Analyst

MINIREVIEW

View Article Online
View Journal

Cite this: DOI: 10.1039/c3an01677c

Biosensor-based microRNA detection: techniques, design, performance, and challenges

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The current state of biosensor-based techniques for amplification-free microRNA (miRNA) detection is critically reviewed. Comparison with non-sensor and amplification-based molecular techniques (MTs), such as polymerase-based methods, is made in terms of transduction mechanism, associated protocol, and sensitivity. Challenges associated with miRNA hybridization thermodynamics which affect assay selectivity and amplification bias are briefly discussed. Electrochemical, electromechanical, and optical classes of miRNA biosensors are reviewed in terms of transduction mechanism, limit of detection (LOD), time-to-results (TTR), multiplexing potential, and measurement robustness. Current trends suggest that biosensor-based techniques (BTs) for miRNA assay will complement MTs due to the advantages of amplification-free detection, LOD being femtomolar (fM)–attomolar (aM), short TTR, multiplexing capability, and minimal sample preparation requirement. Areas of future importance in miRNA BT development are presented which include focus on achieving high measurement confidence and multiplexing capabilities.

Received 4th September 2013
Accepted 23rd January 2014

DOI: 10.1039/c3an01677c

www.rsc.org/analyst

1. Introduction

MicroRNA (miRNA) is small non-coding RNA, typically containing ~21–24 bases, which plays an important role in post-transcriptional regulation of gene expression.[1–4] To date, greater than one thousand potential miRNA targets have been identified, many of which are promising biomarkers, since their aberrant expression correlates with disease and stress states.[5–21] For example, miRNA *let-7a* has been shown to regulate the *MYC* oncogene in lymphoma cells,[22] and *let-7* family miRNA have been shown to be de-regulated in various cancers.[23] Although a number of excellent reviews have been reported on their biological function[5,13,24] and assay by traditional non-sensor amplification-based molecular techniques (MTs), such as polymerase- or microarray-based methods,[25–31] very few reviews have focused on emerging amplification-free biosensor-based techniques for miRNA assay (BTs) which provide useful characteristics for application in both fundamental research and clinical settings.

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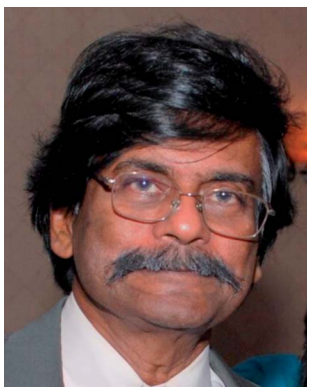

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Early miRNA assay was primarily done using reverse transcriptase polymerase chain reaction (RT-PCR),[32–35] Northern blot,[36–38] and microarray[25,39–45] MTs which employ a selective molecular probe, but not an integrated transducer element. Such approaches satisfy the definition of molecular techniques (MTs) which use a selectively binding molecular probe to quantitatively measure one component of a sample.[46] Importantly, miRNA MTs require extensive sample preparation, including amplification steps, which can increase time-to-results (TTR) and introduce measurement bias. When combined with the challenges arising from miRNA susceptibility to degradation, variability in both melting temperature and relative expression levels, and high sequence similarity, there is a need to examine alternative assay strategies which may offer advantageous measurement characteristics.

Over the past decade, BTs have arisen as a compliment to traditional MTs. BTs combine the use of a selective molecular probe used in traditional MTs with a highly sensitive transducer design in sensors. This gives BTs the ability to achieve rapid and highly sensitive results in complex matrices, such as blood and urine, without the use of polymerase-based amplification steps. These attractive features have led to the recent development of BTs for miRNA assay which is the focus of the current review. We first examine various miRNA biosensor designs based on alternative transduction mechanisms, including electrochemical,[47] electromechanical,[48] and optical,[40] each of which has achieved femtomolar sensitivity levels to date. We highlight recent sensing achievements of miRNA biosensors which now exhibit limit of detection (LOD) at attomolar (aM) levels with multi-log dynamic range and short TTR. We also briefly discuss additional aspects of miRNA BTs, including biosensor surface functionalization, measurement format, multiplexing potential, and thermodynamic constraints in measurement. A comparison with traditional MTs for miRNA assay is also provided which suggest areas of future research in BTs.

2. Classification and performance of MiRNA biosensors

A biosensor is a device which provides quantitative analytical information using a biorecognition element in direct contact with a transduction element.[49] The selective biorecognition element in miRNA biosensors is typically a DNA probe of complementary sequence to the miRNA target. As shown schematically in Fig. 1A, DNA probe hybridization with the miRNA target is transduced through changes in a measurable output signal. Thus, biosensors are categorized based on their transduction mechanism which can be electrochemical-, electromechanical-, or optical-based. As illustrated in Fig. 1B–E, miRNA biosensors using electrochemical, electromechanical, and optical transduction mechanism have been examined to date. In Fig. 1, we show a variety of miRNA biosensors which employ different transduction mechanisms. Fig. 1B shows a nano-gapped electrochemical sensor,[50] Fig. 1C shows a silicon nanowire electrochemical sensor,[51] Fig. 1D shows a silicon photonic micro-ring resonator optical sensor,[52] and Fig. 1E shows a resonant piezoelectric cantilever electromechanical sensor.[48] Although different in appearance, they share the defining feature of a biosensor, an integrated molecular recognition agent and transduction element. We next discuss the individual transduction mechanisms, their influence on biosensor design, and miRNA biosensing achievements.

