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A Label-Free Biosensor for Electrochemical Detection of Femtomolar MicroRNAs

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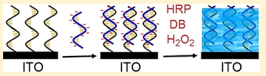
Supporting Information

ABSTRACT: A simple and ultrasensitive label-free microRNA (miRNA) biosensor, based on hybridized miRNA-templated deposition of an insulating polymer film and electrochemical impedance spectroscopic detection, is described in this report. The biosensor is made of a monolayer of charge-neutral morpholino capture probes on an indium tin oxide (ITO)-coated glass slide. Upon hybridization, the neutral surface of the biosensor is converted to anionic by the hybridized miRNA strands. The deposition of the insulating polymer film, poly(3,3′-dimethoxybenzidine) (PDB), is then carried out by the horseradish peroxidase-catalyzed polymerization of 3,3′-dimethoxybenzidine in the presence of H_2O_2. The cumulative nature of the PDB deposition process significantly enhances the sensitivity of the biosensor. Under optimized conditions, miRNA expression profiling can be performed label-freely from 5.0 fM to 2.0 pM with a detection limit of 2.0 fM. The biosensor is applied to the detection of circulating miRNAs in blood and miRNAs in total RNA extracted from cultured cells.



Ten years after the discovery of the first microRNA (miRNA) lin-4 in *C. elegans*, along with the identification of many more miRNAs, their physiological functions and possible implications to human health have started to attract great attention. As a group of noncoding small RNAs that target over 50% of human protein-coding genes, miRNAs are believed to be one of the important groups of post-transcriptional regulators involved in various physiological and developmental processes.[1,2] Increasing evidence has suggested that a large number of genetic diseases are associated with miRNAs dysregulation and miRNA expressions are closely associated with the pathogenesis of most human malignancies. For example, a global down-regulation of miRNA expression is an emerging feature in cancer and specific dysregulation of certain miRNAs is seen in specific cancer types.[3,4] Therefore, microRNA expression profiles can be used as biomarkers in cancer diagnosis, prognosis, and therapy. However, miRNA expression profiling is extremely demanding, because of the unique attributes of miRNAs, such as their short lengths, inherently different melting temperatures, and high similarities among members of a miRNA family. Northern blotting and cloning were first adopted to study miRNAs in the early days.[5,6] Both techniques have offered great help in ascertaining how miRNAs are spatially and temporally expressed. Unfortunately, laborious procedures and the lack of sensitivity of the two techniques prompted researchers to look for high throughput and more-sensitive alternatives.

As the number of miRNAs has grown significantly in the past five years, to meet the urgent need for multiplexing assays, an avenue that was explored is the development of miRNA microarrays.[7,8] Undeniably, microarrays have emerged to be an excellent choice for profiling miRNAs on a global scale, since they offer the highest multiplexing capability. However, because of the extremely short lengths, the conventional polymerase chain reaction (PCR) is ineffective in amplifying miRNAs and simultaneously labeling the amplicons. Various chemical labeling and biological ligation strategies have been exploited by using ^{33}P radioisotopes[7] and fluorophores[8] as miRNA labels. In addition to their multiple-step procedures, the suggested microgram-sample size and unrealistically lengthy hybridization time restrict miRNA microarrays in centralized laboratories.[7,8] To alleviate the limitations pertinent to miRNA microarrays, researchers turned their attention to quantitative PCR (qPCR). On top of improving sensitivity and specificity, other motivations are to reduce technical complexities, turnaround time, and the cost of miRNA assay. Shi and Chiang were the first to report the qPCR-based approach for miRNA analysis.[9] For successful PCR amplification, a target miRNA was first lengthened by ligating a poly(A) tail and then reverse-transcribed with a poly(T) adapter into cDNA. A miRNA-specific forward primer and reverse primer with sequence complementary to the poly(T) adapter were used for subsequent qPCR.[9] Later, two ligation-free miRNA lengthening approaches, namely, primer extension and the use of stem-looped primers were proposed.[10,11] The use of extended primers, instead of direct miRNA reverse transcription and PCR amplification, has the advantage that the synthesized cDNA is more suited for PCR amplification, thereby increasing the PCR efficiency and the number of amplified target. Moreover, the lengthened cDNA also offers better specificity. Currently, qPCR is slowly becoming the popular choice in miRNA expression profiling. Despite its excellent performance, the qPCR approach is yet to be adaptable for point-of-care use. The main obstacle lies in the requirements for special laboratory skills in miRNA sample preparation and for the complex and costly instruments in signal readout. In addition, as compared to microarray and other hybridization-based

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assays, qPCR is more prone to external variations, since handling imperfections can also be amplified.

