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Review

Nanoarchitecture Frameworks for Electrochemical miRNA Detection

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With revolutionary advances in next-generation sequencing, the human transcriptome has been comprehensively interrogated. These discoveries have highlighted the emerging functional and regulatory roles of a large fraction of RNAs suggesting the potential they might hold as stable and minimally invasive disease biomarkers. Although a plethora of molecular-biology- and biosensor-based RNA-detection strategies have been developed, clinical application of most of these is yet to be realized. Multifunctional nanomaterials coupled with sensitive and robust electrochemical readouts may prove useful in these applications. Here, we summarize the major contributions of engineered nanomaterials-based electrochemical biosensing strategies for the analysis of miRNAs. With special emphasis on nanostructure-based detection, this review also chronicles the needs and challenges of miRNA detection and provides a future perspective on the presented strategies.

Highlights

miRNAs play crucial roles in different aspects of many diseases including cancers, suggesting that miRNAs could potentially serve as cancer biomarkers.

Considerations that need to be measured to triumph the full functionality of the miRNAs as diagnostics and prognostics markers are discussed.

Grand challenges associated with the current miRNA detection strategies are addressed.

Multifunctional activity (i.e., porous architecture, magnetic property, enzyme-like activity, conductivity and biocompatibility) of engineered nanomaterials is the key to the functionality and simplicity of many current electrochemical miRNA biosensors.

Using the concept of porous architectures, a new class of ferric-oxide-based nanomaterials has recently been developed and explored as (i) dispersible capture agent, (ii) electrocatalyst, and (iii) nanoenzyme in miRNA detection are discussed.

miRNA: Next Generation of Diagnostic and Prognostic Biomarkers

Cell transcriptomes comprising various RNA species, such as mRNA, **miRNA** (see Glossary), rRNA, tRNA, siRNA, and **long noncoding RNA**, have emerged as prominent noninvasive or minimally invasive **biomarkers** due to their widespread role in a number of pathophysiological phenomena and cellular processes, including cell-cycle regulation, apoptosis, and post-transcriptional gene regulation [1–4]. A growing body of evidence has suggested that dysregulated RNA molecules play a central role in the initiation and progression of several diseases, including cancer (Box 1) [3,5,6]. Various RNA species are not only upregulated or downregulated in cancer, but different genomic anomalies such as copy number variations, SNPs, and mutations are also reflected through them [4,7]. Therefore, monitoring the RNA component can be a powerful diagnostic tool for cancer management.

Ever since the first discovery of *lin-4* in *Caenorhabditis elegans* [8], miRNAs have become recognized as important regulators of cellular function. It has been suggested that miRNAs modulate >30% of mammalian genes and participate in almost all known cellular processes [9], including development, DNA repair, cell cycle regulation, differentiation, apoptosis, as well as malignant cellular transformation and host–pathogen interaction [10]. In addition to their well-known regulatory function, recent studies have revealed a novel role of miRNAs, that is, intercellular and interorgan communication both via miRNA transfer mediated by extracellular vesicles (e.g., exosomes) as well as non-membrane-bound miRNAs [11]. miRNAs effectuate their regulatory function through post-transcriptional repression of gene expression. However, the mechanisms that underlie miRNA-mediated repression are different in animals and plants [12]. With the exception of at least one miRNA (*mir-196*), whereby gene regulation is carried out via RNA-induced silencing complex (RISC)-induced mRNA cleavage (explained later) [13],

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Box 1. RNA dysregulation in human diseases

RNAs exist in cells as ribonucleoprotein complexes comprising one or more RNAs and typically several RNA-binding proteins. In addition to their primary role as carriers of amino acid sequence information, the so-called coding fraction of transcriptome (mRNAs), gene regulation by both coding and noncoding RNAs has increasingly been recognized as essential for cellular functioning. Dysregulated expression of messenger as well as regulatory RNAs is involved in the initiation and progression of several diseases including cancers, hinting at their potential as disease biomarkers.

While altered expression levels of corresponding proteins is the main outcome of dysregulated mRNA expression, other aberrations such as 'alternative splicing' are also known to disrupt various cellular regulatory pathways such as chromatin modifications, cell adhesion, cell cycle [1]. During mRNA biogenesis (i.e., transcription), the pre-miRNA transcripts generate the mature mRNA via splicing at intron–exon junctions. Any alterations in this splicing pattern, known as 'alternative splicing', may trigger the production of miscellaneous mRNA isomers, which further generates diverse protein variants including oncoproteins, and thereby may lead to abnormal cell proliferation in cancer [3,4]. However, mRNA represents only a small percentage of the total transcriptome.

The noncoding RNA fraction comprises housekeeping (tRNA and rRNA) and regulatory RNAs [e.g., miRNA and long-noncoding RNA (lncRNA)]. Among them, researchers have focused on miRNAs (19–25 bases) and lncRNAs (200 bases to 100 kilobases) as potential biomarkers owing to their strong capability in gene regulation. Though they do not participate in the translation process (i.e., protein synthesis), noncoding RNAs actively regulate the post-transcriptional gene expression and remodeling of the epigenome, such as histone modification and DNA methylation [5,12]. They are crucial for a range of biological processes such as cell cycle regulation, pluripotency, retrotransposon silencing. These processes may be affected and eventually could contribute to cancer development due to noncoding RNA-related anomalies such as altered expression levels, copy number variations, single-nucleotide polymorphism, and mutations [6,7,9]. MicroRNA dysregulation in cancer was first reported in 2002, when a cluster of two microRNAs, miR-15 and miR- 16, was identified at 13q14.3, a frequently deleted region in chronic lymphocytic leukemia [18]. Since then microRNA dysregulation has been recognized as ubiquitous in almost all cancers, highlighting their potential as universal cancer biomarker which could be exploited for the clinical management of cancers.

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miRNAs identified in the animal kingdom in most cases bind to their complementary sites in the 3′ untranslated region (UTR) of their target genes and negatively regulate their expression. In contrast, binding sites for plant miRNAs are not restricted to the 3′ UTR and can be found anywhere across the entire length of target mRNA. Therefore, plant miRNAs regulate gene expression via cleavage of target mRNA through RISC. RISC is a ribonucleoprotein complex that incorporates a small single-stranded (ss)RNA (e.g., miRNA) and cleaves target mRNA in the middle of small ssRNA–mRNA duplexes [9]. RNAi is an evolutionarily conserved gene regulation phenomenon that was identified as an endogenous defense mechanism against viral infection, whereby foreign nucleic acids entering the cells are destroyed by RISC-mediated cleavage [12]. RNAi has been exploited as a possible therapeutic strategy against human diseases including cancer. A recent review of literature noted that >14 RNAi-based therapeutic programs are currently at various stages of clinical trials [14]. For example, clinical trials are underway for Miravirsen (SPC3649), a locked nucleic acid (LNA) ribonucleotide-based anti-miRNA drug candidate. Miravirsen targets mir-122, a liver-specific miRNA known to play important role in hepatitis C virus life cycle [15].

Although most types of RNA are reported to have potential applications in diagnostics, prognostics, and therapeutics, this current review focuses on miRNA-based cancer diagnostics. Being a critical regulator of post-transcriptional gene expression, miRNAs, a large group of small (~22 nucleotides) noncoding RNAs, have attracted considerable recent interest as diagnostic biomarkers [1,5,16]. Dysregulation of these highly conserved regulatory RNAs can potentially impact progression and prognosis of cancer [17]. Several studies have demonstrated that in comparison with normal cells from the same tissues, altered miRNA expression is a common feature of all human tumors [18]. In 2008, Lawrie *et al*. first reported in a seminal study the diagnostic role of circulating miRNAs in B cell lymphoma [19]. The levels of miR-21 and miR-155 were reported to be significantly higher in the serum samples of

lymphoma patients compared to those from normal individuals. Since then a number of studies have demonstrated that miRNAs circulating in body fluids can be used as disease and therapy response indicators [20]. As diagnostic and prognostic biomarkers, circulating miRNAs offer several distinct advantages, such as the provision of early detection, better stability, and the potential for a minimally invasive **liquid biopsy**-based monitoring of cancer and other diseases.

Challenges and Considerations

Despite the huge potential for miRNAs in the diagnosis and prognosis of cancer, their current detection approaches are generally confined to classical nucleic acid detection methods such as northern blotting, microarray, quantitative (q)RT-PCR, and next-generation sequencing, as well as robust **biosensor**-based miRNA sensing. Although miRNA detection technologies have been advancing, several major inherent and technical challenges remain. In addition, several recent studies have also challenged the merits of miRNA as an effective biomarker (see Outstanding Questions). The section that follows underlines these major challenges and considerations that need to be addressed to achieve the full functionality of miRNA diagnostics.