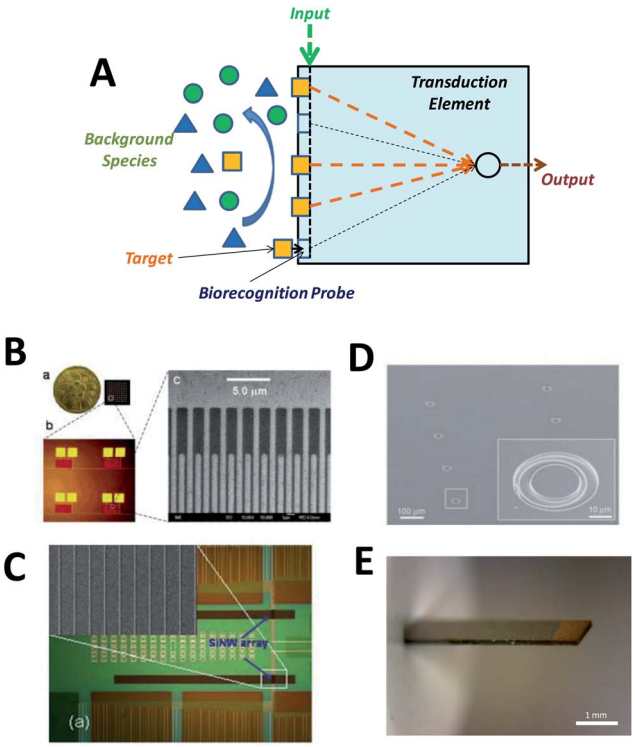

Fig. 1 (A) Schematic of biosensor working principle. (B) Nano-gapped electrochemical miRNA biosensors reprinted with permission.[50] (C) Silicon nanowire miRNA biosensors reprinted with permission.[51] (D) Silicon photonic micro-ring resonator miRNA biosensors reprinted with permission.[52] (E) Resonant piezoelectric cantilever miRNA biosensors reprinted with permission.[48]

2.1 Transduction mechanism and design variation

2.1.1 Electrochemical MiRNA biosensors. The transduction element in electrochemical miRNA biosensors is an electrode, most often a gold (Au)-coated one. Sensing is accomplished by measuring changes in the electrode or interfacial properties upon hybridization between the immobilized DNA probe and the miRNA target. Transduction often relies on an electrochemically-active reporter species, whose activity is used to infer changes in electrode properties caused by miRNA hybridization. As shown in Table 1, commonly used reporters include both small molecules and enzyme–substrate pairs. For example, ferric compounds represent the former, while horseradish peroxidase (HRP)–hydrogen peroxide (H_2O_2) represents the latter. Transduction in electrochemical miRNA biosensors has been investigated *via* amperometric,[53–56] potentiometric,[53,56–60] resistive,[51] and impedance-based[53,55,61,62] approaches. In Table 1, we summarize the electrochemical biosensors examined on the basis of their design, reporter used,

Table 1 Summary of electrochemical miRNA biosensors. Abbreviations: nanoparticle (NP), gold NP (Au NP), peptide nucleic acid (PNA), locked nucleic acid (LNA), horseradish peroxidase (HRP), hydrogen peroxide (H_2O_2), palladium (Pd), ferricyanide/ferrocyanide ($Fe(CN)_6^{3-/4-}$), potassium ion (K^+), chloride ion (Cl^-) and not available (N/A)

Description	Geometry	Enhancer	Reporter	LOD	Ref.
Indium tin oxide electrode	Planar	Osmium dioxide NP-labeled probes	Hydrazine	80 fM	73
Interdigitated microelectrodes	Planar with gaps	Aniline nanowire polymerization	HRP, H_2O_2	5 fM	50
Graphite electrode using inosine-modified probes	Planar	None	None	5 nM	112
PNA, LNA, and DNA probe modified micro-beads	Planar	None	HRP, hydroquinone, ferrocene derivatives	51 pM	113
Silicon nanowire	Wire	None	None	1 fM	51
Pencil graphite electrode	Planar	None	Alkaline phosphatase, α-naphthyl phosphate	N/A	57
Electrode chip	Planar	None	Gap hybridization using esterase 2 and *p*-aminophenol	2 pM	63
Nanopore using viral protein p19	Pore	None	K^+ and Cl^- ions	N/A	64
Nanopore using alpha-hemolysin	Pore	None	K^+ and Cl^- ions	100 fM	65
Nuclease digestion on Au electrode	Planar	None	Glucose oxidase, glucose	10 fM	54
Ruthenium oxide NP-labeled probes	Planar	3,3′-Dimethoxybenzidine polymerization	HRP, H_2O_2	3 fM	61
Tetrahedron probes on Au electrode	Planar	None	HRP, H_2O_2	10 fM	66
Molecular beacons on Au electrode	Planar	Silver nano-clusters	H_2O_2	67 fM	47
Microelectrode array	Planar	None	None	2 pM	60
Au NP-modified Au electrode	Planar	None	Hemin G-quadruplex, $Fe(CN)_6^{3-/4-}$	~4 pM	62
Ruthenium oxide NP-based assay	Planar	Aniline polymerization	H_2O_2	2 fM	58
Graphene and dendritic Au nano-structured glassy carbon electrode	Planar	None	HRP, hydroquinone, H_2O_2, $Fe(CN)_6^{3-/4-}$	60 fM	53
Molecular switching amperometry on graphene-modified Au electrode	Planar	None	HRP, H_2O_2, hydroquinone, $Fe(CN)_6^{3-/4-}$	400 fM	55
Indium tin oxide-coated glass electrode	Planar	3,3′-Dimethoxybenzidine polymerization	HRP, H_2O_2	2 fM	74
Nano-structured microelectrode chips	Planar	$Fe(CN)_6^{3-}$	$Ru(NH_3)_6^{3+}$	10 aM	72
Nafion–thionine–Pd NP electrode	Planar	None	H_2O_2	~2 pM	59
Allosteric molecular beacons on Au electrodes	Planar	None	HRP, H_2O_2, 3,3′,5,5′-tetramethylbenzidine	~3 pM	56

and LOD. As shown in Table 1, device size widely varies from millimeter (mm)[47,63] to nanometer (nm).[51,64,65] Electrode geometry has taken the shape of planar surfaces,[47] nano-textured surfaces,[50] nanopores,[64,65] and nanowires.[51] For example, in Fig. 2A, we show a planar electrode used in the context of a cyclic voltammetry-based approach.[66] Fig. 2B shows a nanopore electrode which was successfully used in amperometric measurement.[65] Fig. 2C highlights a nanowire-based biosensor with resistive transduction.[51] Note the right panels of Fig. 2 show the sensor output data, which may be collected either in a batch or continuous mode. The continuous biosensor measurement is especially attractive as it provides rate information of the surface miRNA hybridization reaction and verifies sensor steady state response.

