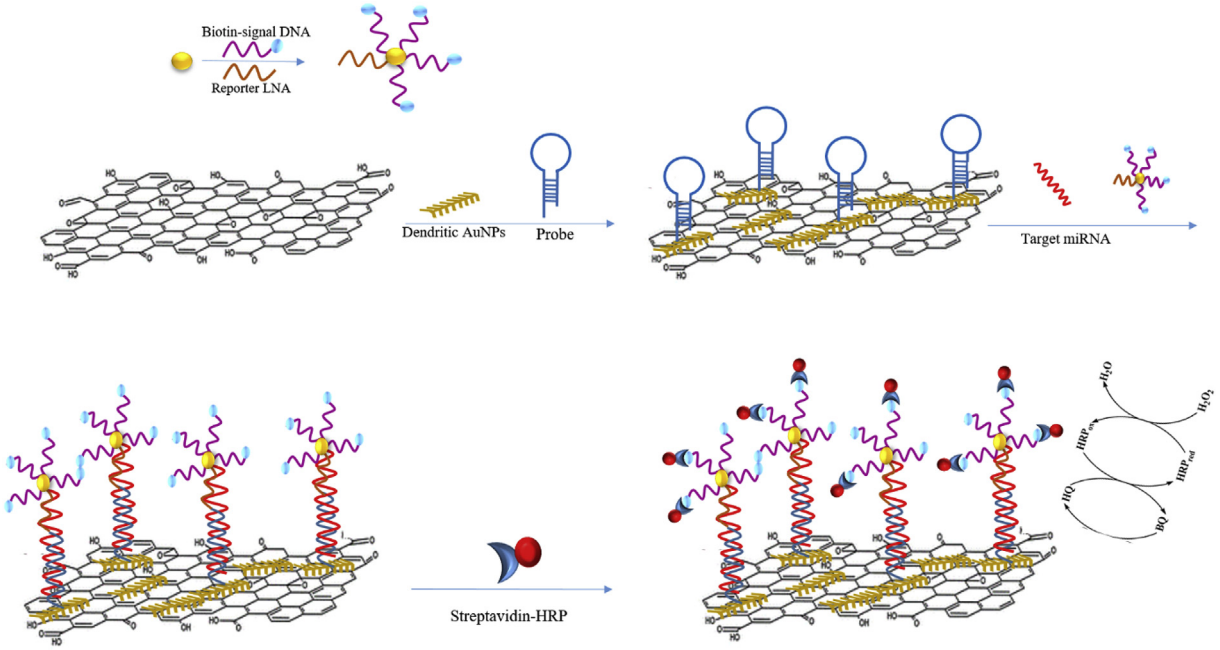

Fig. 9. Electrochemical determination of miRNA based on LNA molecular beacon, dendritic AuNPs immobilized on graphen.

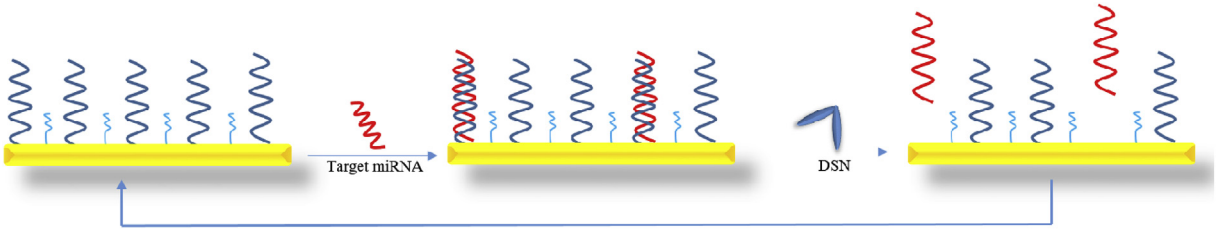

Fig. 10. A sensitive and selective electrochemical biosensor using DSN amplification.

