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A Highly Sensitive and Selective Electrochemical Biosensor for Direct Detection of MicroRNAs in Serum

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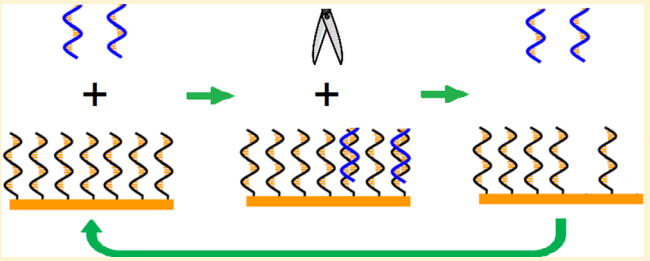
ABSTRACT: On the basis of hybridized target microRNA (miRNA) strand initiated cleavage of hybridized deoxyribonucleic acid (DNA) capture probes (CPs) by a duplex-specific nuclease (DSN), a highly sensitive and selective label-free miRNA biosensor is developed in this article. Briefly, thiolated DNA CPs are immobilized onto a gold electrode through self-assembly. The electrode is then hybridized to a sample solution containing the target miRNA. The hybridized CPs in the miRNA-CP duplexes are simultaneously cleaved by the DSN, releasing the target miRNA strands back to the sample solution. The released target miRNA strands again hybridize with the remaining CPs on the electrode, thus forming an isothermal amplification cycle. The distinct difference in electrochemical impedance between a control and the DSN cleaved biosensor allows label-free detection of miRNA down to femtomolar levels. The mismatch discrimination ability of the DSN permits miRNA expression to be profiled with high selectivity. The exceptional amplification power of the DSN along with the simple assay protocol makes direct miRNA expression profiling possible in real-world samples with minimal or no sample pretreatments. Attempts are made in direct profiling circulating miRNAs in serum and miRNAs in total RNA extracted from cancer cells.



MicroRNAs (miRNAs) are short nocoding RNAs found in animals and plants.[1] The first miRNA lin-4 was discovered in *C. elegans* in 1993 by Lee and co-workers.[2] Numerous studies have confirmed that miRNAs are a group of important post-transcriptional regulators that function through base-pairing to the 3′-untranslated regions of their target messenger RNAs (mRNAs), resulting in gene silencing via translational repression or target degradation.[3] It is estimated that over 60% of all protein encoding genes in humans are regulated by miRNAs through a combinatorial regulation mode; a single miRNA may regulate multiple mRNAs, and one mRNA may be targeted by multiple miRNAs.[4] Apart from their regulatory role in gene expression, increasing evidence has suggested that miRNA dysregulation is related to a large number of diseases and disorders. For instance, aberrant levels of miRNAs have been shown to be closely associated with pathogenesis of most human malignancies,[5,6] virus-induced disorders,[7] and neurodegeneration.[8] A global down-regulation of miRNA expression is an emerging feature in cancer, and specific dysregulations of certain miRNAs are seen in specific cancer types.[9,10] Also, miRNAs are currently being evaluated as potential biomarkers in cancer diagnosis, prognosis, and therapy.

With the fast growing number of miRNAs, over 2000 miRNAs have been identified in humans;[11] to meet the urgent need for miRNA expression analysis, the laborious and insensitive technologies used in miRNA study in the early days like Northern blotting[12] and molecular cloning[13] quickly yielded to more powerful and sensitive techniques such as microarrays[14,15] and quantitative polymerase-chain reaction (qPCR).[16,17] In a miRNA microarray, single-stranded oligonucleotide capture probes (CPs) are immobilized at predefined locations of the microarray. Hybridization of the CPs and target miRNAs is frequently measured by fluorescence. The miRNA microarray is the ideal tool for profiling miRNAs of physiological/pathological samples on a global scale because it offers the utmost multiplexing capability.[14] Although considerable improvements have recently been achieved through novel CP design and miRNA labeling procedures,[18] the complexity of miRNA labeling, low sensitivity, unrealistically lengthy hybridization time, challenges to standardization, and excessive variations between protocols largely restrict the use of the miRNA microarray in centralized laboratories.[19] To significantly improve sensitivity and shorten assay time, several qPCR procedures have been developed for miRNA expression profiling.[16] Quantitative PCR offers the highest sensitivity and covers the broadest dynamic range. Currently, qPCR is slowly becoming the popular choice in miRNA expression profiling.[16,17] Similar to the miRNA microarray, the use of qPCR for miRNA expression profiling is restricted to centralized laboratories due to the requirements for special laboratory skills in miRNA sample preparation and for the complex and costly instruments in signal readout. In addition, the short length of miRNA poses a significant limitation on the flexibility of primer design. Also, the thermal amplification in qPCR implies that qPCR only works with highly purified RNA