2.1.2 Electromechanical MiRNA biosensors. Transduction in electromechanical biosensors occurs *via* hybridization-induced changes in either a static or dynamic property of the electromechanical system. For example, monitoring deflection or resistance represents the former, while monitoring resonant frequency, phase shift, or impedance represents the latter. The resonant frequency-based transduction present in cantilever miRNA biosensors represents a dynamic measurement principle (see Fig. 3B).[48] Dynamic transduction was also employed in a tapping-mode atomic force microscope (AFM)-based miRNA sensing approach which monitored hybridization-associated changes in surface morphology (see Fig. 3A).[67] One notes that AFM is not a traditional biosensor by definition because the biorecognition probe and the transducer (the cantilever) are not integrated. Nonetheless, we include it here as its detection mechanism is mechanically-based unlike MTs.

Alternatively, an example of a static mechanical transduction principle is the deflection-based measurement principle encountered in microcantilevers which arises from the differential surface stress associated with hybridization. However, miRNA biosensors with such a transduction principle have not yet been investigated. Similar to electrochemical biosensors, electromechanical biosensor size varies widely from mm [48] to nm scale.[67] Most importantly, as shown in Table 2, size variation appears to correlate with TTR, in terms of shorter TTR associated with the macro-device.

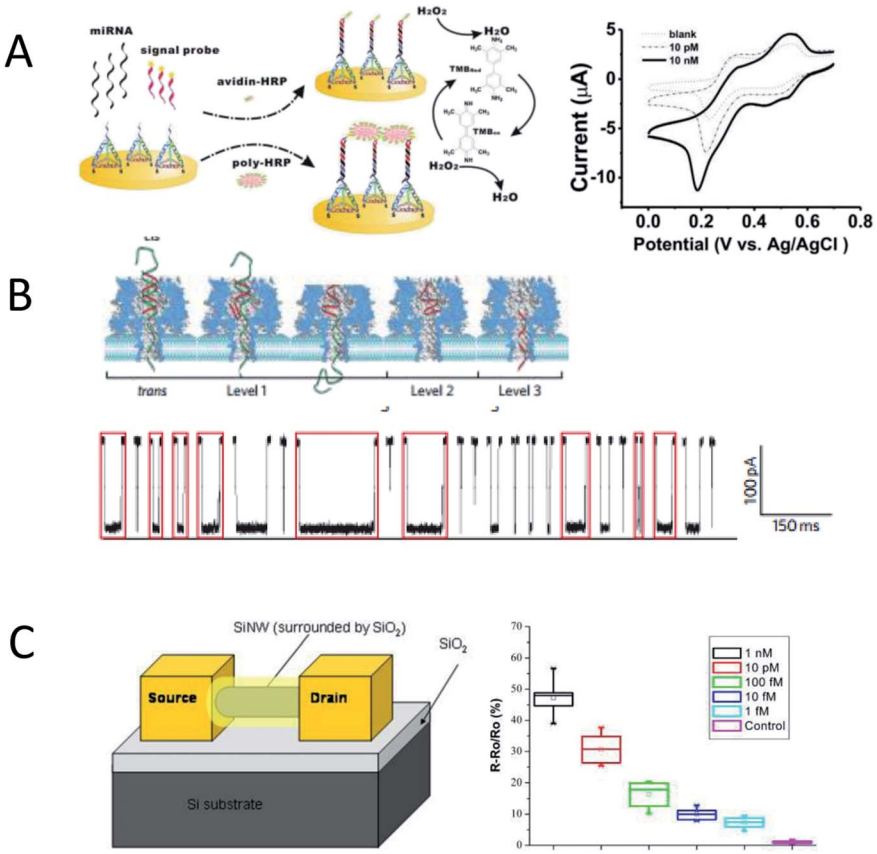

Fig. 2 Examples of electrochemical miRNA biosensor designs. (A) Planar electrode-based design reprinted with permission.[66] (B) Nanopore-based design reprinted with permission.[65] (C) Nanowire-based design reprinted with permission.[51]

2.1.3 Optical MiRNA biosensors. Optical biosensors transduce miRNA hybridization through associated changes in the absorbance or fluorescence of an optically-active reporter linked to the DNA probe. Unlike the redox reporters used in electrochemical biosensors, the optical reporters are sometimes directly conjugated to the DNA probe. For example, dye-labeled probes[68] and quantum dots[69] have been used as reporters in optical miRNA biosensors. Such reporters are less prone to inhibitory effects unlike the enzyme reporters commonly used in electrochemical biosensors and MTs, such as reverse transcriptase and HRP, and thus, optical biosensor-based miRNA assay also avoids extensive sample preparation and