Stability and Proper Storage of miRNAs

The ribonuclease (RNase)-associated degradation of RNAs at room temperature is considered one of the bottlenecks in RNA analysis. During incubation, both endogenous and exogenous RNases progressively degrade miRNAs, thereby affecting the accuracy of detection [1]. The use of RNase inhibitors is considered a potential solution to this problem [21]. However, for circulating miRNAs, RNase degradation is not as much of a major consideration, as these miRNAs are either confined inside of microvesicles (e.g., exosomes, apoptotic bodies, or microparticles), or in other cases are found to be associated with lipoproteins or RNA-binding proteins in the circulation, making them resistant to RNases. Moreover, they are stable even under extreme conditions including long-term storage, freeze–thaw cycles, etc., and are thus considered as stable biomarkers for disease diagnosis [11,22]. A study of serum storage conditions showed that miRNA in pure serum can be stored at −20 °C for at least 2–4 years [23]. Nevertheless, preprocessing time may influence stability significantly as *in vitro* hemolysis can release miRNAs from peripheral blood cells [24]. As no standard control is available for normalization of RNA level, it has been suggested that the blood samples should be processed within 2–4 h of collection in EDTA tubes [25]. Moreover, RNase-free solutions and accessories should be used for miRNA experiments in order to prevent and minimize RNase mediated degradation.

Sample Source Selection and Optimization of RNA Extraction

To ensure the quality of miRNA expression profiling, standardization of sample sources and extraction procedures are important, as a little discrepancy there could substantially affect the final results. For example, Wang *et al.* reported that the miRNA concentration was higher in serum than in the plasma of the same individual [26]. The presence of platelet-derived miRNAs obtained from the blood coagulation process could presumably be the cause of this inconsistency. Several studies also showed that there is a significant difference in expression profiling of tissue-bound and circulating miRNAs [27].

EDTA is a commonly used anticoagulant but long-term storage of blood samples in EDTA-coated tubes may lead to inaccurate miRNA profiling [28]. However, standardization of the holding period may help to overcome this challenge. In addition, careful attention should be paid to retaining the small RNA fraction while extracting it from the complex biological samples to avoid a false-positive readout [29].

Glossary

Biocompatibility: materials that are not harmful, toxic, or injurious, to living tissues or a living system, and do not cause immunological rejection are recognized as biocompatible.
Biomarker: any naturally occurring chemical, metabolite, molecule, gene, cell, characteristic, or factor that can be reproducibly measured and evaluated as an indicator of normal biological condition or a disease state or pharmacological responses.
Biosensor: platform made up of a molecular recognition layer and a signal transducer that can be coupled to an appropriate readout device.
Electrocatalysts: these materials possess the ability to modify and increase the rate of electrochemical reactions without being consumed in the process. They are either bound on the electrode surface, or the electrode surface itself can work as an electrocatalyst.
Electrochemical transducers: provide a suitable platform that facilitates the formation of probe-target complex (i.e., specific recognition event) in such a way that the binding event triggers a useable signal for electrochemical readout.
Lab-on-a-chip: miniaturized device that integrates one or several laboratory functions (both biological and chemical) on a single chip.
Liquid biopsy: simple alternative to invasive surgical biopsies in which analytes are collected from body fluids such as blood, urine, and saliva, and can demonstrate the properties of a tumor or disease state.
Long noncoding RNA: large class of transcribed RNA with a length of >200 nucleotides, which act as important regulators of gene expression and have diverse functions in cellular and developmental processes.
Metal–organic frameworks (MOFs): a class of crystalline materials consisting of transition-metal cations and multidentate organic linkers with a variety of geometries, sizes, and functionalities that are used for separation, purification, catalysis, as well as sensing applications.

Varying Size of RNAs and Associated Challenges

Amplification-based detection of miRNAs is highly challenging. Primers cannot anneal with the small miRNA sequences because of the similar size of traditional primers and miRNAs, and thus they cannot amplify them. To solve this problem, miRNAs are enzymatically polyadenylated using oligo(dT) primers to generate longer primer binding sequences for the reverse transcription step in qRT-PCR [30]. Primer size matching can also be avoided by generating the reverse transcription priming site with a stem-loop primer [31].

Poor Discrimination of Homologous miRNAs

Isolating a specific miRNA in the background of homologous miRNAs (high sequence similarity) originating from the same miRNA family is difficult to achieve reliably. For example, let-7a is underexpressed in lung cancer, while let-7c is downregulated in breast cancer [32]. let-7a and let-7c have only a single base mismatch. An assay that is unable to differentiate this single base difference can thus easily compromise the diagnostic accuracy. One of the approaches that have been used to overcome this problem is to use thermostable LNA probes. When incorporated into DNA probes, these nucleotide analogs increase the melting temperature and therefore binding affinity of the strand. This increases the specificity and sensitivity of the assay [33]. The nonspecific adsorption of RNAs onto the nanoparticle (NP) surface is also responsible for biased signals (see later discussion), which can be overcome by designing target-specific nanoparticulates or engineering target-specific signaling events [34]. Recently, a 2D NP-based combinatorial nanosensor array was reported for analysis of sets of homologous miRNAs using a target-specific bind-and-release model, where the signal was achieved through the release of capture-probe-quenched fluorophores through the formation of specific target–probe complexes, avoiding signals from any nonspecific adsorption or bindings of biomolecules to the sensor surface or NPs [35].

Nonspecific Responses in the Detection Platforms

In biosensor-based strategies, the clinical samples may have a complex biological matrix containing large amounts of unrelated biomolecules such as proteins and non-target nucleic acids, which could nonspecifically be adsorbed on the sensor surface, resulting in a false readout. The use of a suitable blocking/antifouling agent such as mercaptohexanol, mercaptoethanol, poly(ethylene glycol), or bovine serum albumin is highly recommended to prevent nonspecific binding. Additionally, RNA molecules are also prone to aggregation via UV-induced crosslinking with other RNA or proteins [36]. When nanomaterials are integrated into a sensor surface or are in solution for signal generation, interference might also come from nonspecific adsorption (low specificity of nanomaterials for highly similar miRNAs) and/or from an aggregation of NPs (when the amount of nanomaterials is much higher than analytes) [37]. Therefore, along with making nanomaterials biocompatible and dispersed in aqueous media, there is a need to utilize a specific recognition probe, keeping away the sensors from a UV source and optimizing the amount needed to be incorporated into sensors.

Assay Inconsistency Due to Platform-Dependent Variations

Substantial interplatform differences (variation between different detection methods as well as between different laboratory set-ups) during miRNA analysis are gradually becoming a major concern for clinical applications. Readers may refer to the critical reviews by Mestdagh *et al.*, Witwer, and Singh *et al.* for the vast body of knowledge on this issue [38–40]. Several works have reported contradictory specificity and inconsistent reproducibility of miRNA detection platforms. The enzymatic reaction or amplification steps during preparation or signal enhancement, the strength of hybridization, complementary probe design, laboratory practices, and detection techniques could be accountable for such interplatform miRNA quantification variations.

miRNA: small endogenous noncoding RNAs of 22–24 nucleotides that function in RNA silencing and post-transcriptional regulation of gene expression by targeting specific mRNAs- Their expression levels are altered in certain disease conditions presenting as promising diagnostic and prognostics biomarkers.
Nanostructures: nanoparticles, nanorods, nanowires, and nanosheets are promising nanostructure building blocks that exhibit size- and shape-dependent intrinsic properties for biosensing, catalysis, imaging, and therapeutics.
Rolling circle amplification (RCA): an isothermal enzymatic process, which amplifies a small DNA or RNA primer to a long ssDNA or RNA using a circular DNA template and special DNA or RNA polymerases.

Requirement for a Highly Sensitive Assay to Analyze Clinically Relevant miRNA Concentrations

Detecting circulating miRNAs is challenging due to their low abundance in plasma and serum (e.g., femtomolar to picomolar levels/few molecules per cell). Therefore, the development of a sensitive miRNA assay platform is crucial. To enable this, various enrichment and amplification steps are generally used in conventional miRNA assays, whereas different novel transduction schemes and nanomaterial-based signal enhancement strategies can be incorporated into the sensing strategies [41].

Nanoarchitecture for miRNA Detection

With recent advances, **nanostructured** materials are transitioning to applications in the fields of biosensing, medicine, and biotechnology. These materials are being used in developing novel methods and devices for diagnosis and monitoring of specific diseases via detecting levels of disease-specific biomolecules. Nanostructured materials generally possess flexible and modular structures, small size, high surface area, low toxicity, enzyme mimicking, superparamagnetic behavior, and **biocompatibility** [42,43]. In the past decades, they have widely been used to develop many devices and methods with unprecedented power to comprehensively interrogate genetic, epigenetic, transcriptomic, proteomic, and cellular information from many diseases including cancer [44]. In comparison with the bulk materials of the same mass, nanostructured materials exhibit expressive advantages in molecular diagnostics, particularly in disease diagnosis applications: (i) the smaller size of nanostructured materials breaks down the barrier for structural miniaturization of diagnostic platforms, leading to the prospect of designing small-size, inexpensive, portable, low-cost tools in point-of-care diagnostics; (ii) nanostructured materials enable direct contact with the sensing environments (electrolyte, labeler, etc.), thereby accelerating signal transduction, and consequently boosting the robustness and sensitivity of the analysis and lowering the detection limit; (iii) they have the potential to provide reagentless biosensing (via immobilizing receptor molecules), biomimetic, in vivo detection; (iv) high surface area and superparamagnetic activity of nanostructured materials allows them to be used as carrier or capture vehicles for loading a large number of specific biological probes, and work as dispersible capture agents for isolating and capturing circulating disease-specific markers; (v) optical [surface-enhanced Raman spectroscopy (SERS), surface plasmon resonance (SPR), and florescence] and electrochemical properties of NPs can be exploited to adopt many novel transduction schemes; and (vi) peroxidase-like activity of many materials has found a wide range of applications in ELISA-like sensing approaches. These unique properties of nanostructured materials have also been widely explored for achieving sensitive biosensing of miRNAs [45]. Figure 1 depicts the common nanostructures widely used in miRNA biosensing.