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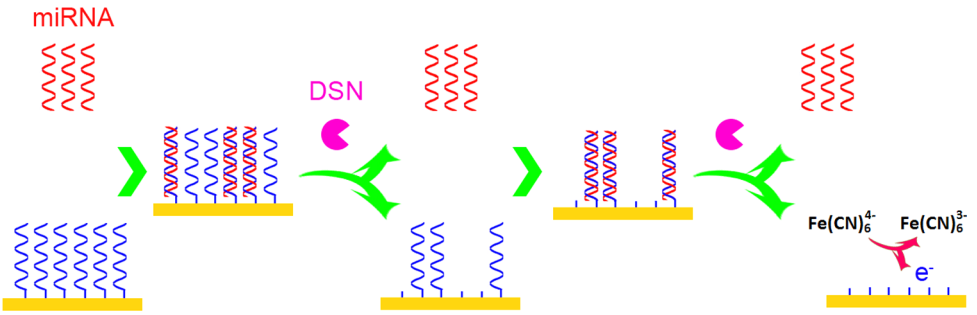

Figure 1. Schematic illustration of the working principle of the label-free electrochemical biosensor.

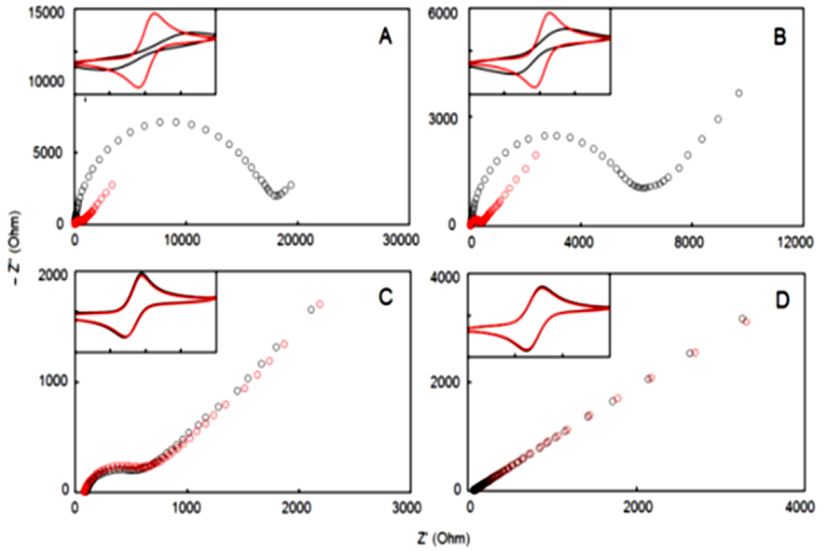

Figure 2. EIS spectra of a biosensor (red) and a control (black). 60-min hybridization/DSN incubation in 10 pM target miRNA (let-7b) sample solution containing 0.2 U of the DSN. EIS tests were conducted in 0.10 M Na_2SO_4 containing 5.0 mM of (A) $Fe(CN)_6^{3-/4-}$, (B) $W(CN)_8^{3-/4-}$, (C) $Co(phen)_3^{2+/3+}$ (phen = 1,10-phenothroline), and (D) $Ru(NH_3)_6^{2+/3+}$. The EIS spectra were collected at their formal potentials with a 5-mV sinusoidal voltage perturbation, respectively. Insets: Corresponding voltammograms of the biosensor (red) and control (black) in the respective EIS testing solutions.

samples as the polymerase is easily deactivated by thermally unstable coexisting materials such as proteins in pristine biological samples.