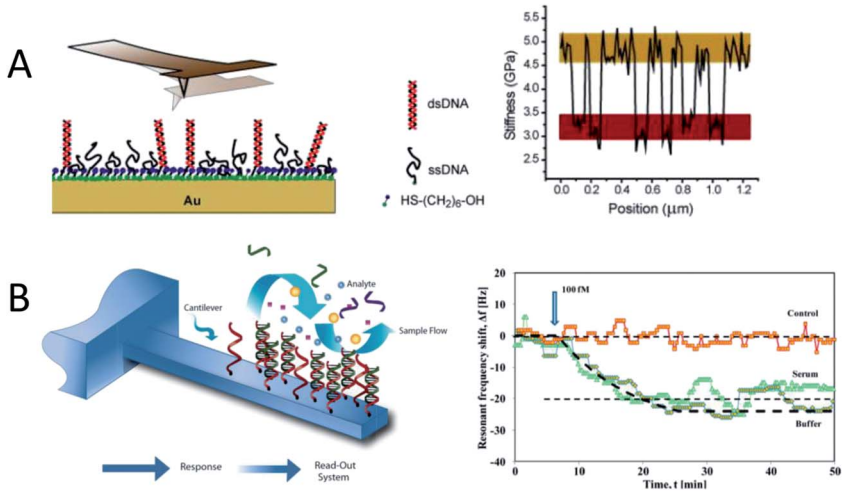

Fig. 3 Examples of electromechanical miRNA biosensor designs. (A) AFM-based biosensor design reprinted with permission.[67] (B) Resonant-cantilever-based biosensor design reprinted with permission.[48]

Table 2 Summary of electromechanical miRNA biosensors. Abbreviations: atomic force microscope (AFM), gold nanoparticle (Au NP), verification strategy (V), and not available (N/A)

Description	Transduction principle	Measurement format	TTR	Enhancer	LOD	Ref.
AFM	Surface nano-structure characterization	Batch	6–8 h	—	1 aM	67
Piezoelectric cantilever	Frequency shift of electromechanical resonance	Continuous flow	<1 h	Au NP-labeled probes V: dehybridization and fluorophore-labeled probes	5 fM	48

normalization steps. Although fluorescence-based approaches are most common, alternative strategies based on changes in the absorbance[70] or the intensity and frequency of optical resonance fields[40,71] have also been examined (Table 3). For example, surface plasmon resonance (SPR)-based miRNA detection is a common example of the latter strategy.[40,71] Similar to electrochemical and electromechanical biosensors, designs vary widely among optical miRNA biosensors. For example, the probe may be either surface- or solution-based as in SPR[71] or quantum dot[69] applications, respectively. While probes are surface-bound in electrochemical and electromechanical biosensors, those used for optical biosensors are sometimes solution-based. For example, in Fig. 4A a solution-based miRNA biosensor approach which employs a DNA probe-integrated photo-active protein complex is shown.[69] In Fig. 4B a biosensing strategy that uses fluorescence modulation of grapheme oxide upon surface binding is illustrated.[68] One notes that although graphene oxide is suspended in solution, the probe-target complex is immobilized. In both cases, the selective probe and transduction element are integrated which constitutes a biosensor.

2.2 Sensing achievements of MiRNA biosensor-based assay

2.2.1 Electrochemical MiRNA biosensors. Impressive sensitivity ranging from aM–nM levels have been reported using electrochemical miRNA biosensors (see Table 1). For example, an electrochemical biosensor with nano-structured electrode and Ru^{3+} reporter exhibited LOD of 10 aM.[72] In another study, use of osmium dioxide (OsO_2) nanoparticles (NPs) gave signal enhancement which provided LOD of 80 fM.[73] Another strategy based on target-guided polymer wire formation in nano-gaps *via* aniline polymerization exhibited LOD of 5 fM.[50] A similar technique involving target-guided film formation has also been examined and has shown comparable sensitivity near 2 fM.[74]

In some electrochemical biosensor studies, secondary reaction steps were used for enhancing response signal (see Table 1). Such an enhancement feature improves measurement reliability, reduces false negatives, and improves LOD. We define an enhancement or verification strategy as one in which the initial target hybridization provides a measurable signal and the second step occurs only if the first step is indeed a sensing response, else it would only constitute another assay step which does not significantly improve measurement reliability. For example, sensor response enhancement was accomplished using Ag nano-cluster-labeled DNA probes[47] and aniline polymerization.[58] We note that such features are desirable in a practical diagnostic setting where reliable measurement is required.

In addition to millimeter scale biosensor designs, nano-scale electrochemical miRNA biosensors are being currently investigated as potential miRNA biosensors. For example, nanowires and nanopores have given LOD of 1 fM [51] and 100 fM,[64,65] respectively. The major drawback of nano-scale devices is low sensor mass transfer rates which increases the time-to-results (TTR). In some cases, such techniques require pre-amplification to achieve reliable detection in background of other nucleic acids,[64] which can increase assay TTR and complexity. Thus, they can resemble microarray-based MTs which often use polymerase chain reaction (PCR) for amplification and labeling

Table 3 Summary of optical miRNA biosensors. Abbreviations: surface plasmon resonance (SPR), gold nanoparticle (Au NP), cadmium telluride (CdTe), cadmium selenide (CdSe), verification strategy (V), and not available (N/A)

Description	Transduction principle	Enhancer	LOD	Ref.
SPR	Surface plasmon	Au NP-labeled probes	10 fM	40
Au NP-labeled probes	Absorbance	Silver enhancement	10 fM	70
ATPase-integrated probes and CdTe quantum dots	Fluorescence	None	N/A	69
CdSe ionic nanocrystal-labeled probes	Fluorescence	None	35 fM	114
SPR	Surface plasmon	DNA–RNA duplex-recognizing antibodies	2 pM	71
Silicon photonic micro-rings	Wavelength shift of optical resonance	V: RNase duplex cleavage	~1 nM	52
Silver nano-cluster DNA probes	Fluorescence	None	~20 nM	115
Capillary electrophoresis using drag-tag probes	Fluorescence	None	~100 pM	116
Silicon photonic micro-rings	Wavelength shift of optical resonance	DNA-RNA duplex-recognizing antibodies	10 pM	76
Water-soluble carbon nanoparticles	Fluorescence	V: DNase effect	3.5 nM	117
Graphene-oxide with dye-labeled probes	Fluorescence	None	2.1 fM	68
Magnetic beads	Fluorescence	None	20 fM	118
Poly(vinylidene fluoride)	Fluorescence	None	10 nM	119