Gold Nanoparticles

Gold nanoparticles (AuNPs) have found multifaceted applications in biosensing systems. In addition to their ease of synthesis, the affinity of AuNPs surface with a variety of ligands has helped in the development of highly stable AuNPs and facilitated facile bioconjugation with a variety of biomolecules [46]. Furthermore, tunability of AuNP characteristics like conductivity, redox behavior, and catalytic activity make them excellent candidates for interfacing nucleotide recognition events with electronic signal transduction [47,48]. Pioneering AuNP-based DNA detection was reported by Mirkin and colleagues, who utilized a distance-dependent change in the extinction spectrum of AuNPs to detect target DNA in a separation-free manner. Two different capture probes, noncomplementary to each other, were attached to two separate batches of AuNPs. Hybridization of target DNA molecules containing sequences complementary to both the surface-bound probes leads to particle aggregation and color change [49]. Since then, AuNPs have been integrated in electrochemical nucleic-acid-sensing systems,

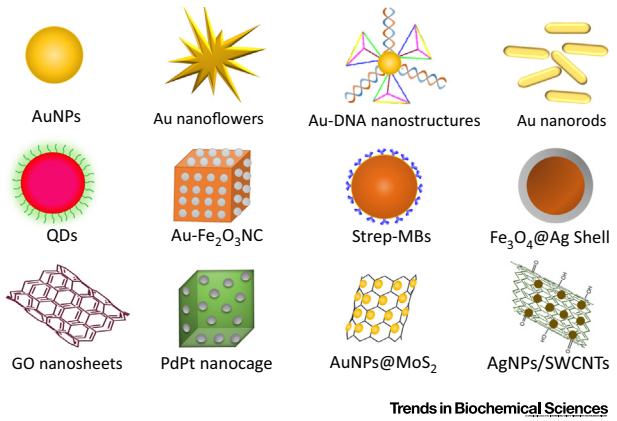

Figure 1. Commonly Used Nanoarchitecture in miRNA Biosensing. Schematic representation of nanostructured materials widely used in developing electrochemical miRNA biosensors. Abbreviations AgNPs/SWCNT, silver nanoparticles/single-walled carbon nanotubes nanohybrid; Au-Fe_2O_3NC, Au-loaded iron oxide nanocube; AuNPs, gold nanoparticles; AuNPs@MoS_2, AuNPs decorated over molybdenum disulfide; Fe_3O_4@Ag Shell, core-shell nanostructure with magnetic Fe_3O_4 as core and Ag metallic shell; GO, graphene oxide; PdPt, palladium platinum; QD, quantum dot; Strep-MB, streptavidin-magnetic bead.

including miRNA-detection platforms, in various roles such as: (i) tags attached to oligonucleotides that can be electrochemically detected after acidic dissolution through stripping voltammetry; (ii) carriers/enhancers of other electroactive labels; (iii) as electrocatalytic signal amplifiers; and (iv) as dispersible nanoelectrodes that seek out the analyte, thereby substantially enhancing the rate of analyte mass transfer to the transducer [48,50].

Strong thiol–Au interaction provides the basis of many nucleic acid biosensors in which thiol-derivatized nucleic acid fragments are used to modify AuNPs [51]. Furthermore, direct adsorption of both single-stranded and double-stranded nucleic acids has also been observed and several nucleic acid biosensors have been developed based on this principle [52,53]. Direct adsorption arises from electrostatic interactions, hydrophobic forces, and sequence-dependent (DNA base–Au) affinity interactions. Over the past few years, several electrochemical miRNA sensors have been developed by our group as well as other researchers, in which magnetically purified target miRNAs are directly adsorbed onto the Au electrode surfaces for subsequent electrochemical interrogation in a highly sensitive manner [54–58]. In addition to these functionalities, several amplification strategies coupled with AuNPs have been devised and have achieved ultrasensitive miRNA detection [59,60].

Silver Nanoparticles

Silver nanoparticles (AgNPs) are another class of metal NPs that have found application in a range of electrochemical and optical sensor platforms owing to their easy signal amplification, higher extinction coefficients, higher scattering to extinction ratio, and high field enhancements. AgNPs and their network structure are easily oxidized and show well-defined amplified signals even at a lower potential, enabling their use as redox tags for electrochemical detection [61]. For instance, AgNP aggregates have been used as labels on electrode surfaces for label-free (signal-on) electrochemical miRNA sensors [62]. Moreover, AgNPs demonstrate higher extinction coefficients, as well as stronger Raman and fluorescence enhancement compared to those of AuNPs of the same size, leveraging several developments in miRNA-sensing technologies. These approaches are based on plasmonic properties of AgNPs, for example, platforms incorporating SERS or localized SPR or metal-enhanced fluorescence readouts.

Oligonucleotides and ligand-modified Ag nanocluster (NC) building blocks or tags, having luminescent properties and biospecificity for nucleic acids, have been used in electrochemiluminescent miRNA sensors [63]. Additionally, positively charged AgNPs can also be integrated with negatively charged Au-loaded DNA nanostructures, resulting in an increased resonance shift for amplified SPR signals [62]. Recently, based on Ag nanostructures such as the DNA-mediated Au–Ag nano-mushrooms, as well as Au@R6G@AgAuNPs (rhodamine-6-G attached to AuNPs and then encapsulated in Ag–Au alloy shell NPs) as SERS signal reporters, several miRNA sensors have been reported for simultaneous and multiplexed detection of numerous RNA targets [64–66].

Carbon and Carbon-Based Nanomaterials

Carbon (C) nanomaterials such as C nanotubes (CNTs), graphene, graphene oxide (GO), C nanofiber, and C quantum dots (QDs) offer attractive opportunities for developing novel sensors and refining the analytical performance of already existing platforms. The intrinsic characteristics of CNTs such as their hollow core, high elastic modulus, as well as diverse electron transport capability make this material an ideal candidate as a building block, sensing layer, a surface modifier, or dispersible capture agent for loading a large number of biomolecules or assembling labels for signal amplification [67]. In addition, CNTs are fluorescent in the near-IR spectral region (tissue-penetrating region) and their emission wavelength and intensity are sensitive up to single-molecule attachment, permitting perturbations at the nanotube surface to be transduced via modulation of their emission. Based on such single-walled C nanotube (SWCNT) modulation, an *in vivo* miRNA sensor has been fabricated for multiplex detection by means of multiple nanotube chiralities [68]. In addition, graphenes [GO, reduced graphene oxide (rGO), and graphite-GR] have unique electronic, adsorption, and fluorescence properties, and are emerging as powerful key foundations of miRNA sensors [69–71]. The GO surface possesses many carboxylic acid and hydroxyl groups, making it more water-soluble and suitable for simple covalent functionalization and biomolecule adsorption [72]. Therefore, many recent biosensors have utilized GO and GO-loaded metal composites to establish miRNA-sensing platforms [73].

Quantum Dots

QDs are fluorescent semiconductor nanocrystals that have gained increasing application in the construction of electrochemical biosensors mainly because of their high electron transfer efficiency and high surface reaction activity. Their robust photostability and size-tunable light emission spectra also make them ideal labels for optical and chemiluminescent sensors. Several miRNA-detection methods based on photophysical properties of QDs have been reported [74,75]. Zhang *et al.* used graphene QDs as a substrate to immobilize horseradish peroxidase onto a target miRNA sequence containing double-stranded (ds)DNA structure [76]. Similarly, owing to their user-friendly biolabeling capability, QDs have found application in paper-based biosensors. Deng *et al.* utilized QDs in a target recycled non-enzymatic-amplification-strategy-based strip (paper) sensor for obtaining efficient, reproducible, and portable detection of miRNA [77].