On the other hand, the development of label-free miRNA assays would substantially simplify the procedure and allow miRNAs to be profiled with high confidence, particularly for diagnostic purposes at point-of-care. Toward this goal, in recent years several label-free approaches, such as colorimetry,[20] silver nanorod arrays with surface-enhanced Raman spectroscopic detection,[21] stacking-hybridized universal tag-based[22] and structure-specific RNA-binding protein-assisted fluorescence microarray,[23] single-stranded DNA binding protein-assisted capillary electrophoresis,[24,25] and electrochemical biosensors,[26−30] have been proposed. Among them, the high portability and affordability of electrochemical biosensors could allow miRNAs expression to be profiled in a decentralized setting such as at point-of-care. So far, the lowest detection limit of 10 aM was obtained by leveraging on the high catalytic activity of a polymerized streptavidin-horseradish peroxidase (HRP) conjugate with up to 400 HRP moieties per conjugate.[30] However, the multistep procedure may impede its further development. In this article, a simple and yet ultrasensitive miRNA biosensor was described. Through incorporating a cumulative signal amplification mechanism by a duplex-specific nuclease (DSN), label-free detection of miRNAs down to 1.0 fM was realized through measuring the electrochemical impedance of the biosensor.

RESULTS AND DISCUSSION

Detection Scheme. Figure 1 depicts the working principle of the label-free electrochemical biosensor for miRNAs. A monolayer of thiolated deoxyribonucleic acid (DNA) CPs is self-assembled onto a gold electrode, acting as target miRNA capturing interface. To improve the quality and stability, defective sites of the monolayer are filled with thioglycolic acid. The DNA CPs form duplexes with target miRNA strands during hybridization. Concurrently, all hybridized CP strands (DNA-miRNA duplexes) are cleaved off the biosensor by the DSN, exposing part of the substrate electrode, and releasing the target miRNA strands are recycled (released back to the sample solution) for further hybridization, thus forming an isothermal signal amplification cycle through which one target miRNA strand cleaves thousands of the CP strands during hybridization/DSN incubation. Previous studies have demonstrated that the DSN selectively cleaves DNA−DNA and DNA−RNA duplexes with little activity toward single-stranded nucleic acids and RNA−RNA duplexes.[31] Finally, an electrochemical impedance spectroscopic (EIS) test is performed. The magnitude of the charge-transfer resistance (R_{ct}) of $K_3Fe(CN)_6/K_4Fe(CN)_6$ redox probes inversely reflects the number

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of DNA−RNA duplexes formed or the CP strands being cleaved off the biosensor during hybridization/DSN incubation and hence the concentration of the target miRNA.

Feasibility Study. As the removal of the CP strands from the biosensor surface is exclusively associated with the target miRNA concentration, unlike other EIS-based biosensors where the upper limit of R_{ct} is largely limited by the capacity of the EIS instrument,[32] the upper and lower limits of R_{ct} are set by the control biosensor (0% CP removal) and the biosensor after the CP strands are completely removed by the DSN, respectively. Therefore, the redox probes should be extremely sensitive to minute changes of the impeding power of the CP monolayer to detect traces of miRNAs. Ideal redox probes should produce a considerably large R_{ct} at the control and yet be highly sensitive to any hybridization related removal of the CP strands from the biosensor. In addition, the formal potential of the redox probes should fall between −0.5 and 0.5 V (vs Ag/AgCl) so as not to damage the CP monolayer during EIS tests.

To safeguard the performance of the biosensor, several redox probe pairs commonly used in EIS were first tested, and the results were summarized in Figure 2. As seen in Figure 2, cationic redox probe pairs showed negligible difference in R_{ct} between the biosensor and the control. Evidently, the anionic CP monolayer accumulates the cationic redox probes through strong electrostatic attraction. In turn, the accumulated redox probes in the CP monolayer form an electron relay network, readily allowing electron exchange between the redox probes in solution and the substrate electrode. Indeed, as demonstrated in Figure 2, instead of impeding the charge transfer process, the CP-redox probe network displayed little charge-transfer impeding power and in some cases even facilitate the charge-transfer process between solution species and the substrate electrode.[33] The EIS responses of the cationic probes were further confirmed by their voltammetric behavior under identical conditions. Practically identical voltammograms were obtained at the biosensor and the control (Figure 2, insets). On the contrary, due to the removal of the CP strands from the biosensor, significant changes in R_{ct} were observed when anionic redox probes were tested (Figure 2). Between the two pairs of the anionic redox probes, $K_3Fe(CN)_6/K_4Fe(CN)_6$ showed a much greater change in R_{ct} between the biosensor and the control. This is probably due to their moderate electron-transfer rate, as minute changes after hybridization are reflected by the changes in R_{ct}. Again, supporting evidence was found in the voltammetric tests of the respective systems under identical conditions (Figure 2, insets), reflected by the drastic changes in peak-to-peak potential separation (ΔE_p) as ΔE_p scales down with the decrease of R_{ct}. The EIS responses of the latter suggest that the CP monolayer effectively impedes the charge-transfer process between the anionic redox probes and the substrate electrode. Consequently, their impedance is largely controlled by the interfacial charge transfer across the CP monolayer, manifesting itself by the appearance of the semicircle and the diameter of which is R_{ct}. More importantly, a considerably large R_{ct} was observed at the control, which offers an excellent opportunity for the development of an ultrasensitive miRNA biosensor and provides the necessary prerequisite for a high signal/noise (S/N) ratio and a wide dynamic range. In this case, R_{ct} is determined by the intrinsic electron transfer rate of the redox probes in conjunction with the charge-transfer impeding power of the CP monolayer. The anionic redox probes must now penetrate the CP layer to reach the substrate electrode. The presence of a large amount of negative charges in the monolayer further suppresses the access of the anionic redox probes to the substrate electrode. From the above tests, it seemed that $K_3Fe(CN)_6/K_4Fe(CN)_6$ are the ideal redox probes for our biosensor. Besides the large R_{ct} produced at the control, their relative low redox potential of 0.15 V (vs Ag/AgCl), low cost, and good compatibility with the biosensor (aqueous system) are advantageous over other redox probes.