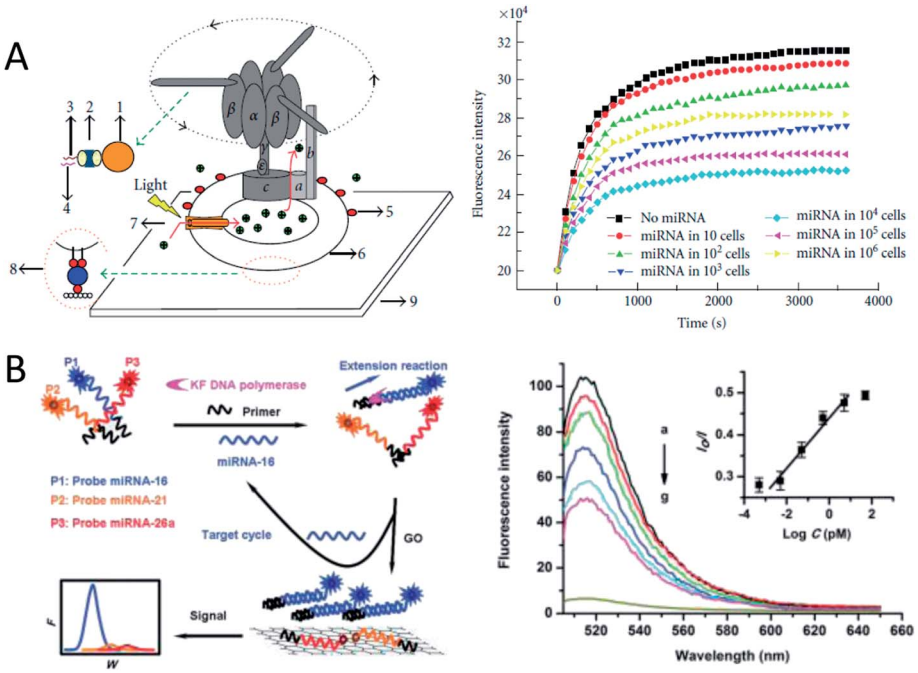

Fig. 4 Examples of optical miRNA biosensor designs. (A) Solution-based optical technique reprinted with permission.[69] (B) Surface-based optical technique reprinted with permission.[68]

prior to the array-based screening. However, nano-scale biosensing approaches are promising as high sensitivity and multiplexing capability have been demonstrated.

2.2.2 Electromechanical MiRNA biosensors. Electromechanical biosensors are among the most sensitive classes of miRNA biosensors with sensitivity ranging from aM–fM levels. For example, a resonant piezoelectric-excited millimeter-sized cantilever (PEMC) sensor exhibited 5 fM LOD in human serum with minimal sample preparation.[48] Another biosensing strategy based on atomic force microscopy also exhibited high sensitivity with LOD $\sim$ 1 aM.[67] It should also be noted that, although not yet applied to miRNA, the aforementioned static-mode microcantilevers which transduce hybridization into cantilever deflection have been shown to exhibit sensitivities at 100 picomolar (pM) for detection of short oligonucleotides strands in a continuous flow-based measurement format.[75] We note that continuous flow format was also used in piezoelectric cantilever (PEMC) biosensors which are also known to facilitate detection of miRNA[48] with less than 1 hour TTR (see Fig. 3B). On the other hand, the AFM approach which both lacks flow and has small device length scale results in increased TTR.[67]

2.2.3 Optical MiRNA biosensors. LOD in optical miRNA biosensors ranges from nM–fM levels. The results of various investigations are summarized in Table 3. Surface plasmon resonance (SPR) has provided sensitivity at low pM levels.[71] Photonic resonance in silicon micro-rings has also been investigated and gave LOD near 1 nM,[52] albeit with excellent signal-to-noise ratio suggesting higher sensitivity could be achieved. In fact, LOD was improved to 10 pM using a DNA–RNA duplex-recognizing antibody secondary binding step.[76] Such a technique was also used in SPR-based miRNA sensing for enhancing detection response.[71] We note that nanoparticle-based enhancement has also been investigated in SPR-based miRNA sensing for improving LOD.[40] Similar to the sensing format of resonant cantilever miRNA biosensors, SPR-based miRNA detection is attractive due to its flow-based format and associated short TTR.

Multiplexing capability is desirable in miRNA assay and has been an important aspect that drives research in PCR- and microarray-based MTs. Multiplexing capability has just begun to be investigated in miRNA biosensors. For example, silicon photonic micro-ring resonators represent a class of optical miRNA biosensors with multiplexing capability and low pM sensitivity levels.[76] As shown in Table 3, optical biosensing approaches are commonly based on arrays suggesting they have excellent multiplexing potential. Although still evolving, we note that biosensor multiplexing is an active area of study.

3. Functionalizing biosensors for MiRNA assay

Although the previous section illustrates that miRNA biosensor working principle and design vary, all miRNA biosensing platforms share the requirement of immobilizing a selective capture probe on a functionalized transducer surface. Linear probes are commonly used in miRNA biosensors. Padlock and hairpin probe varieties are also common, but are used more frequently in MT-based approaches. Probe immobilization on the biosensor surface is traditionally facilitated by reactions between surface and probe functional groups, such as sulfhydral,[48,77] or amine and carboxyl groups.[25,41] Strategies based on

biotinylation have also been used.[41] Tetrahedral thiol linkers,[66] organosilanes,[51] and carbodiimide-based chemistry,[57] have also been used for immobilizing miRNA biorecognition probes.