Magnetic Nanoparticles

Integration of magnetic materials with conventional NP-based miRNA detection strategies enhances their sensitivity and selectivity and offers a low-cost miniaturization platform. In the absence of an external magnetic field, magnetic NPs show negligible magnetic moment (superparamagnetism), which allows the formation of a stable colloidal suspension that can be manipulated and engineered by applying a magnetic field without any reversal aggregation. Having such superparamagnetic properties, easy biofunctionalization, and high surface-to-

volume ratio, magnetic NPs facilitate magnet-based enrichment, capture, isolation, and purification of the target from a complex biomatrix. In addition, they can be directly used in cost-effective electrode surface modification for biomolecule immobilization, as **electrocatalysts** for significant signal enhancement by increasing the electron transfer rate, and as a signaling label for sensitive biorecognition and transducing events [78]. Moreover, certain magnetic nanomaterials have been shown to possess intrinsic peroxidase-like activity, which has fueled their application as labels in colorimetric miRNA sensing [79,80]. In the past few years, we have used gold- and GO-loaded superparamagnetic iron oxide nanocubes as transducer surfaces and electrocatalysts for ultrasensitive miRNA detection [53–56,81]. Recently, Tavallaie *et al.* utilized redox-labeled probe DNA modified magnetic NPs for sequence-specific hybridization with a target [50]. However, while the rate of analyte capturing depends on the affinity towards the biomolecules and the total surface of magnetic particles, a higher amount of nanomaterials may increase the probability of particle aggregation and risk of nonspecific adsorption. Therefore, a lesser amount of particles with a high surface-to-volume ratio (e.g., porous nanomaterials) and high bioaffinity could be an excellent choice for biosensing.

Metal–Organic Frameworks

Metal–organic frameworks (MOFs) are a large family of crystalline materials with organic multidentate ligands as linkers and metal ions as nodes, which can form 3D frameworks with unique properties, including high pore volume, large surface area, and extraordinary thermal stability. MOFs can bind with the negatively charged probe DNA sequences through electrostatic interactions and/or π stacking, presenting them as sensitive tracer labels for biosensing. Moreover, they are highly advantageous in biosensing as they provide high loading of probe DNA and show resistance against probe DNA degradation [82]. MOFs are widely used as quenching labels for disease-specific nucleic acid detection. In such strategies, the organic ligands tightly bind the probe DNA while the metal ions working as a coordination center activate their intrinsic fluorescent quenching capability through a photoinduced electron transfer process [83]. Moreover, the presence of various functional groups, such as –COOH and $–NH_2$, makes MOFs an excellent platform for loading different signaling metal substrates and biological ligands [84]. The structural modification of MOFs with other functional materials such as AuNPs, AgNPs, copper NPs (CuNPs), palladium NPs (PdNPs), or ruthenium NPs (RuNPs) adds many superior characteristics to the combined material compared with pure MOFs, owing mainly to the synergistic effects [85,86] Among them, Cu-MOFs have received special attention in electrochemical applications because of their well-defined configuration, unique electrical conductivity, and superior catalytic activity [87]. For example, Wang and coworkers fabricated a paper-based electrochemical miRNA sensor using hairpin assembly target recycling for signal amplification and AuNP-modified Cu-based MOFs for catalysis [88].

Nanoarchitecture-Based Electrochemical miRNA Detection

Electrochemistry-based readouts have stimulated great interest in recent years, mainly due to their inherent sensitivity, specificity, simplicity of operation, short response time, and broad dynamic range (over nine orders of magnitude greater than many nucleic acid biosensors). Additionally, electrochemistry-based methods are portable and highly amenable to miniaturization, which can prove to be beneficial in achieving massively multiplexed electrochemical devices. Thus, electrochemistry-based devices have the potential to solve most of the analytical challenges hampering the development of a point-of-care device for miRNA biomarkers. In these devices, electrode materials play the critical role in obtaining high-performance data via various electrochemical techniques such as voltammetry [cyclic voltammetry, differential pulse voltammetry (DPV), linear sweep voltammetry (LSV), etc.], coulometry, amperometry, and impedance spectroscopy. Incorporating engineered nanomaterials with common electrode surfaces gives synergistic

effects related to biocompatibility, magnetism, catalysis, and conductivity, and provides accelerated signal transduction and amplified biorecognition events, resulting in an ultrasensitive biosensing platform. Over the last few decades, significant research has been reported on the construction of various nanomaterials for the fabrication of functional electrode surfaces, as signaling tags or as electrocatalysts [89–91]. Here, we review the pros and cons of recent (2015 to the present) NPs-integrated electrochemical miRNA assays. Within this timeframe, all strategies from the literature are discussed, based on their intrinsic properties towards biosensing. Table 1 summarizes the most commonly used nanomaterials and their applications in miRNA detection in this timeframe and Figure 2 schematically outlines the major nanoarchitecture-based electrochemical strategies for miRNA detection.

Nanostructures as Capture Agent or Vehicles for miRNA Isolation and Purification

Nanostructures with superparamagnetic properties are becoming promising tools for capture and separation of biomolecules from their complex biological matrices. These tools offer significant advantages over conventional separation techniques such as chromatography, because they possess enhanced functional area, can readily disperse in solution, and are quickly localized or retrieved using an external magnet. The materials can be synthesized at low cost and can be reused several times in bioassays [92]. Over the last 3 years, we have developed several electrochemical sensors employing streptavidin-labeled magnetic beads (Dynabeads and MyOne) for isolating target miRNAs [53–58]. The 1-μm beads possess a large surface area, high probe-binding capacity due to strong avidin–biotin interaction (dissociation constant, $K_d = 10^{-15}$), proficient magnetic pull, and slow sedimentation during incubation. In such bead-based isolation and purification steps (Figure 2), the magnetic beads are functionalized with complementary capture probes through biotin–avidin interactions, followed by the hybridization of target miRNAs. The bead-bound target miRNAs then undergo multiple washing and purification steps. The target is then heat released and subjected to electrochemical readouts [57]. Trau *et al.* reported a method for amplification-free detection (fluorescence and electrochemical) of RNA species, including miRNA, using parallel magnetic isolation of individual RNA targets, and *in situ* poly(A-T)-templated CuNB synthesis [93]. Recently, electric-field-induced reconfigurable Au-coated magnetic NPs (Au@MNPs) as dispersible electrodes were reported by Tavallaie *et al.* for direct miRNA analysis [Figure 3(i)]. In this remarkable report, the thiol-modified cDNA probes were mixed with an excess amount of Au@MNPs followed by mixing with samples for target miRNA hybridization [50]. Au@MNPs–DNA/miRNA hybrids were then separated magnetically from unhybridized sequences and other biomatrices and collected onto an Au microelectrode surface. The square-wave voltammetric responses from the electronic reconfiguration of Au@MNPs before and after hybridization were taken to analyze the target present in samples. However, magnetic-nanostructure-based separation and purification depend on the shape and size of the magnetic cores and the functional surfaces. The isolation and purification process requires time-consuming multiple incubation steps. Nanostructures with high superparamagnetism (to avoid residual magnetism), enlarged functional surface (porous structure) area, and higher target affinity could be a possible route for robust miRNA isolation and purification.

Nanostructures for miRNA Immobilization

Immobilization of reporter or target miRNA on the transduction surfaces is one of the key requirements for designing electrochemical sensors. This immobilization step can be affected by denaturation, low hybridization efficiency, or poor transducing capabilities. Engineered nanostructures have been integrated on electrode surfaces as they provide a high surface area, a high density of capture sites, and high hybridization moiety for faster and efficient analyte binding, and enhanced amplification agent incorporation. Nevertheless, immobilization steps need to be carefully optimized to protect the nanostructured surface from undesired adsorbents and to