Optimization. To leverage on the amplification power of the DSN for sensitivity enhancement and to shorten the assay time, the working temperature of the DSN was fixed at its optimal temperature of 50 °C.[31] Thereafter, the simple procedure of the biosensor infers that only two experimental variables, the concentration of the DSN and the hybridization/DSN incubation time, need to be optimized. However, the synchronized hybridization and DSN incubation protocol implies that the response of the biosensor is interdependent on the miRNA and DSN concentration, making the selection of optimal concentration of the DSN and the determination of the dynamic range of the biosensor somewhat arbitrary. Nonetheless, our attention is focused on the detection of ultralow levels of miRNAs (femtomolar miRNAs or even lower). With this preset goal, the optimization of experimental conditions became much easier.

Because R_{ct} is the key parameter, for a successful adaptation of the DSN amplification strategy in developing an ultrasensitive miRNA biosensor, R_{ct} must be exclusively dependent on the target miRNA, preferably a linear dependence on the concentration of the target miRNA. To exploit the amplification power of the DSN for sensitivity improvement, the removal of the CP strands from the biosensor should be solely associated with the concentration of the target miRNA. In other words, the R_{ct} of the biosensor must be solely controlled by hybridization efficiency. This desired relationship can easily be realized with a high enough concentration of the DSN to ensure that the CP cleavage process by the DSN will never be the rate-determining step. Under this circumstance, R_{ct} of the biosensor would scale down with the miRNA concentration and its hybridization time until the CP strands on the biosensor are completely removed. The EIS response of the biosensor was therefore first examined with respect to the concentration of the DSN in which a let-7b biosensor was used to analyze 2.0 pM of a synthetic let-7b miRNA together with a control biosensor where the CPs are completely noncomplementary to let-7b miRNA. As shown in Figure 3, at both 0.05 and 0.2 U, the R_{ct} of the biosensor diminished with the proceeding of the DSN incubation, indicating the removal of charge-transfer impeding materials (CP strands) from the biosensor. At 0.05 U, the correlation between R_{ct} and the incubation time was largely nonlinear throughout the entire incubation period of 80 min. This is likely due to the complexity in the kinetics of the CP removal. The removal rate is probably jointly controlled by both hybridization and DSN cleavage. On the other hand, it was found that R_{ct} diminishes rapidly and essentially linearly at first with the proceeding of the DSN incubation at 0.2 U, but then slowly levels off until R_{ct} reaches its minimal level—the complete removal of the CP strands from the biosensor. Practically, the same responses were observable with higher concentrations of the DSN. To maximize the sensitivity, lower the cost, and have a reasonable turnaround time, it was found that a period of 60 min incubation with 0.2 U DSN is sufficient for the detection of