Probe immobilization conditions and surface passivation method affect assay results as they control surface density,[78,79] probe accessibility,[80] and chemical reactivity of the passivated surface,[81] all of which affect the heterogeneous DNA–miRNA hybridization reaction. For example, over-packing of DNA probes has been found to inhibit hybridization.[78,82] It has also been shown that surface electric charge and potential,[83] as well as choice of buffer surfactant,[84] affect hybridization extent in surface-based measurements. Probe length, secondary structure, and sequence variation have also been shown to affect hybridization.[85,86] For example, in one study it was found that miRNA hybridization rate using DNA probes was slower than that observed with PNA probes.[87] It has also been shown that hybridization rates in solution were more rapid than those occurring on surfaces.[80] The above experimental observations suggest thermodynamic and rate considerations in miRNA biosensing are especially important and directly impact practical biosensor characteristics of sensitivity, TTR, and measurement reliability.

4. Thermodynamic constraints in miRNA biosensing

Excellent computational tools are now available for predicting free energy of hybridization between selective probes and RNA targets in solution phase (ΔG°)[88–90] with ability to account for ionic strength,[91,92] presence of locked nucleic acids (LNAs),[93,94] and secondary structure effects.[95,96] Thus, the hybridization extent of the homogeneous hybridization reaction can be well characterized through the equilibrium constant. Alternatively, given the additional parameters which affect the heterogeneous hybridization reaction present in BTs, such as probe–probe, probe–backfiller, and probe–surface interactions, the equilibrium constant can be significantly different.[97] Interestingly, the use of peptide nucleic acids (PNAs)[51] and locked nucleic acids (LNAs)[36–38] in miRNA sensing has become important for optimizing probe design as they lower duplex free energy (*i.e.* raise melting temperature, (T_m)). Such effects have shown the ability to significantly increase hybridization extent at a given temperature *versus* the use of native bases,[98] and thus, LNAs could provide means of enhancing sensitivity of miRNA BTs by increasing hybridization extent. Although a detailed discussion is beyond the scope of this review, an interested reader is referred to previous excellent discussions on nucleic acid analogs.[99,100]

Another important thermodynamic consideration in miRNA detection is the hybridization reaction driving force $\Delta T = (T_m - T)$, where T is the assay temperature and T_m is the melting temperature. Thus, when multiple different miRNA targets are analyzed simultaneously the inherent variance in T_m among different species given their sequence difference causes different relative driving force which creates bias that often requires further data normalization. Thus, considerations of variable T_m is required for meaningful interpretation and comparison of experimental results, especially if polymerase-based amplification steps are used. This can be in part addressed by use of native endogenous or spiked exogenous controls. We also note that since T also governs the reaction rate, assays with high T will have the advantage feature of reduced TTR. Thus, strategies for increasing T_m of probe–miRNA target pairs, as well as considerations of T_m variance within assay, are of importance to BTs and MTs alike.

5. Amplification-based molecular techniques (MTs) for miRNA assay and their highlights

Given the discussion of BTs for miRNA assay, we briefly summarize molecular techniques (MTs) for miRNA sensing to draw comparison between the alternative approaches. MTs fall into several categories, primarily including polymerase amplification, microarray, spectroscopy, sequencing, cloning, and Northern blotting. It should be noted that although sensor-based microarray platforms have been developed, they are primarily used in combination with a polymerase-based amplification step. Thus, here we classify microarray as a MT, although we acknowledge that it has been adapted to sensor-based application in other cases. Such methods-based approaches, referred to here as MTs, do not satisfy the definition of a biosensor because the transduction element and the DNA probe are not directly integrated. Thus, MTs differ widely from BTs in terms of protocol and detection principle which are summarized in Table 4. Various excellent reviews can be found on MTs for miRNA,[30,31,101,102] and therefore, we restrict our discussion here to a brief summary with the purpose of comparing with BTs for miRNA. The MT protocols share the following characteristics: (1) sample preparation, (2) amplification, and (3) data normalization. In Fig. 5, we compare the general assay steps used in two commonly used MTs, RT-PCR and microarray, with that of BTs. Note, both MT and BT miRNA assays involve multiple steps, but use of BTs can provide minimal associated sample preparation which is especially desirable for providing short TTR.

Sample preparation for removing extraneous species, especially proteins, is often essential for MTs, such as those which employ polymerase-based protocols. Preparation includes: isolation, pre-concentration, and labeling. Labeling, in particular, typically constitutes a major aspect of many MTs and is accomplished *via* a number of direct and indirect means. We note that a detailed discussion on labeling approaches is beyond the scope of this review, and many excellent discussions can be found elsewhere.[30,101,103] As shown by the MTs highlighted in Table 4, common labels include radioactive elements,[104] fluorophores,[104] and enzymes.[105] Ultimately, the need for isolation, pre-concentration, and labeling potentially increases loss of target, increases assay complexity, lengthens TTR, and alters the selectivity of binding. Thus, label-based approaches are undesirable and their absence in BT-based miRNA assay is an important advantage. It should also be noted

Table 4 Summary of molecular techniques (MTs) for miRNA detection. Abbreviations: reverse transcriptase polymerase chain reaction (RT-PCR), rolling circle amplification (RCA), RNA-primed array-based Klenow enzyme (RAKE), surface-enhanced Raman spectroscopy (SERS), resonance energy transfer (RET), fluorescence-RET (FRET), locked nucleic acid (LNA), advantages (A), disadvantages (D), and not available (N/A)