Table 1. Electrochemical miRNA Detection Based on Nanoarchitecture

Nanoarchitecture	Function	Principle	miRNA	LOD	Refs
AuNPs	Selective preconcentration of AuNPs	Differential adsorption ability of nucleic acids on AuNPs	let-7A	16 fM	[107]
AuNPs-MMB and Fc-capped AuNPs	AuNPs-MMB for probe DNA immobilization, target recognition, and magnetic separation; and Fc capped AuNPs for target recognition event amplifications	Cyclic voltammetric detection of miRNA based on AuNP-MMBs and Fc-capped AuNPs signal amplification	miR-182	0.14 fM	[115]
Au-NP-modified Cu-MOFs	Immobilization of probe DNA and signal amplifications	Target miRNA triggered DPV signal amplifications	miR-21	0.35 fM	[88]
N-doped graphene/AuNPs and flower-like $MoSe_2$	$MoSe_2$ electrode surface enables loading of AuNPs for probe DNA and NG-AuNPs used as electrocatalyst towards the oxidation of hemin	Target miRNA assisted supersandwich structure for hemin redox signal amplifications	let-7a	0.17 fM	[116]
PTh/rGO Au/TMC/Fe_3O_4 CdSe@CdS/TMC/Fe_3O_4	PTh/rGO serves as immobilizer for probe DNA and augmented electrode conductivity; and both nanocomposites acted as selective labels for two miRNAs	Nanocomposite-based dual signal amplification for simultaneous detection of two miRNAs	miR-106a let-7a	0.06 fM 0.02 fM	[103]
Cd^{2+} functionalized titanium phosphate nanosphere (TiP–Cd^{2+})	TiP nanospheres containing large amount of Cd^{2+} acted as stripping label	Target assisted sandwich structure for capturing signal tags.	miR-21	0.76 aM	[117]
(+)AuNPs	Positively charged AuNPs stoichiometrically bind with the negatively charged miRNA and attached ferrocene for SWV detection	Dual mode (EIS and SWV) electrochemical detection	miR-45	0.37 fM	[59]
Flower-like gold nanostructures	Hierarchical gold nanostructures for immobilization of large amount of cDNA	Stoichiometric amounts of $Ru(NH_3)_6^{3+}$ bound to anionic phosphate of DNA/miRNA sequences provide electrochemical readout.	miR-21	1 fM	[98]
AuNPs@MoS_2	As both electrode modifier and nanoamplifier	Nanocomposite-based sandwich structure empowers electrochemical signals using $[Fe(CN)_6]^{3-/4-}$ and $[Ru(NH_3)_6]^{3+}$	miR-21	0.78 fM	[118]
CNT	Solid substrate for solid-state RCA strategy	Target miRNA unfolded the stem-loop structure and triggered CNT based RCA process	let-7 family	1.2 fM	[99]
AuNPs and hemin–rGO	Achieving electrochemical transducing interface on GCE	Decrease in the DPV response of hemin resultant dsDNA obtained via hybridization of probe DNA with complementary DNA	cDNA	0.14 aM	[119]
$CoFe_2O_4$ MNPs	Nanoelectrocatalyst for toluidine blue catalysis	Target miRNA hybridizes with the padlock probe, resulting in the generation of padlock exponential rolling circle amplification (P-ERCA) products for circular exponential signal amplification	miR-21	0.3 fM	[113]
Fe_3O_4 NPs	To carry a large number of redox signaling molecules thionine and Fc for obtaining target specific DPV readout	Target-triggered HCR strategy for capturing large amounts of specific magnetic probes for simultaneous detection of miRNAs	miR-141 and miR-21	0.28 fM 0.36 fM	[112]

Table 1. (continued)

Nanoarchitecture	Function	Principle	miRNA	LOD	Refs
MoS_2-Thi-AuNPs nanocomposites	Functionalization of GCE to probe DNA-RNA complex	Hybridization of target with probe DNA greatly hinders the electron transfer on the electrode, resulting in decrease of thionine signals	miR-21	0.26 pM	[120]
Au@NPFe_2O_3NC	Immobilizing target miRNAs on AuNPs on nanocube and electrocatalysis of surface-bound Ru $(NH_3)_6^{3+}$	Nanocube-assisted electrocatalytic chronocoulometric reduction of target miRNA-bound $Ru(NH_3)_6^{3+}$	miR-21	1 pM	[33]
GO-loaded–iron oxide hybrid materials	Immobilizing target miRNAs on GO and GO/IO based electrocatalysis of surface-bound $Ru(NH_3)_6^{3+}$	Chronocoulometric readout of charge-compensating $[Ru(NH_3)_6]^{3+}$ followed by an enhancement in CC charge display through the Ru $(NH_3)_6]^{3+}$/$[Fe(CN)_6]^{3-}$ system	miR-21	100 aM	[56]
DNA–Au@MNPs	Au@MNPs as dispersible electrodes	Square-wave voltammetric responses from electric-field-induced reconfiguration of target-bound DNA–Au@MNPs	miR-21	10 aM	[50]
PdNPs@Fe-MOFs	Nanocarriers for signal probe immobilization and redox probes and electrocatalysts for signal enhancement	Streptavidin–PdNPs@Fe-MOFs as tracer anchored on target based sandwiched structure for electrocatalytic oxidation of TMB (in presence of H_2O_2)	miR-122	0.003 fM	[86]

Abbreviations: CdSe@CdS, cadmium diselenide–cadmium sulfide; CNT, carbon nanotube; $CoFe_2O_4$, cobalt ferrite; Fc, ferrocene; MMBs, magnetic microbeads; MNPs, magnetic NPs; MOFs, metal-organic frameworks; MoS_2, molybdenum disulfide; $MoSe_2$, molybdenum diselenide; PdNPs, palladium NPs; PTh, polythiophene; rGO, reduced graphene oxide; Thi, thionine; TMC, N-trimethyl chitosan polymer.

maintain the ambient environment. The merits of the nanostructured electrode surface greatly depend on the size and morphology, however. The Kelly research group elaborately leveraged the effect of nanostructured features on the substrate angle of deflection, hybridization efficiency, and biomolecule–electrode connections [94–96]. In 2009, they reported highly nanostructured Pd microelectrodes, which were modified with peptide nucleic acid (PNA) probes and then exposed with total RNA for hybridization. The microelectrode system was coupled with an electrocatalytic Ru^{3+}/Fe^{3+} reporter system and achieved a detection limit of 10 aM for miR-21 electrochemically [97]. In the following year, by manipulating the deposition time, applied potential, metal concentration, and electrolyte, three nanostructured Pd (II) electrodes; smooth hemispherical, moderately nanotextured (100–150 nm size), and finely nanotextured (20–30 nm), were engineered [94]. The highly nanostructured electrode with a surface coverage of ssDNA ($\sim 9 \times 10^{12}$ molecules/cm^2) exhibited a high hybridization efficiency, while smooth hemispherical ones showed lower hybridization efficiency. Recently, Su *et al.* manufactured shape-controlled hierarchical flower-like Au nanostructures on indium tin oxide electrodes for miR-21 detection from human lung cancer cells (A549) and achieved a limit of detection (LOD) of 1 fM [98]. Although these nanostructures presented an excellent platform for sensing miRNA, the chemistry involved in fabrication steps limits their application in robust and multiplex point-of-care devices.

The nonmetallic nanostructures such as C derivative materials (CNT, SWCNTs, graphene, and GO) have also become promising choices for the fabrication of recognition platforms, owing to their easy functionalization, fast electron transfer, physisorption capability, for example, π–π noncovalent interactions, etc. For instance, CNTs as an excellent electron transfer electrode material were used as a signal transducer and solid primer for DNA hairpin probe immobilization for