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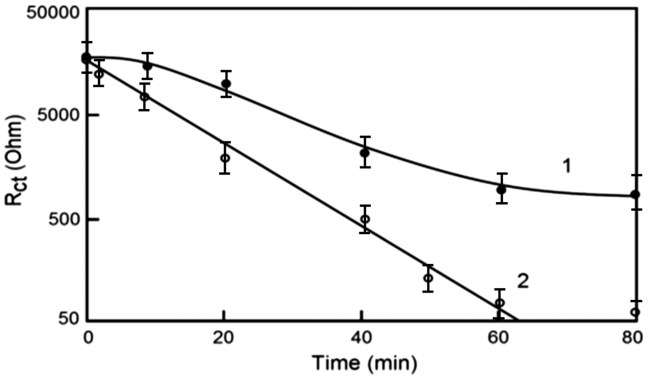

Figure 3. Effect of (1) 0.05 and (2) 0.20 U DSN on R_{ct} of a let-7b biosensor at different hybridization/DSN incubation times. 2.0 pM let-7b miRNA. Other conditions are as for Figure 2A.

miRNA down to femtomolar levels. Nonetheless, the cumulative nature of the DSN amplification strategy offered a highly flexible means to significantly improve the sensitivity of the biosensor at will.

Analytical Performance of the Biosensor. Apart from the dependence on the hybridization/DSN incubation time, R_{ct} is also closely linked to the target miRNA concentration. Because the removal of the CP strands is dictated by the hybridization efficiency in the presence of a sufficient amount of the DSN, a linear correlation between the concentration of miRNA and R_{ct} should exist. Furthermore, a sufficiently long period of hybridization/DSN incubation favors the detection of miRNA at considerably low levels. Indeed, as depicted in Figure 4, a linear relationship between R_{ct} and the concentration of the target miRNA was obtained between 2.0 fM and 2.0 pM with a detection limit of 1.0 fM at a S/N of 3.0. The standard

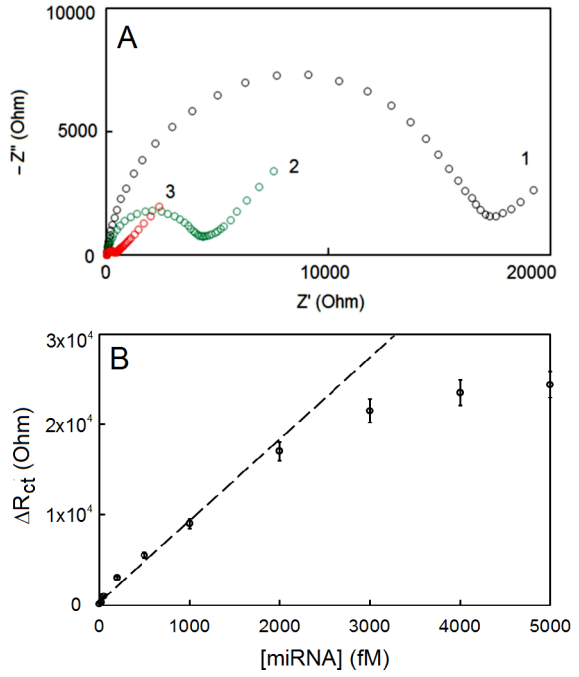

Figure 4. (A) EIS responses of a let-7b biosensor to (1) 2.0 fM, (2) 200 fM, and (3) 2.0 pM of let-7b miRNA, and (B) the corresponding calibration curve for let-7b. Experimental conditions are as for Figure 2A.

deviations of eight duplicated tests at 2.0 fM and 2.0 pM were found to be ~16% and ~10%, respectively.

The capability in discriminating closely related miRNAs in a miRNA family is crucial in miRNA expression profiling. In addition to its high efficiency in cleaving DNA–DNA and DNA–RNA duplexes, the DSN is capable of discriminating between complementary and mismatched short DNA–DNA and DNA–RNA duplexes.[31] Therefore, the miRNA biosensor provided an excellent avenue to evaluate the specificity of the DSN amplification strategy. Three representative members of let-7 family were selected to evaluate the specificity of the biosensor. As revealed in Figure 5, selectivity factors of 50 and

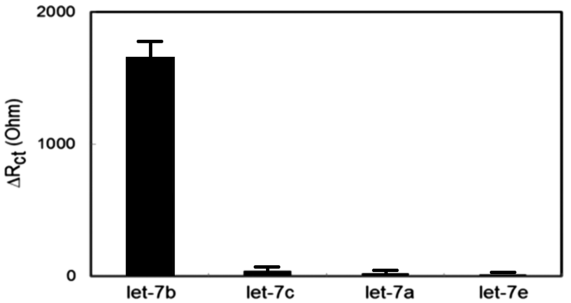

Figure 5. EIS responses of the let-7b biosensor to 100 fM let-7a, let-7b, let-7c, and let-7e, respectively. Experimental conditions are as for Figure 2A.