Molecular technique	Description	Ref.	LOD Range	Advantages, Disadvantages, & TTR[30,31]
Polymerase-based	Transcription-mediated amplification	120	fM–aM	A: – high sensitivity
	End point and looped RT-PCR	33		Quantitative
	Stem-loop probes	35, 121–123		Multiplexing
	MiRNA-specific hexamers	34		D: – enzyme-based
	Branched RCA	124		High cost
	RCA using padlock probes	125		Detailed data analysis
	RCA with capillary electrophoresis	126		TTR: ~1 day
	Isothermal ramification amplification	127		
	Encoded gel microparticle RCA	128		
	Dumbbell probe RCA	129		
	Primer-extension PCR	130		
Microarray	Label-free two-temperature hybridization	131	nM–pM	A: – high throughput
	T4 RNA ligase labeling	45		Simple working principle
	Polyadenylation	132		D: – low sensitivity
	RAKE assay	25		Semi-quantitative
	Reverse transcription-based labeling	32, 42, 44, 133		Long TTR
	Dual-channel arrays	45		TTR: >1 day
	CodeLink™ array platform	108		
	Quantum dot and nanogold label	41		
Spectroscopic	SERS	134	μM–fM	A: – vetted technology
	Modified invader assay FRET	135		D: – complex instrumentation
				TTR: ~1–5 h
	Single-molecule FRET	136		
	Duplex-binding p19 fusion protein	137		
	Fluorescence correlation spectroscopy	138		
	Signal-amplifying ribozymes	139		
Enzyme-based	Calorimetric-based	140	pM–fM	A: – rapid
	Chemiluminescence RET	141		Moderate sensitivity
	Bioluminescent enzyme	105		D: – potential inhibition effects
	Ligase-chain reaction	142		TTR: ~1 h
	Splinted ligation	143		
Cloning and sequencing based	Cloning and RAKE assay	39	N/A	A: – novel miRNA identification
	Pyrosequencing	144		D: – time-consuming data analysis
				TTR: N/A
Northern blot	LNA probes	36–38	nM–pM	A: – vetted methodology
				D: – Low sensitivity and TTR
				TTR: >1 day
In situ hybridization	Bioluminescent reporter gene and fluorescent molecular beacon	145	N/A	A: – yields localized miRNA expression
	Digoxigenin-labeled probes	146		D: – low sensitivity and TTR
	Ultramer-extension	147		TTR: ~1 day
	RNA oligonucleotide probes	148		
	Fluorescent metal nano-shell probe	149		
	Tyramide signal amplification using LNA probes	150		

that dependence on enzymes for amplification in MT-based miRNA assay can contribute to biased expression levels.[102]

The effects discussed above in part describe the need for normalization in MT protocols. Normalization has been accomplished by use of naturally endogenous or spiked species.[106] For example, spiked miRNA from *C. elegans* was used to normalize expression levels in lung cancer patients using nanopore miRNA biosensors.[65] We note that although normalization is not typically examined in biosensing, it is likely important for future biosensor-based assay given T_m variance among miRNA is an inherent thermodynamic challenge. However, we note that corrections would not be as large as for MTs which suffer the added variation due to both sample preparation and polymerase-based amplification.

6. Comparison of biosensor-based miRNA assay with conventional molecular techniques

Biosensor-based techniques for miRNA assay (BTs) and MTs can be compared in terms of desirable features listed in Table 5. The ordering of importance may vary with application, but in the final analysis, sensitivity, protocol design, TTR, and

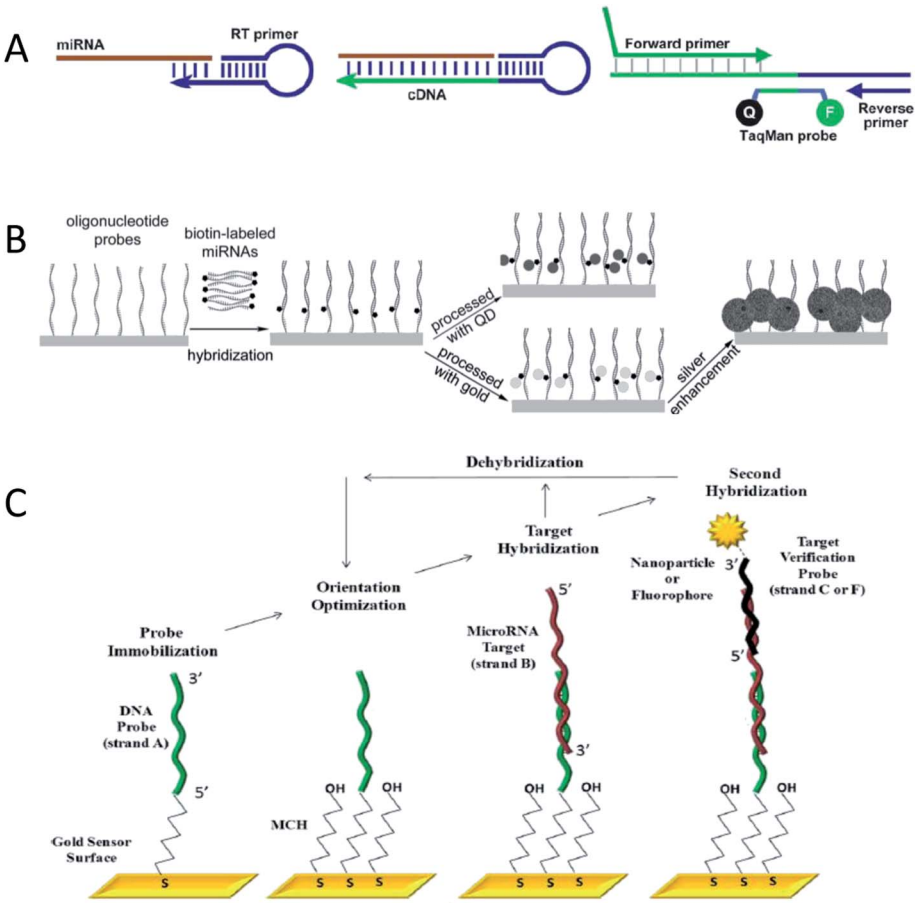

Fig. 5 Comparison of assay design and detection protocol between MTs and BTs. (A) Protocol for reverse transcriptase polymerase chain reaction (RT-PCR) MT reprinted with permission.[35] (B) Protocol for microarray MT reprinted with permission.[41] (C) Protocol for BT reprinted with permission.[48]

multiplexing potential are the most important ones in miRNA sensing.