Each of the above-mentioned techniques has advantages and disadvantages, and some, if not all, of the above-mentioned factors may influence the method of choice. Specifically, the requirement for labeled miRNAs can introduce limitations in terms of reagent cost and assay time; it may also introduce additional bias. Therefore, label-free technologies represent attractive alternatives for miRNAs analysis, enabling quantitative, multiplexed miRNA expression profiling without requiring additional assay reagents and with minimal sample pretreatment. Therefore, the development of label-free electrochemical miRNA assays is probably one of the promising ways to solve some of the problems encountered with miRNA analysis. The high portability and affordability of electrochemical instrument could allow miRNAs to be tested in a decentralized setting such as at the point-of-care. To date, several label-free miRNA biosensing platforms have been reported (seeTable S1 in the Supporting Information). For instance, based on guanine oxidation consequent to the hybrid formation between a target miRNA and its inosine substitute capture probe (CP), a straightforward label-free voltammetric approach was reported.[12] A detection limit of 5.0 nM (0.10 pmol in 20 μL) was observed. To further enhance the sensitivity, a gap hybridization coupled with an enzyme amplifier[13] and a triple-signal amplification scheme[14] were proposed for the construction of amperometric miRNA biosensors. In the former, the gap between capture and detection probe is filled in the presence of the target miRNA and the hybridized duplex, together with the amplifier, is greatly stabilized. A detection limit of 2 pM was obtained. In the latter, brought about by the synegetic action of gold nanoparticles, oligonucleotide probes, and horseradish peroxidase (HRP), the triple amplification produced a detection limit of 60 fM. However, the multistep procedure and complex amplification strategy may hinder its further development. More recently, Dong and co-workers described a label-free voltammetric biosensor for the detection of miRNAs.[15] A target miRA first hybridized with a stem-looped CP, unlocking the stem loop. A second hybridization was then carried out to the bottom segment of the unlocked CP in a solution containing silver nanocluster-tagged detection probes, bringing the detection probe together with the silver nanoclaster onto the biosensor, Electrocatalytic reduction of H_2O_2 by the silver nanocluster was utilized to detect miRNAs from 100 fM to 10 nM.

On the other hand, electrochemical impedance spectroscopy (EIS)-based detection is one of the promising candidates for the development of label-free miRNA biosensors. Besides the above-mentioned attractive features of electrochemical detection, EIS offers additional advantages, such as being largely nondestructive and highly sensitive to the presence of minute amounts of electron-transfer impeding material on the biosensor surface. In this report, we presented a simple and yet highly sensitive EIS biosensor for label-free detection of miRNAs. By engaging a cumulative signal generation process, amplified miRNA detection was realized through measuring the impedance of the biosensor after a sufficient period of incubation. Without labeling and the use of secondary amplifiers, the proposed biosensor may offer a viable alternative for miRNA analysis, where sensitivity, simplicity, and assay time have priority over throughput.

EXPERIMENTAL SECTION

Materials and Apparatus. Amine-terminated morpholino capture probes (MCPs) used in this work were custom-made by Gene Tools (Philomath, OR). Synthetic target miRNAs were from Integrated DNA Technologies (Coralville, IA). Sequence information of the MCPs and miRNAs used in this work is listed in Table S2 of the Supporting Information. HRP (200 units/mg) was purchased from Boehringer (Germany). All other chemicals and reagents were obtained from Sigma–Aldrich and used without further purification. Total RNA extraction kit was purchased from Invitrogen (Carlsbad, CA). Indium tin oxide (ITO)-coated glass slides were obtained from Delta Technologies (Stillwater, MN). A pH 8.5 10 mM Tris-HCl–1.0 mM EDTA–0.10 M NaCl (TE) buffer was used as the hybridization and washing buffer. To minimize the effect of RNases on the stability of RNA, all solutions were treated with diethyl pyrocarbonate, and all surfaces were decontaminated with RNaseZap (Ambion, TX).

All electrochemical measurements were performed on a Zennium electrochemical workstation (Zahner Elektrik, Germany). A conventional three-electrode system, consisting of a 2.0-mm ITO-based biosensor, a nonleak Ag/AgCl (3.0 M NaCl) reference electrode (Cypress Systems, Lawrence, KS), and a platinum foil counter electrode, was used in all electrochemical measurements. EIS experiments were conducted at −0.11 V ($E^{0\prime}$ of $Ru(NH_3)_6^{2+/3+}$) over a frequency range from 10 mHz to 1.0 MHz with a 5-mV sinusoidal voltage perturbation in 0.10 M sodium acetate containing 2.5 mM $Ru(NH_3)_6Cl_2$/2.5 mM $Ru(NH_3)_6Cl_3$. All electrochemical experiments were carried out at room temperature and all potentials in this report were referred to the Ag/AgCl reference electrode. UV–vis tests were performed with a UV-570 spectrophotometer (JASCO, Japan). An epifluorescence microscope (Carl Zeiss, Germany) was used in fluorescence microscopic experiments.