245 were obtained when let-7c and let-7a were tested by the let-7b biosensor, respectively. In other words, selectivity factors of 50 and 245 were obtained with one-base mismatched (let-7c) and two-base mismatched (let-7a) miRNAs, respectively. Moreover, negligible responses were observed when three (let-7e) or more (let-7g) bases were mismatched. This level of specificity is much better than those of previously reported miRNA biosensors,[26−30] due apparently to the additional mismatch discrimination ability brought to the system by the DSN.

Sample Analysis. In addition to its high sensitivity and selectivity, the simplicity of the one-step label-free approach makes the proposed biosensor particularly attractive for routine miRNA expression profiling in decentralized settings. Attempts were thus made in utilizing the biosensor for profiling circulating miRNAs in blood and miRNAs in total RNA samples extracted from blood and cancer cells. As shown in Table 1, the results obtained with the biosensor were in good

Table 1. Analysis of let-7 miRNAs in Total RNA Extracted from Hela Cells and Circulating miRNAs in Serum

sample	let-7a (10^6 copy/μg)	let-7b (10^6 copy/μg)	let-7c (10^6 copy/μg)
Hela cells	1.7 ± 0.22	2.7 ± 0.33	2.2 ± 0.34
	(2.1 ± 0.24)[a]	(2.9 ± 0.41)	(2.4 ± 0.27)
serum (extracted RNA)	2.2 ± 0.38	3.3 ± 0.42	2.0 ± 0.26
	(2.5 ± 0.30)	(3.5 ± 0.42)	(2.3 ± 0.28)
serum (direct analysis)[b]	1.6 ± 0.41[c]	2.2 ± 0.53[c]	1.2 ± 0.32[c]
	2.3 ± 0.26[d]	3.5 ± 0.39[d]	2.2 ± 0.28[d]

[a]Data in brackets were obtained by qPCR. [b]Data were converted to copy numbers of miRNA in equivalent RNA. [c]Data were obtained by calibration curve. [d]Data were obtained by standard addition.

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agreement with those obtained by qPCR and consistent with published data,[34] thus confirming the practical value of the biosensor. To date, all miRNA assays have to work with highly purified RNA samples. As compared to the PCR-based thermal amplification strategies, the isothermal amplification via the CP cleavage by the DSN together with its excellent selectivity is an additional advantage. The freedom from miRNA labeling and thermal amplification greatly improves the suitability in direct profiling miRNAs with minimal or no sample pretreatments. The proposed biosensor therefore offered a rare opportunity for direct profiling miRNAs in pristine biological samples such as serum and whole blood, following the great success of enzyme-linked immunosorbent assay (ELISA) for proteins.[35] As a proof-of-concept, let-7 miRNAs in serum was directly profiled by the biosensor. Acceptable results were obtained in the direct miRNA expression profiling in serum, although they were persistently 25−40% lower than those found in the extracted total RNA samples (Table 1). Undoubtedly, the high protein content in serum interferes with the detection of miRNA by fouling the biosensor through nonspecific adsorption, thus producing a false negative response. A possible solution to overcome the surface fouling problem is to analyze the serum sample by the standard addition method. As seen in Table 1, substantial improvements were obtained, and the results were now in good accordance with those obtained by qPCR. However, the low throughput of the standard addition method is a major drawback. Another possible solution is to coat the biosensor with a protein-resistant thin film such as polyethylene glycol-based materials for CP immobilization instead of direct self-assembly. The search for a suitable antifouling interface while maintaining most of the attractive features of the biosensor is currently underway in our laboratory.

CONCLUSION

In summary, in this article, we demonstrated a novel isothermal signal amplification strategy for the construction of a simple and label-free miRNA biosensor. In principle, the much simplified procedure suggests that the proposed miRNA biosensor can substantially be upscaled by adopting the complementary metal−oxide semiconductor technology for biosensor/array fabrication and by replacing the manual operation with a microfluidic cartridge. As compared to other miRNA assays, the proposed electrochemical biosensor exhibited several distinct advantages: (1) the simplicity of the one-step label-free detection; (2) isothermal amplification process; (3) excellent sensitivity, selectivity; and (4) the freedom from miRNA labeling and thermal amplification. It greatly improves the suitability in direct profiling miRNA expressions with minimal or no sample pretreatments. The proposed biosensor is therefore an attractive candidate for the development of a simple, robust, and ultrasensitive miRNA expression profiling platform for uses at point-of-care.