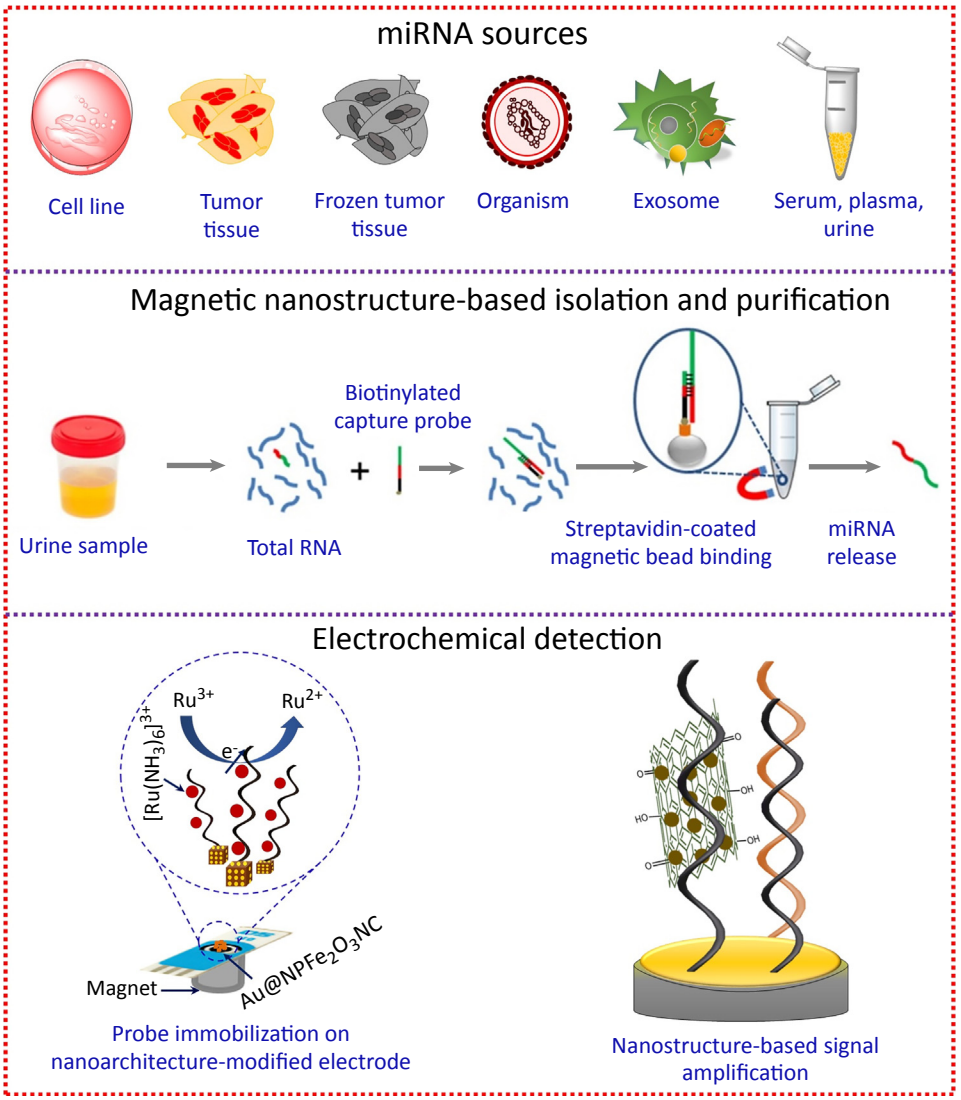

Figure 2. Nanoarchitecture in miRNA Biosensing. Top: miRNAs can be extracted from various biological samples such as cell lines, tumor tissues, organisms, exosomes, serum, plasma, and urine samples. Middle: total small RNAs (i.e., pool of miRNAs) were obtained from a patient sample (urine) using a commercial extraction-kit-based procedure followed by hybridization of target miRNA with a sequence-specific capture probe; adapted, with permission, from [57]. After hybridization, the target miRNAs were purified and isolated using streptavidin-coated magnetic beads prior to heat release of miRNAs for subsequent electrochemical quantifications. Bottom: nanostructure material acts both as (left) electrode surface modifier and (right) signal amplification tag/label. In the left figure, an Au@NPFe$_2$O$_3$NC is magnetically bound onto a screen-printed C electrode. A target miRNA is directly adsorbed onto the surface of the electrode-bound Au@NPFe$_2$O$_3$NC via affinity interaction between the Au and miRNA nucleobases. In the right figure, target-specific capture probes preimmobilized onto an electrode surface and interact with a graphene nanosheet containing a large number of electroactive molecules, resulting in an enhanced electrochemical response. In the presence of target miRNAs, a successful hybridization event between the immobilized capture probes and target miRNAs reduces the attachment of graphene-nanosheet-based electroactive molecules onto the electrode surface, leading to a significant reduction in electrochemical response. Abbreviations: Au@NPFe$_2$O$_3$NC, Au-loaded nanoporous ferric oxide nanocube.

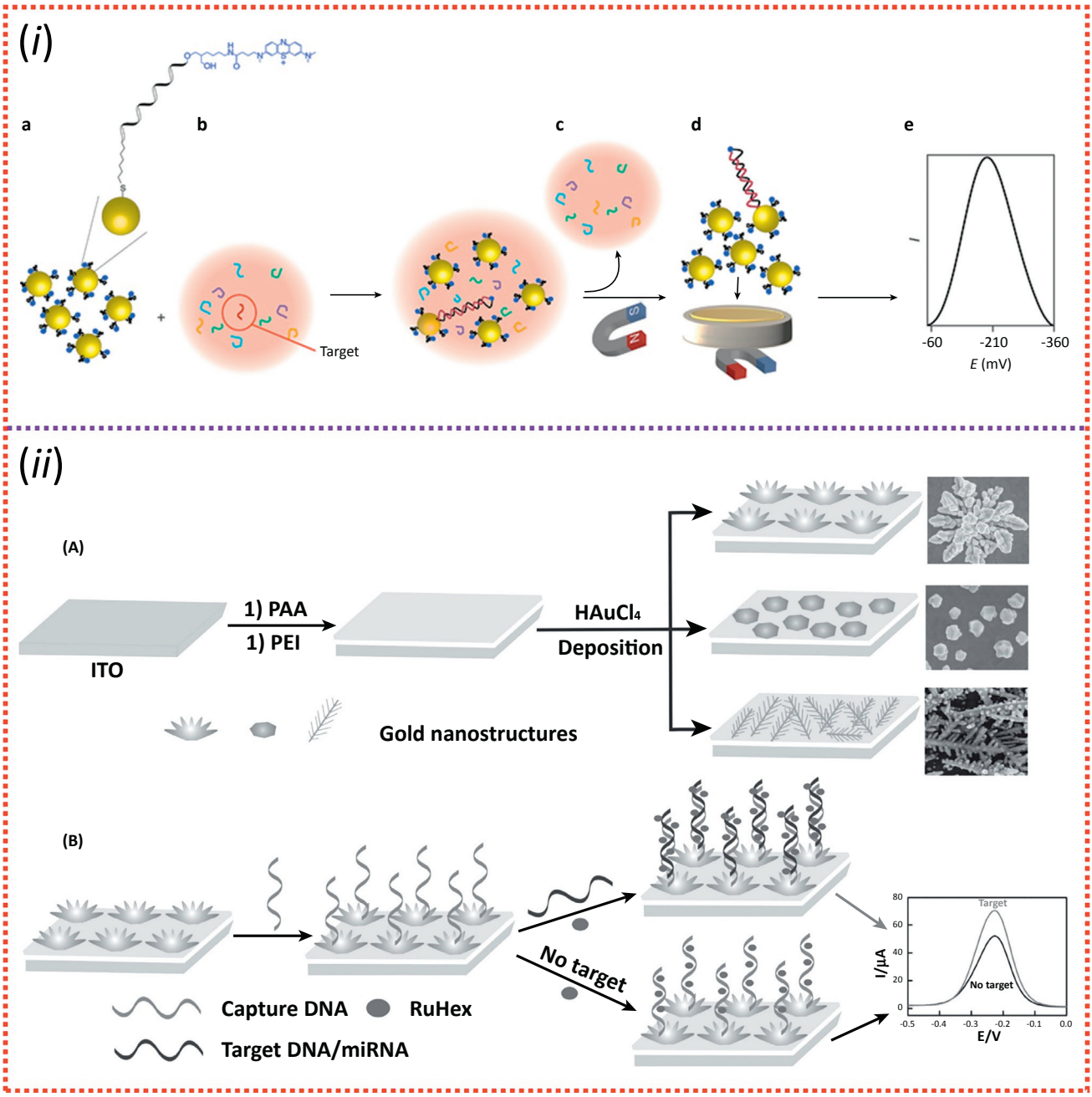

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Figure 3. Nanoarchitecture-Based Electrochemical miRNA Biosensing. (i) Representation of Au@MNPs dispersible electrode based electrochemical miRNA detection. The Au@MNPs modified with a methylene-blue-labeled probe DNA (a), followed by mixing with the sample to capture target miRNA (b). After magnetic isolation and purification of miRNA hybrid (c), Au@MNPs are magnetically collected on to the electrode surface (d), for electrical reconfiguration of Au@MNPs through square-wave voltammetry (e). (ii) Schematic illustration of Au nanostructure synthesis and electrochemical nucleic acid sensor design; (ii-a) represents the preparation of four different sizes and morphologies of gold nanostructures electrode by electrodeposition on ITO; (ii-b) demonstration of smooth HFGN gold nanostructure-based electrochemical biosensor for miR-21 detection employing $Ru(NH_3)_6^{3+}$ as electrochemical signal molecule. $Ru(NH_3)_6^{3+}$ molecules were stoichiometrically bound with the HFGN/ITO electrode attached anionic phosphate of DNA/miRNA strands thereby resulting in enhanced square wave voltammetry signals for target miR-21. Adapted, with permission, from [50,98]. Abbreviations: Au@MNPs, Au-coated magnetic nanoparticles; HFGN, hierarchical flower-like gold nanoparticle; ITO, indium tin oxide; PAA, poly(acrylic acid); PEI, poly(ethyleneimine); $Ru(NH_3)_6^{3+}$, hexaammineruthenium(III) ion; RuHEx, hexaammineruthenium(III) chloride.

miRNA detection [99]. GO contains carboxylic acid, and carbonyl and epoxy groups, and it has better solubility in aqueous and organic solvents. This material has been utilized for the modification of glassy C electrodes (GCEs), pencil electrodes, or screen-printed C electrodes (SPCEs). The GO-modified electrode surface offers a significantly enhanced surface for better interaction between capture probes and target miRNAs. The capture probes are easily immobilized at the GO surface and facilitate hybridization with target miRNAs [100]. Due to the high content of oxygenated functional groups, however, GO needs to be reduced to form rGO to retrieve the sp^2 network and facilitate electron transport. This functionality can be easily achieved by reducing the GO electrochemically [101]. The electrical conductivity can be further enhanced by introducing N atoms into the lattice of C during the reduction steps. Recently, an N-doped rGO was coupled with chitosan for increased adsorption capability to obtain a synergetic sensing platform for miRNA detection [102]. In another recent report, the rGO electrode surface conductivity was improved by modifying SPCEs with polythiophene (PTh) and rGO [103]. In this work, the PTh- and rGO-coated SPCEs provided an optimized platform for successful immobilization of capture probes and subsequent hybridization, signal amplification, and electrochemical readout steps. The combination of GO and gold nanorod (GNR) was also used for enhancing the sensitivity of nanobiosensors, as both GO sheets and GNR provide high electron transport and a large surface area, while GNR facilitates easy and stable probe immobilization. Azimzadeh *et al.* introduced a thiolated probe-functionalized GNR decorated GO sheet on the surface of the GCE for miR-155 detection [104]. In this work, the increase in current responses to the intercalating agent Oracet Blue upon hybridization of the target miRNA was measured by DPV.