7. Limitations of electrochemical miRNA nano-based biosensor for translating to clinical settings

According to the current research, nanotechnology has a considerable role in advancement of different dimensions of electrochemical miRNA biosensing. Applying various sorts of nano-materials in electrochemical systems is accompanied with several significant analytic characteristics that are actually interesting to note in miRNA diagnostic sensitivity [117–119]. Nevertheless, some undeniable problems exist regarding the provision of these biosensors to be applied in point of care (POC). For instance, making these systems portable is a major challenge which needs to be dealt with. Moreover, employment

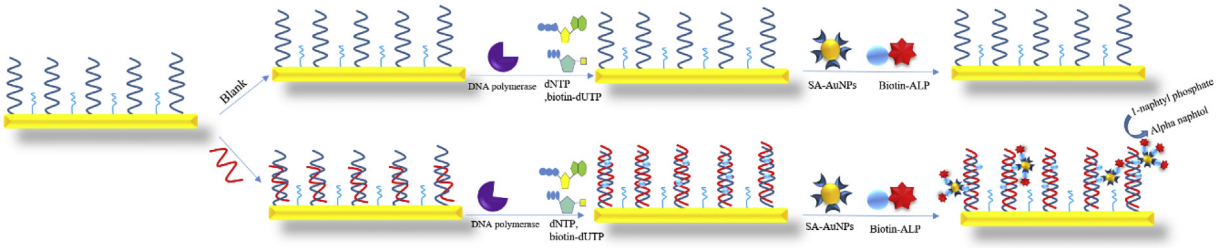

Fig. 11. Electrochemical detection of miRNA based on streptavidin-AuNPs and enzymatic amplification.

of nano-materials has brought about several issues associated with security, adaptability, and environmental friendly traits [120]. Moreover, provision of ideal nano-materials at the large scale in order to be utilized in analytic areas would not have favorable economic outcomes or the potential to decrease the costs of screening or diagnostic processes [120].

Eventually, stability in the features of sensitivity and specificity are the required criteria for many nano-biosensors which have not been addressed yet, although stable storing potential for a long time can be considered as one of the basic characteristics of POC systems [23].

Thus, these novel systems are still in their initial phases of application, even though these days numerous studies have been carried out on the devise of electrochemical transducers at nano-scale with an aim to integrate the necessary components into a practical tool [121].

Regardless these issues, increasing development of electrochemical nano-platforms along with the endeavors dedicated to the study and progress of miRNA diagnostic process may meet the future expectations. As a result, acquiring a quick and cheap small tool for POC examination would be important from clinical point of view and may be introduced in near future.

8. Conclusion

Due to the biological function of miRNAs in cancer prevention, they were extensively used for cancer diagnosis, prognosis and treatment over the past decade. Numerous miRNAs have different expression patterns and can be up-regulated or down-regulated in tumor tissues. According to the current view, it is predicted that miRNAs can regulate 40–50% of mammalian mRNAs in translational level that reveal their great impacts in biological processes. Therefore, there is an immediate need to develop reliable and ultrasensitive testing platforms/tools for miRNAs detection and quantification as well. Today, miRNAs expression is measured by methods such as northern blot, RT-PCR, microarrays, and miRNA-seq. Owing to the inherent advantages of electrochemical transduction methods including excellent compatibility with advanced semiconductor technology, ease of miniaturization and low cost, electrochemical detection of nucleic acids has the potential to be served in high performance device at low cost with simple miniaturized readout, and thus are reason from the challenges related to other detection systems [122].

On the whole, enanobiosensors are currently developing which have led to a novel category of inexpensive, strong, dependable, convenient, and very sensitive diagnostic tools through connecting the benefits of electrochemical biosensing and nanotechnology. These unraveled characteristics have provided more sensitivity, precision, and less limitations in diagnosis. Therefore, elevated sensitivity would rely on more efficient sensors, while the nano-dimensions could be compared with the size of target biomolecules, the highly large proportion of the surface to volume which makes interactions much more convenient, along with dependency on higher capture efficacy of the sensors.

At the end, this review focused on the recent advances in electrochemical miRNA sensors. These methods may have an impact on pathogens detection, genetic mutations and pharmacogenomics targets and industrial sectors attention in near future.

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