EXPERIMENTAL SECTION

Materials and Apparatus. Thiolated DNA CPs, synthetic miRNAs, and all other oligonucleotides of PCR purity were purchased from Proligo (Singapore). The DSN was supplied by Genomax Technologies Pte Ltd. (Singapore). All other reagents were purchased from Sigma-Aldrich and used without further purification. A pH 8.0 TMD buffer (50 mM Tris-HCl + 20 mM $MgCl_2$ + 1.0 mM DTT) was used as the hybridization/incubation medium. To minimize the effect of RNase on the stability of miRNAs, all glassware and centrifuge tubes were autoclaved, all solutions were treated with diethylpyrocarbonate, and all surfaces were decontaminated with RNaseZap (Ambion, TX). RNase-free ultrapure water was used throughout. Reference values of miRNA expression levels in the samples were obtained by using a commercial miRNA qRT-PCR kit from Agilent Technologies (Santa Clara, U.S.).

Electrochemical experiments were conducted with an IM6 electrochemical workstation (Zahner Elektrik, Germany). A three-electrode system, consisting of the biosensor, an Ag/AgCl reference electrode, and a platinum foil counter electrode, was used in all electrochemical measurements. EIS tests were performed in 0.10 M Na_2SO_4 containing 2.5 mM of $K_3Fe(CN)_6$ and 2.5 mM $K_4Fe(CN)_6$, over a frequency range from 1.0 MHz to 0.10 Hz with a 5-mV sinusoidal voltage perturbation and a bias potential of 0.17 V (vs Ag/AgCl). $K_3Fe(CN)_6/K_4Fe(CN)_6$ in the 0.10 M Na_2SO_4 serve as redox probes so as to perform EIS. All potentials reported in this work were referred to the Ag/AgCl reference electrode.

Biosensor Fabrication and MiRNA Detection. Before CP immobilization, a gold electrode was thoroughly polished with 0.05 μm alumina slurry and successively sonicated in water and ethanol for 10 min. The CPs were introduced to the cleaned gold electrode via self-assembly as follows: immediately before CP immobilization, the thiolated CPs were treated with 0.10 M DTT for 60 min and then purified using a NAP-10 column.[36] An aliquot of 5 μL of the DTT treated CPs was applied to the gold electrode and incubated in an environment chamber for 12 h at room temperature. After a thorough washing, the gold electrode was further incubated in 2.0 mg/mL thioglycolic acid for 2 h. The surface coverage of the immobilized CPs, estimated electrochemically using $Ru(NH_3)_6Cl_3$,[37] was found to be in the range of 6.9−10.1 pmol/cm^2. The hybridization of the target miRNA and its EIS detection were performed as follows: First, the biosensor was incubated in the TMD buffer containing the target miRNA for 60 min. Thereafter, the biosensor was rinsed thoroughly with a blank TMD buffer at the hybridization temperature. Finally, EIS tests were performed, and R_{ct} values were extracted from the EIS spectra by fitting the spectrum to the Randles equivalent circuit[38] using a complex nonlinear regression least-squares program (Zahner Elektrik, Germany).

Sample Analysis. MicroRNAs (total RNA) in cultured cancer cells were extracted using a RNA extraction kit from Qigen according to the recommended procedure, and miRNAs in the total RNA were enriched by using a cleanup kit (Qigen). Circulating miRNAs in serum were extracted using the miRNeasy RNA isolation kit from Qiagen. Briefly, 5 mL of Qiazol solution was added to 0.50 mL of serum. The mixture was thoroughly mixed and incubated for 10 min at room temperature. After 1.0 mL of chloroform was added, the mixture was vortexed for 60 s and centrifuged at 14 000 g for 15 min at 4 °C. Isolation and purification of miRNAs in the aqueous phase was achieved following the recommended procedure. Finally, miRNAs in the extracted total RNA samples were analyzed as described above. Reference values of miRNAs were obtained by qPCR (the gold standard). Direct profiling of circulating miRNAs in serum was conducted after spiking aliquots of concentrated TMD buffer to serum samples to have a final TMD buffer composition of 50 mM Tris-HCl, 5.0 mM $MgCl_2$, and 1.0 mM DTT.

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Notes

The authors declare no competing financial interest.

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