Biosensor Fabrication. After a thorough cleaning, an ITO-coated glass slide was silanized with aldehyde-functionalized silane according to a published procedure.[16] A patterned 1-mm-thick adhesive insulating pad (Sigma–Aldrich) was assembled on the already-silanized ITO slide to form an array of 20–50 2.0-mm-diameter ITO electrodes. Aliquots (10 μM) of the amine-terminated MCP in pH 6.0 0.10 M acetate buffer were dispensed onto the individual ITO electrodes and incubated for 4 h at room temperature in an controlled environment. The ITO electrodes were then thoroughly rinsed with 0.10% SDS and water. Free aldehyde moieties on the ITO electrodes were blocked by ethanolamine. Finally, the ITO electrodes were soaked in 2.5 mg/mL sodium borohydride in phosphate buffered saline (PBS, pH 7.4)/ethanol (3/1) to reduce imine groups. The electrodes were ready for use after a 2-min cleaning in vigorously stirred, copious amounts of hot water.

Hybridization and EIS Detection. As depicted in Figure 1, hybridization and EIS detection of a target miRNA were carried out in two steps. First, the biosensor was placed in a water bath maintained at 60 °C. Aliquots (5.0 μL) of the target miRNA standard solutions or total RNA samples dissolved in the TE buffer were applied to the biosensor. After 60 min, the hybridized biosensor was thoroughly rinsed with the TE buffer. Deposition of PDB onto the biosensor was carried out as follows: To a solution of 1.0 mM DB in pH 4.5 0.10 M acetate buffer, 5.0 μg/mL HRP and 5.0 mM H_2O_2 were added and the solution was thoroughly mixed. The hybridized biosensor was

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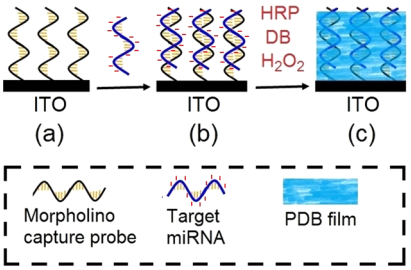

Figure 1. Schematic illustration of the working principle of the label-free miRNA biosensor.

then incubated in this PDB deposition cocktail for 30 min at room temperature. After a brief rinsing with water, EIS tests were performed in 0.10 M sodium acetate containing 2.5 mM $Ru(NH_3)_6Cl_2$/2.5 mM $Ru(NH_3)_6Cl_3$. Data points were reported as averages of six replicates. Synthetic miRNAs with sequence completely noncomplementary to the MCPs and target miRNA-depleted total RNA were used as samples in control experiments.

Isolation of MiRNA. Circulating miRNA in blood (serum) was extracted using the miRNeasy RNA isolation kit from Qiagen, according to the recommended procedure with minor modifications.[17] Briefly, 5 mL of Qiazol solution was added to 0.50 mL of serum. The mixture was vortexed briefly and incubated for 10 min at room temperature to dissociate nucleoprotein complexes. After adding 1.0 mL of chloroform, the mixture was vigorously vortexed for 60 s, followed by a centrifugation at 14 000 *g* for 15 min at 4 °C. Precipitation and purification of RNA in the aqueous phase (upper phase) was performed according to the recommended protocol. Total RNA in cultured cells was extracted by the Invitrogen extraction kit, following the recommended protocol. The yield and quality of total RNA were confirmed by UV−vis spectrophotometry.[18] Control RNA samples (let-7 miRNAs depleted total RNA) were prepared by removing let-7 miRNAs with magnetic beads coated with fully complementary capture probes. The miRNA and the control samples were stored in a −20 °C freezer.