Nanostructures for Electrochemical Signal Amplification

Functional nanostructures generate a synergetic effect involving catalytic activity, conductivity, and biocompatibility to accelerate the signal transduction, and amplify biorecognition events with specifically designed signal tags, leading to a highly sensitive detection performance. Gao and Yang first utilized isoniazid-capped OsO_2 NPs as a tag for obtaining enhanced signals for electrocatalytic oxidation of hydrazine and achieved an LOD of 80 fmol/droplet [105]. Following this, numerous NPs have been used in electrochemical sensors as signal-amplifying tags for miRNA detection [59,60,106]. As discussed earlier, the preconcentration of oligonucleotides onto AuNP surfaces or the controlled manipulation of AgNP aggregation in colloidal solution can be integrated with the electrochemical transduction surface to obtain amplified signals for target miRNAs. ssDNA can easily overcome the repulsion between the negative charge of the phosphate backbone and citrate ions of AuNPs and stick to the AuNPs, while the stronger repulsion and rigid structure of dsDNA make the adsorption onto AuNPs unfavorable. Therefore, in the absence of target miRNAs, the probe ssDNA adsorbed onto the AuNPs and ssDNA–AuNPs are unable to bind with a 1,6-hexanedithiol-modified Au electrode (HMAuE), resulting in significantly lower amperometric signals of hexacyanoferrate(III) ($[Fe(CN)_6]^{3-}$) [107]. When the target miRNA hybridizes with probe ssDNA to form a duplex strand, the AuNPs exist in a free state and are attached to HMAuEs, producing a significantly improved amperometric signal. Despite its simple assay design and portability, this assay is limited by poor detection sensitivity (16 fM). Encapsulation of AgNCs onto oligonucleotides adsorbed on AuNPs could enhance the sensitivity, although the encapsulation involves complex and time-consuming chemistry [59]. As an alternative, AgNP aggregates can be used with specific recognition elements as electrochemical reporters. Recently, Liu *et al.* presented a 4-mercaptophenylboronic acid (MPBA)-induced citrate-capped AgNP aggregation-based amplification strategy where boronate ester covalent interaction occurred between the MPBA and *cis*-diol at the 3′ terminus of miRNAs [108]. In this strategy, target miRNAs were hybridized with a thiolated hairpin-like DNA probe assembled on an AuNP-modified electrode. The MPBA was then anchored with the 3′ terminus of miRNAs to capture AgNPs. Meanwhile, free MPBA molecules

in solution induced the *in situ* assembly of AgNPs on the electrode surface, resulting in enhancement of the electrochemical signals. The method could successfully detect a 20 aM level of miR-21 in human serum samples.

Another strategy for obtaining ultra-low miRNA detection is to integrate nanostructures with enzymatic amplification steps such as electrocatalysis [91], PCR [63], rolling circle amplification [109], isothermal amplification [110], hybridization chain reaction, and duplex-specific nuclease (DSN) amplification [63]. Controlled aggregation of AuNPs has been integrated with triple signal amplification involving miRNA triggered DSN-catalyzed cleavage cycles, bridge DNA–AuNPs, and AuNP-based electrochemical species enrichment for ultrasensitive detection of miR-21 [106]. Upon the addition of target miRNA, the hairpin structure of the DNA probe on the Au electrode surface opens, forming a DNA/RNA duplex, thereby facilitating the DSN digestion of duplex DNA (Figure 4). The remaining ssDNA sequence was then hybridized with the single-stranded ends of three AuNPs containing bridge DNA, which adsorbed a significantly large amount of hexaammineruthenium(III) ion ($[Ru(NH_3)_6]^{3+}$) for obtaining enhanced chronocoulometric readouts. This turn-on (as target miRNA activates the signals) assay design achieved an ultra-high sensitivity (LOD 6.8 aM) without the need for tedious thermal cycling or reverse transcription processes. The use of Au composites such as GO–AuNP hybrid as a signal carrier has been reported for electrochemical–chemical–chemical (ECC) detection of miRNA using MgO-nanoflowers/AuNPs as a sensing platform [111]. This strategy is advantageous for achieving high sensitivity as it utilizes triple mode signal enhancement steps (ECC) and has achieved an LOD of 50 aM.

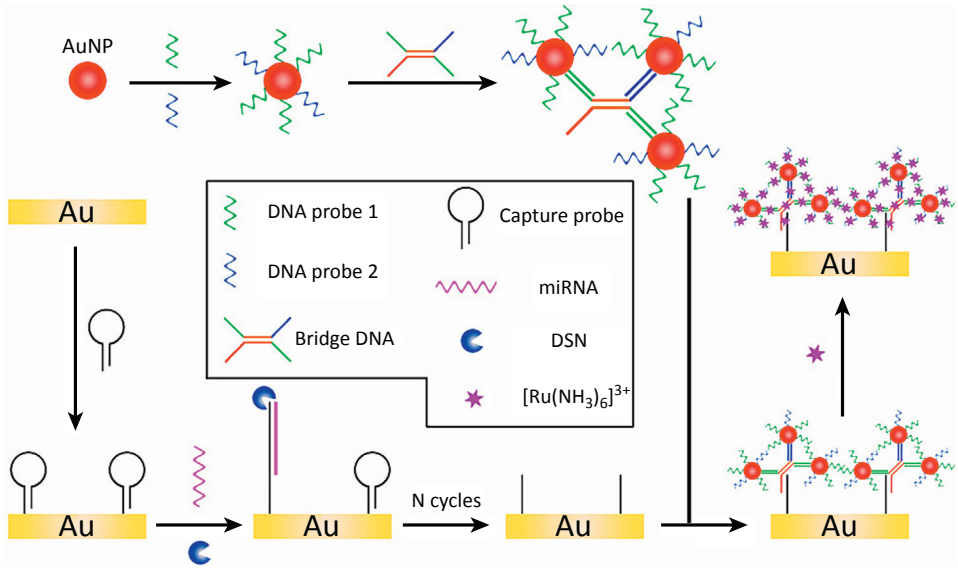

Figure 4. Signal Amplification. Triple signal amplification strategy for ultrasensitive determination of miRNAs based on DSN and bridge DNA–AuNPs; adapted, with permission, from [106]. DNA nanocomposite was formed using specially designed bridge DNA and DNA-probe-modified AuNPs. In the absence of target miRNAs, the hairpin probes attached on an Au electrode were inactivated while the target miRNA opened the hairpin structure, allowing the formation of DNA/RNA duplex and digestion of duplex by a DSN (enzyme that preferentially cleaves dsDNA and DNA in DNA–RNA hybrid duplexes but not single-stranded DNA or single- or double-stranded RNA). The remaining DNA sequence on electrode surface became activated and hybridized with DNA nanocomposites, facilitating the absorption of numerous $[Ru(NH_3)_6]^{3+}$ for generating enhanced chronocoulometric responses. Abbreviations: AuNP, Au nanoparticle; DSN, duplex-specific nuclease; $[Ru(NH_3)_6]^{3+}$, hexaammineruthenium(III) ion.

Due to the enzyme mimetic and nanoelectrocatalyst merits of magnetic nanomaterials, they have been widely used in electrochemical miRNA sensing for catalyzing redox markers. Yuan *et al.* reported two distinguishable magnetic nanoprobes (DNA1/Fe_3O_4 NPs/thionine and DNA2/Fe_3O_4 NPs/ferrocene) for simultaneous detection of miR-141 and miR-21 [112]. Upon the hybridization of the target miR-141 and miR-21 with the thiol-modified hairpin capture probes (HCP1 and HCP2) on the Au electrode surface, the hybridization chain reaction (HCR) generates a large number of sequences for binding magnetic nanoprobes. Boosted DPV responses are then obtained for plentiful magnetic nanoprobes attached to thionine and ferrocene for target miRNAs by target-triggered HCR and the catalytic responses of magnetic nanoprobes. A similar nanocomposite (Au@Co-Fe_2O_4/Tb-Gra composites) tagged padlock exponential **rolling circle amplification** (P-ERCA) assay has also been reported for miR-21 [113]. CNTs can interact with DNA through the π–π stacking of DNA aromatic bases and van der Waals forces, but dsDNA tends to stack less than ssDNA does. Based on this principle, Asadzadeh-Firouzabadi and Zare designed an electrochemical nanogenosensor for miR-25 using an AgNP/SWCNT nanohybrid as a label [114]. AuNP-modified Cu-based MOFs (AuNPs@Cu-MOFs) as nanocarriers and catalyst have been reported for miR-155 detection using a target-triggered signal amplification cycling strategy [88].