6.1 Sensitivity comparison

Although both MTs and BTs contain significant LOD variation ranging from aM–nM levels (see Tables 1–4), it is most meaningful to compare performance of the highly sensitive techniques, as they are important towards practical diagnostics. For example, profiling of the relatively lowest expressed or down-regulated miRNAs in serum has great diagnostic significance, but such measurement would require BTs and MTs with sensitivity at ~fM levels and ability to measure in complex matrix of body fluid.[107] Importantly, as shown in Tables 1–3, BTs for miRNA have achieved such characteristics. We note that comparison between MTs and BTs is often not straightforward as the convention for reporting LOD is different; namely, absolute mass *versus* concentration, respectively. Comparison using absolute mass can sometimes be difficult if sample volume is not reported. For example, if LOD is reported as 1 attomole (10^{-18} mol), and the sample volume was ~1 μL (10^{-6} L), the concentration-based LOD is 1 pM, which would fall slightly outside the range of the most sensitive BTs reported in Tables 1–3. It is the opinion of the authors that concentration is a more meaningful parameter as it correlates with modeling of reaction kinetics, although absolute mass can also be a useful value in other applications.

6.2 Multiplexing potential

Some MTs, such as PCR and microarray, offer attractive multiplexing capabilities not yet demonstrated in biosensors. For example, microarray platforms have been designed to detect

Table 5 Desirable metrics used for comparison of miRNA BTs and MTs. Abbreviations: limit of detection (LOD), time-to-results (TTR)

1	High sensitivity (*i.e.* low LOD)
2	Minimal sample preparation
3	Rapid measurement (*i.e.* short TTR)
4	Selective biorecognition
5	Label-free protocol
6	High-throughput/multiplexing potential
7	Small amount of total RNA required
8	Quantitative results
9	Wide dynamic range
11	Simplicity in implementation/minimal assay steps
12	Low cost
13	Minimal target amplification
14	High mass transfer rates

thousands of different miRNA targets.[108] This represents an excellent model for next-generation miRNA biosensors since understanding the complex role of miRNA in disease and biological processes requires a multiplexed approach. As discussed previously, multiplexed biosensors have not been extensively investigated, but we believe that multiplexing capability can be facilitated with the current state of measurement technology.

6.3 Time-to-results

Sample preparation steps not only contribute to various disadvantages, such as reduced measurement repeatability and sensitivity, but they also increase time-to-results (TTR), cost, and complexity, and thus, should be avoided if possible. For example, as shown in Table 4, TTR associated with MTs is typically on the order of days.[30,31] On the other hand, average TTR for BTs is far less (~1–2 hours). We note that rapid response is an important distinguishing feature of BTs.

7. Future trends in biosensor-based miRNA assay

The evolution of miRNA biosensing will correspond with improvements in the desirable characteristics listed in Table 5; namely, towards sensors with short TTR, high sensitivity, and zero false results. We highlight a few aspects which require further research and development.

7.1 Integrating complementary transduction mechanisms

Integration of a complementary transduction reaction step in a single measurement could potentially reduce or eliminate false negatives. It will improve measurement reliability *via* dual readout signals and facilitate improved understanding of transduction mechanisms which could lead to design advances. For example, electrochemical and electromechanical transduction methods have been integrated in electrochemical-quartz crystal microbalance (E-QCM) which facilitates simultaneous electrochemical and mass sensing.[109]

7.2 Overcoming mass transfer limitations

The ongoing research into miRNA detection with nano-scale sensing elements drives the need for developing methods for overcoming mass transport limitation at small length scales. Examining the effects of flow, electric, and magnetic fields on the transport and sensor response time is likely to receive greater attention. New methods for actuating fluid motion at small length scales and techniques for incorporating them within existing sensing platforms will be an important area for further developments.

7.3 Reducing nonspecific binding effects

Although the need for sample preparation is avoided in miRNA BTs, nonspecific binding of background biologics still presents a significant measurement challenge. Thus, techniques that employ chemical, mechanical, or electrical methods which reduce nonspecific binding are of great interest for sensitive detection in practical samples. Research in this area is ongoing and has led to methods based on chemical[110] and mechanical[48] passivation for reducing nonspecific binding effects.

7.4 Improving reliability of measurement

Although enhancement of response is important as it improves LOD, the subsequent verification step provides a critical design advantage in the diagnostic settings where false negatives are highly undesirable. Interestingly, as shown in Tables 1–3, many investigators have examined enhancement and verification capabilities. These strategies have included: antibody binding steps to the DNA–RNA duplex,[71,76,111] secondary hybridization steps using fluorescently-[48] and nanoparticle-labeled strands,[48] and coupled redox reactions.[101]

8. Conclusions

Electrochemical, electromechanical, and optical miRNA biosensors offer sensitivities competitive with existing molecular techniques at attomolar to femtomolar levels. Comparison of biosensor-based techniques for miRNA assay (BT) and MT-based approaches reveals various advantages for BTs including enhanced reliability of measurement, lower time-to-results, and presence of label- and sample preparation-free protocols. Discussion of measurement format among miRNA biosensors was used to show that macro-scale biosensing platforms offer higher target capture rates relative to nano-scale designs. The most sensitive miRNA BTs have achieved LOD near attomolar levels in practical diagnostic conditions with ~ one hour time-to-results. Future research on integrating dual-mode measurement, overcoming mass transfer challenges, and improving measurement reliability was discussed.

Acknowledgements

The authors are grateful for the generous support of NSF Grant CBET-0828987 which provided the entire funding for the reported work.

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