RESULTS AND DISCUSSION

Feasibility Study. Figure 1 schematically illustrates the three principal steps in the operation of the biosensor: (a) a monolayer of charge neutral MCPs, acting as a bioaffinitive interface, is first immobilized on a silane-activated ITO electrode; (b) hybridization with a target miRNA brings the target miRNA, together with a high density of negative charges on the biosensor surface; and (c) strong interaction of the anionic miRNA strands with DB produces a high density of DB on the biosensor surface.[19] This high concentration of DB greatly facilitates DB polymerization and the deposition of PDB in the presence of HRP and H_2O_2. The reason for using MCPs other than conventional oligonucleotide CPs is 2-fold: on one hand, morpholino has higher affinity toward nucleic acids,[20] which can suppress nonspecific uptake of miRNA; and on the other hand, its neutral backbone can prevent undesired adsorption of DB molecules. To prove this concept, we must first confirm the successful immobilization of MCPs on the silane-activated ITO electrode, and more importantly, the capability of the immobilized MCPs in selective interaction (hybridization) with target miRNAs. Therefore, a mixture of an equal molar ratio of two fluorophore-tagged target miRNAs was tested at two biosensors where the MCPs are fully complementary to the two target miRNAs, respectively. Upon applying the miRNA mixture to the two biosensors, the two targets selectively hybridize to their corresponding MCPs and become fixed on the two biosensor surfaces, respectively. As shown in Figure 2, intensive fluorescence was observed at both

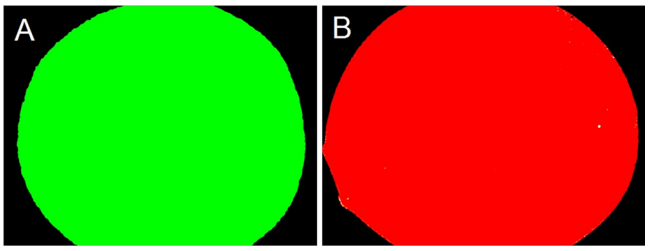

Figure 2. Fluorescence images of (A) 50 nM FAM-tagged and (B) 50 nM Cy3-tagged target miRNAs hybridized to their corresponding MCPs immobilized on ITO electrodes, respectively.

biosensors. And more significantly, the pure green (from FAM tags) and red (from Cy3 tags) fluorescence images unambiguously signified that only complementary target miRNA is able to hybridize to its corresponding MCPs, thus confirming the specificity of the hybridization between the surface-immobilized MCPs and target miRNA as any noticeable nonspecific uptake of the target miRNAs will seriously compromise the purity of the two colors. With this distinct fluorescence responses associated with the targets, additional experiments were performed to establish the relationship between the fluorescent signal and the target concentration. It was found that the MCP-coated ITO slide can be directly used to detect fluorophore-tagged miRNAs in the range of 0.20 to 50 nM with a detection limit of 0.10 nM, similar to that of most fluorescence-based nucleic acid assays.[21] This level of sensitivity is far below the requirement for miRNA detection and an in situ amplification mechanism is inevitable.

Subsequently, the control and one of the two hybridized biosensors were incubated in the PDB deposition cocktail for 30 min. Figure 3A shows typical EIS spectra of the control and the biosensor in 0.10 M acetate containing 2.5 mM $K_4Fe(CN)_6$/2.5 mM $K_3Fe(CN)_6$. As seen in Figure 3A (trace 1), at the hybridized biosensor, a semicircle was obtained in the entire frequency range from 1 MHz to 10 mHz, whereas the EIS spectrum of the control was made of a semicircle at the high-frequency side and a straight line with a phase angle of 45° at the low-frequency side (Figure 3A (trace 2)). The EIS response of the former suggests the presence of a thin insulating film on the biosensor surface, which significantly slows the electron transfer. Consequently, its impedance is controlled by the interfacial charge transfer across the insulating film, manifesting itself by the appearance of the semicircle and the diameter of which is the charge transfer resistance (R_{ct}).[22] Moreover, R_{ct} is the key parameter associated with the hybridization and PDB deposition processes and, hence, the concentration of the target miRNA in the sample solution. R_{ct} can be conveniently extracted from the EIS spectrum by either direct analysis of the spectrum or by fitting the spectrum to a Randle equivalent circuit (see Figure 3A (inset)).[22] The straight line with a 45° phase angle, known as Warburg impedance,[22] is closely associated with the diffusion of the redox probes in solution. The absence of Warburg impedance and the extremely large R_{ct} of the hybridized biosensor imply that a