Recently, we have utilized specially engineered Au@NPFe_2O_3NC for SPCE modification to achieve amplification-free, nonenzymatic, and electrocatalytic miRNA-21 detection using $[Ru(NH_3)_6]^{3+}$ as an intercalating agent, and achieved a 1.0 pM level of detection [54]. Ru^{3+} is an excellent electrochemical reporter, although it does not yield high sensitivity because only one electron can be accepted by each Ru^{3+} acceptor. The limited concentration of Ru^{3+} is also inadequate to produce an enhanced signal. To increase the sensitivity, the $[Ru(NH_3)_6]^{3+}$ system was later combined with $[Fe(CN)_6]^{3-}$, where the electrocatalytic reduction of miRNA intercalated $[Ru(NH_3)_6]^{3+}$ subsequently reduces the solution-phase $[Fe(CN)_6]^{3-}$, as shown in Figure 5

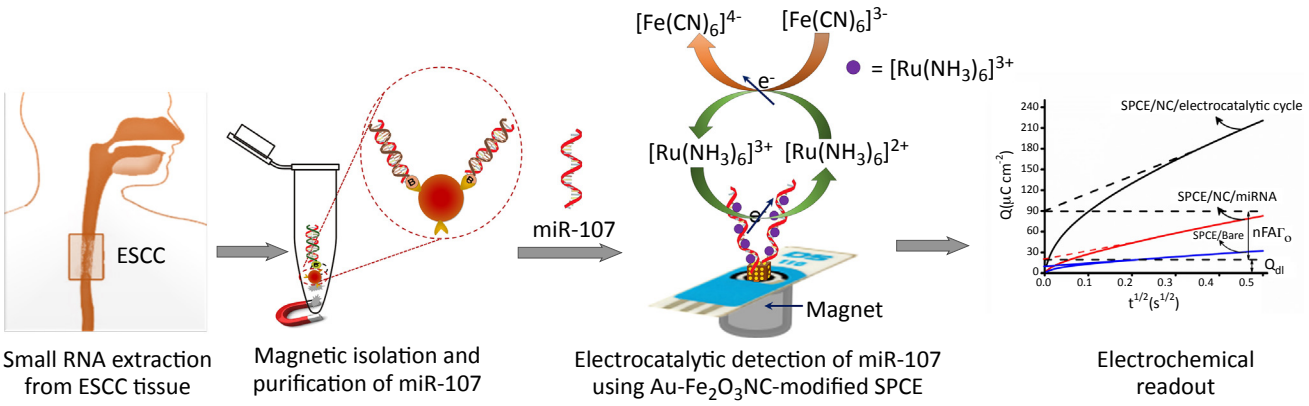

Figure 5. Electrocatalytic Detection via Au-Loaded Magnetic Nanoparticles. Schematic representation of Au-Fe_2O_3NC-catalyzed miR-107 assay using $[Ru(NH_3)_6]^{3+}/[Fe(CN)_6]^{3-/4-}$ electrocatalytic cycle. Target miR-107 was magnetically isolated and purified from ESCC tissue followed by adsorption on an Au-Fe_2O_3NC-modified SPCE. The adsorbed miR-107 was detected by measuring the saturated amount of charge-compensating $[Ru(NH_3)_6]^{3+}$ molecules in the surface-attached miR-107 by CC, as redox charge of the $[Ru(NH_3)_6]^{3+}$ molecules quantitatively reflects the amount of adsorbed miR-107 confined at the Au-Fe_2O_3NC surface. The electrocatalytic activity of Au-Fe_2O_3NC was used for the reduction of $[Ru(NH_3)_6]^{3+}$ bound with target miR-107. The catalytic signal was further amplified using the $[Fe(CN)_6]^{3-/4-}$ system. Right panel, corresponding CC curves obtained for the miR-107-attached Au-Fe_2O_3NC/SPCE in absence (blue line) and presence (red line) of $Ru(NH_3)_6]^{3+}$. In the presence of $[Ru(NH_3)_6]^{3+}/[Fe(CN)_6]^{3-/4-}$ electrocatalytic cycle, an enhanced CC response was obtained (back line). Adapted, with permission, from [55]. Abbreviations: Au-Fe_2O_3NC, Au-loaded iron oxide nanocube; CC, chronocoulometry; ESCC, esophageal squamous cell carcinoma; $[Fe(CN)_6]^{3-/4-}$, potassium hexaferro/ferricyanide; $[Ru(NH_3)_6]^{3+}$, hexaammineruthenium(III) ion.

[55]. The negative charge of $Fe(CN)6]^{3-}$ prevents this complex from reaching the surface of the electrode because of the electrostatic repulsion of the negatively charged phosphate backbone. Thus, the Fe^{3+} complex remains in solution and acts as an oxidant when it interacts with outwardly diffusing Ru^{2+}, regenerating Ru^{3+}. By regenerating Ru^{3+}, one bound Ru^{3+} complex can interact with the electrode on multiple occasions, causing a significant amplification of the target responding chronocoulometric signal (Figure 5). This Au-NPFe_2O_3NC catalyzed and $[Ru(NH_3)_6]^{3+}/[Fe(CN)_6]^{3-}$ redox cycling amplified assay design for miR-107 achieved an LOD of 100 aM. A similar assay for miR-21 detection has also been reported using another GO-loaded iron oxide (GO/IO) hybrid material [56,81].

Concluding Remarks and Future Perspectives

With the increasing recognition of the potential of miRNAs as diagnostic and prognostic biomarkers, recent years have witnessed a rapid surge in novel molecular and nanotechnology-based miRNA detection technologies. The momentous progress on nanostructures as nanocarriers, catalysts, conductive surface modifiers, probe immobilizers, and signal generators and enhancers, as well as the simplicity, robustness, ultrasensitivity, miniaturization, and cost-effectiveness of electrochemical readouts are continuing the dynamic progress on nanostructure-based **electrochemical** miRNA sensors. This review has summarized recently reported (2015–2018) remarkable achievements gained in the application of engineered nanostructures for the development of electrochemical miRNA sensors. Moreover, with a few illustrations of RNA diagnostics and prognostics, the review has described the challenges involved in current miRNA detection strategies as well as offering an outlook for the avenues where future developments may be directed (see Outstanding Questions).

The advances in nanostructure-based electrochemical miRNA quantification reported in the recent scientific literature are a sign of flourishing research, although much work still needs to be performed to make it suitable for a point-of-care platform. To date, most sensors are merely proof-of-concept demonstrations and highly dependent on laboratory-based setup. We suggest that a number of factors, such as proper selection of the miRNA source, the right extraction procedure, designing special primers, avoiding amplification bias, etc., should be carefully considered to avoid inconsistencies in RNA assays. It is also obvious that the majority of the reported nanostructures involve multistep preparation processes that require complex probe functionalization and tagging steps. Moreover, two or more NPs are commonly used to form a composite to achieve nanostructures that have high signal enhancement capacity as well as a large functional surface area. These steps can be simplified by engineering porous composite nanomaterials that have an enlarged surface area. Single-step nanostructure synthesis with *in situ* probe functionalization and easy electrochemical transduction and readout could also be considered for future nanostructure-based miRNA sensing.

Incorporation of target-specific multifunctional nanostructures and miniaturized electrochemical readouts into portable paper-based microfluidics and **lab-on-a-chip** devices in an easy and user-friendly format needs to be explored for reducing the burdens (cost, complication, and time frame) associated with the existing miRNA diagnostic technologies. An automated miRNA diagnostic platform that can perform without any human intervention is the future platform that may find applications beyond the classical laboratory. On that note, we believe, that further developments in miniaturized nanostructure-based electrochemical miRNA analysis platforms will shape the future of personalized disease diagnostics both in research and clinical settings.

Outstanding Questions

Which RNA species can be the best choice as biomarker amongst a variety of clinically relevant coding and non-coding RNA molecules?

Do these RNA biomarkers provide accurate information regarding tumor heterogeneity?

Keeping in view the tumor heterogeneity, can these RNA biomarkers provide accurate clinical information?

Circulating miRNA versus tissue-bound miRNA: which one is preferable?

Can exosomal miRNAs help to overcome the challenges of poor reproducibility and compromised specificity associated with miRNA analysis and prove to be the ultimate biomarker of choice for stably diagnosing diseases?

What are the major challenges that have so far hampered the translation of laboratory-based strategies to off-laboratory point-of-care settings, and how can these be overcome?

Can the tedious extraction procedures for small RNAs be avoided, that is, what is the scope for directly interrogating miRNAs from the complex biological matrix?

How can the analytical performance of the sensor be maintained even after simplifying and miniaturizing the sensor system?

Which strategies should be adopted to design the best biofavorable nanomaterial for the development of a next-generation nanomaterials-enabled integrated electrochemical sensor?

How could a final readout strategy be developed that can be readily used by a non-expert end-user?

What future steps should be considered to translate a proof-of-concept biosensor from the laboratory towards commercialization (for large-scale production at low cost)?

What are the future considerations for the development of a modular automated miRNA profiler that can function with less or no human intervention?

Acknowledgments

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