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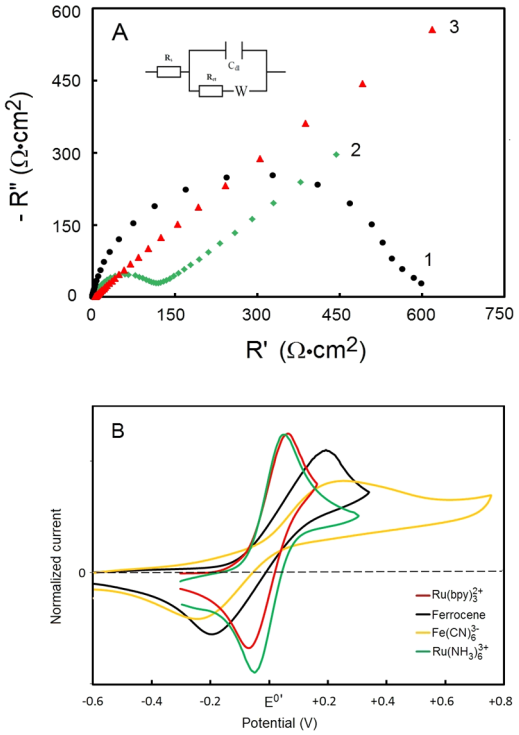

Figure 3. (A) EIS spectra of a biosensor (plot 1) and a control (plot 2 and 3) hybridized to 50 nM target miRNA. Thirty minutes (30 min) of incubation in 1.0 mM DB + 5.0 μg/mL HRP + 5.0 mM H_2O_2 in pH 4.5 0.10 M acetate buffer. EIS tests of spectra 1 and 2 were conducted in 0.10 M acetate containing 2.5 mM $K_4Fe(CN)_6$/2.5 mM $K_3Fe(CN)_6$, and spectrum 3 was conducted in 0.10 M acetate containing 2.5 mM $Ru(NH_3)_6Cl_2$/2.5 mM $Ru(NH_3)_6Cl_3$ at 0.15 and −0.11 V, respectively. For clarity, the EIS spectrum of the biosensor was scaled down 50 times. Inset shows the Randle equivalent circuit: the solution resistance (R_s), the double layer capacitance (C_{dl}), the charge-transfer resistance (R_{ct}), and the Warburg impedance (diffusion) element (W). (B) Normalized cyclic voltammograms of redox probes at the control biosensor. Potential scan rate = 100 mV/s, and all voltammograms of the redox probes were presented with respect to their formal potential ($E^{0\prime}$ = 0).

significantly large amount of charge transfer impeding material is brought to the hybridized biosensor. Unfortunately, a considerable magnitude of R_{ct} was also observed at the control. As we know, the R_{ct} of redox probes is determined by their inherent electron transfer rate and the presence of any charge-transfer-impeding material on the electrode that the redox probes must penetrate to reach the electrode surface,[23,24] The large R_{ct} (background) of the control dictates the sensitivity of the biosensor.

To safeguard a high signal-to-noise (S/N) ratio and high sensitivity, ideal redox probes should produce a negligibly small R_{ct} value at the control and yet be highly sensitive to any hybridization-related uptake of insulating material (PDB). To search for more-favorable redox probes, several well-known redox systems, including $Fe(CN)_6^{3-}$, ferrocene, $Ru(bpy)_3^{2+}$ (bpy = 2,2′-bipyridine), and $Ru(NH_3)_6^{3+}$, were tested at the control by cyclic voltammetry and the results were compiled in Figure 3B. As shown in Figure 3B, $Fe(CN)_6^{3-}$ exhibited the largest peak-to-peak potential separation (ΔE_p) of ∼450 mV whereas $Ru(NH_3)_6^{3+}$ produced the smallest ΔE_p of ∼90 mV. Although $Ru(bpy)_3^{2+}$ (ΔE_p = 110 mV) is not much worse than $Ru(NH_3)_6^{3+}$, $Ru(NH_3)_6^{3+}$ seems to be the best for our biosensor, because

(i) a minimal R_{ct} is expected at the control, since R_{ct} scales down with the decrease of ΔE_p,[23]
(ii) relative low redox potential of −0.11 V, and
(iii) good compatibility (high solubility in water) with the biosensor.

Indeed, EIS tests revealed that $Ru(NH_3)^{2+/3+}$ displays a negligible R_{ct} at the control (Figure 3A (trace 3)), which paves the way for the development of an ultrasensitive EIS biosensor. Therefore, all subsequently experiments were conducted with the $Ru(NH_3)_6^{2+/3+}$ redox probes. Besides incompatible with aqueous systems, ferrocene showed a surprisingly sluggish electron transfer process with a ΔE_p of ∼350 mV at the MCP-coated ITO electrode, although its inherent electron transfer rate is among the fastest.[23] Similar electrochemical behavior of ferrocene and its derivative at bare ITO electrodes has previously been observed.[25,26]

Optimization. The much-simplified assay procedure makes optimization of the biosensor a relatively easy task. Only two variables—hybridization time and PDB deposition time—need to be optimized. Nonetheless, for a successful adaptation of the EIS biosensor in ultrasensitive miRNA expression profiling, R_{ct} must be exclusively associated with the concentration of the target miRNA in a straightforward manner, preferably a simple linear relationship. The response of the biosensor was first examined with respect to the hybridization time. A let-7c biosensor was used to analyze the synthetic let-7c miRNA at 50 fM and 2.0 pM, respectively. When the biosensor was treated with the let-7c miRNA solutions, immediate increases in R_{ct} were observed (Figure 4A). As presented in Figure 4A, at 2.0 pM R_{ct} rose rapidly and almost linearly at first as the

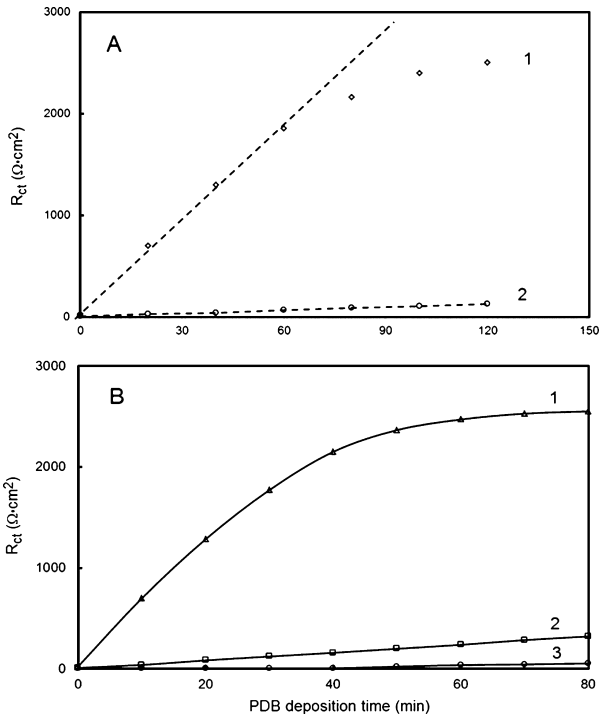

Figure 4. (A) Dependence of R_{ct} of 2.0 pM target miRNA (plot 1) and 50 fM target miRNA (plot 2) on hybridization time. PDB deposition time = 30 min. (B) Dependence of R_{ct} on PDB deposition time (2.0 pM target miRNA at the biosensor (plot 1), 100 fM target miRNA at the biosensor (plot 2), and (3) 2.0 pM target miRNA at the control (plot 3)); hybridization time = 60 min.

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hybridization proceeded, but then slowly leveled off until maximum hybridization was reached. More than 70% of the final response was achieved in the first 60 min of hybridization. In contrast, a practically linear correlation between the hybridization time and R_{ct} was observed at 50 fM throughout. According to the hybridization model on a solid surface,[27] the hybridization time needed to produce a measurable signal is inversely proportional to the target concentration.[27] A considerably long period of hybridization is necessary for the detection of ultralow levels of target. To maximize the sensitivity and to have a reasonable turnaround time, it was found that 60 min of hybridization is sufficient to detect miRNA down to femtomolar levels.

To leverage the cumulative nature of the PDB deposition process for sensitivity improvement, the formation of the insulating film must be solely controlled by the number of hybridized miRNA strands on the biosensor surface. This much-simplified relationship can only be realized with the highest possible concentrations of DB and H_2O_2, and a sufficient amount of HRP to ensure that all processes occurring in the cocktail are much faster than the PDB deposition process. However, the low solubility of DB limits the other two components in the PDB deposition cocktail. At its saturated concentration of ~1.0 mM, 5.0 mM H_2O_2 and 5.0 μg/mL HRP are found to be sufficient for the deposition of PDB. As shown in Figure 4B, similar to the effect of hybridization time, an immediate increase in R_{ct} was observed in all cases, because of the deposition of PDB. And, initially, R_{ct} was found to be proportional to the PDB deposition time. The deviations from linearity after prolonged incubation is due to the fact that the electron-transfer impeding power of the PDB film is enhanced to such an extent that further growth has significantly less impact on R_{ct}. Under extreme circumstances, when the incubation time is sufficiently long and the target concentration is sufficiently high, the R_{ct} of the biosensor may reach a steady state or hit the upper limit of EIS. Taking into consideration both the signal intensity and dynamic range, the optimal incubation time should be at the high end of the linear increase phase of the high target concentration. In practice, a PDB deposition time of 30 min was chosen to ensure sufficiently high sensitivity and good signal-to-noise (S/N) ratios. For comparison, a totally noncomplementary miRNA (control) at 2.0 pM was also analyzed at the let-7c biosensor. As illustrated in Figure 4B, negligible R_{ct} ($\leq$3 Ω cm^2) was observed within the first 30 min of incubation, suggesting that there is little uptake of the noncomplementary miRNA. However, R_{ct} of the control became visible after a much-prolonged period of incubation, probably due to the nonspecific deposition of PDB. For instance, the R_{ct} of the control increased to ~40 Ω cm^2 after 60 min of incubation, which significantly lifted the detection limit from femtomolar to picomolar levels.

Analytical Characteristics of the Biosensor. As demonstrated above, utilizing the hybridized target miRNA-templated deposition of PDB film as the signal generation and amplification strategy for EIS detection of miRNA, R_{ct} is primarily dependent on the amount of PDB deposited on the biosensor surface. A very attractive feature of this strategy is the cumulative nature of the PDB deposition process. Besides the dependence on the target miRNA concentration, R_{ct} is also closely associated with the incubation time in the PDB deposition cocktail. Significantly higher sensitivity can be conveniently realized if the deposition of PDB is allowed to proceed for a considerably longer period of time. Figure 5A shows representative R_{ct} values obtained from the biosensor treated with target miRNA standards of increasing concentrations. Under optimized experimental conditions, a linear relationship between R_{ct} and the target miRNA concentration was obtained between 5.0 fM and 2.0 pM with a detection limit of 2.0 fM at S/N = 3.0. At 2.0 pM, the standard derivation of 10 duplicated tests was found to be ~11% and ~18% was observed when 10 fM target miRNA was tested.

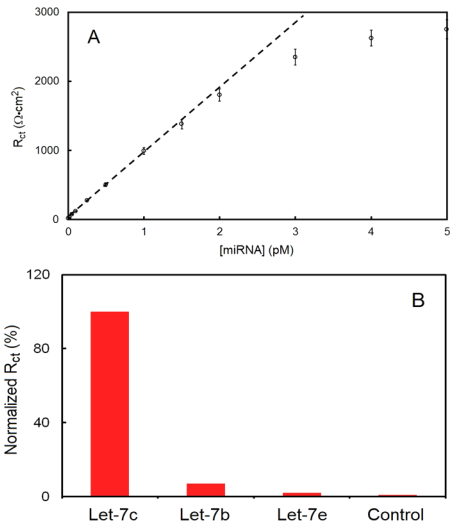

Figure 5. (A) The calibration curve for let-7c. (B) Normalized R_{ct} obtained at the control and the let-7c biosensor after hybridizing with 100 fM let-7c, let-7b, and let-7e. EIS conditions are as described in the Experimental Section.

The capability of the biosensor in discriminating members of a miRNA family was evaluated by analyzing three reprehensive members of the let-7 family, namely, fully complementary (let-7c), one-base-mismatched (let-7b), and two-base-mismatched (let-7e). As depicted in Figure 5B, the normalized R_{ct} (taking the R_{ct} obtained with let-7c miRNA as 100%) decreased to ~7% when let-7b was tested on the let-7c biosensor and a further decrease to ~2% was observed when let-7e was tested. In other words, selectivity factors of 14 and 50 were observed with one-base mismatched and two-base mismatched miRNAs, respectively. This level of selectivity between the complementary and mismatched sequences is better than those of many other hybridization-based biosensors,[28,29] probably because of the beneficial effect of the MCP on the hybridization selectivity.[20]

Apart from its high sensitivity, as compared to other electrochemical miRNA biosensors, the much-simplified label-free approach makes the proposed biosensor particularly attractive for routine miRNA analysis.[30,31] To date, all miRNA assays must work with highly purified RNA samples. The freedom from chemical/biological ligation and PCR amplification greatly improves the suitability in direct profiling miRNAs with minimal or no sample pretreatment. Attempts were thus made in utilizing the biosensor for analyzing circulating miRNAs in blood and miRNAs in total RNA extract from culture cells. The same sample (except serum) was simultaneously analyzed by the biosensor and by qPCR. As presented in Table 1, good agreements between the results obtained by the biosensor and qPCR on the same sample confirmed the practical value of the biosensor. In addition, the results obtained were also consistent with published data.[32,33] Because of its high sensitivity, the amount of RNA needed for a

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Table 1. Analysis of Circulating MiRNA in Serum and miRNA in Total RNA Extracted from Cultured Cells

sample	let-7a (× 10^6 copy/μg RNA)	let-7b (× 10^6 copy/μg RNA)	let-7c (× 10^6copy/μg RNA)
HeLa cells	7.4 ± 1.3 (8.2 ± 1.4)[a]	25 ± 3.6 (23 ± 3.4)	6.8 ± 0.92 (7.4 ± 0.89)
lung cancer cells	1.8 ± 0.25 (2.2 ± 0.25)	2.9 ± 0.39 (2.7 ± 0.42)	2.4 ± 0.38 (2.1 ± 0.29)
serum (extracted RNA)	2.7 ± 0.38 (2.9 ± 0.32)	3.8 ± 0.59 (3.6 ± 0.48)	2.0 ± 0.29 (2.3 ± 0.27)
serum (direct analysis)[b]	1.2 ± 0.46	1.9 ± 0.58	1.0 ± 0.50

[a]Data in brackets were obtained by qPCR. [b]Data were converted to copy numbers of miRNA in equivalent RNA.

successful miRNA detection was in the range of 3−10 ng. In addition, a direct analysis of let-7 miRNA in serum was attempted. Unfortunately, persistently lower values with much larger variations (30%−50%) were obtained. The results were even worse when RNA extraction efficiency is taken into consideration. Evidently, the high protein content and other blood constituents in serum severely interfere with the detection of miRNA, probably by fouling the biosensor through nonspecific adsorption. Efforts in developing an antifouling interface while maintaining most of the attractive features of the biosensor are underway.

At the present time, this work only serves as a proof-of-concept through rather labor-intensive manual operation. Nonetheless, as with other electronic/electrochemical biosensors, both low- and high-density sensor arrays can, in principle, be constructed by adopting the standard microfabrication technology and the complementary metal-oxide semiconductor technology that require no additional processes other than a simple switching mechanism. Two prominent examples demonstrating the power and capacity of the electronic/electrochemical detection systems are the electrochemical microarray developed by combimatrix[34] and the Ion Proton Sequencer recently developed by Life Technologies.[35] For example, up to 12 million individual sensors can be fabricated on a single chip in the Ion Proton Sequencer. Compared to other label-free electrochemical biosensors,[12−15] the much simplified procedure implies that the throughput can also be significantly upscaled by upgrading the manual operation to a microfluidic cartridge. Of course, similar to all other hybridization-based systems, great care must be taken when profiling multiple miRNAs sinutaneously, since there is very little room to fine-tune the hybridization conditions for all miRNAs.

CONCLUSION

In summary, in this report, we have demonstrated that hybridized target miRNA-templated deposition of PDB can be employed to devise a simple and ultrasensitive biosensor for label-free detection of miRNAs. Moreover, the amplification strategy developed in this work can be extended to other detection techniques, such as surface plasmon resonance and cantilevers. It is possible that this biosensor may be suited to profile miRNAs in completely untreated samples such as whole blood or serum. Without engaging tedious miRNA extraction and tagging procedures, the proposed biosensor is an attractive candidate for the development of a simple and robust miRNA expression profiling platform for point-of-care and field uses if the unpopularity of EIS biosensors can be relinquished. Such a tool may open a new paradigm in routine miRNA analysis. Encouraging progresses are being made, reflected by the successful commercialization of EIS-based systems—xCELLigence, by ACEA Biosciences, and SLA IA-2 Biosensor System, by Sharp Laboratories of America.

ASSOCIATED CONTENT

Supporting Information

This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Friedman, R. C.; Farh, K. K.; Burge, C. B.; Bartel, D. P. *Genome Res.* **2009**, *19*, 92−105.
(2) Bartel, D. P. *Cell* **2009**, *136*, 215−233.
(3) Volinia, S.; et al. *Proc. Natl Acad. Sci. U.S.A.* **2006**, *103*, 2257−2261.
(4) Lu, J.; et al. *Nature* **2005**, *435*, 834−838.
(5) Valoczi, A.; Hornyik, C.; Varga, N.; Burgyan, J.; Kauppinen, S.; Havelda, Z. *Nucleic Acids Res.* **2004**, *32*, e175.
(6) Takada, S.; et al. *Nucleic Acids Res.* **2006**, *34*, e115.
(7) Liu, C.-G.; Calin, G. A.; Meloon, B.; Gamliel, N.; Sevignani, C.; Ferracin, M.; Dumitru, C. D.; Shimizu, M.; Zupo, S.; Dono, M. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 9740−9744.
(8) Thomson, J. M.; Parker, J.; Perou, C. M.; Hammond, S. M. *Nat. Methods* **2004**, *1*, 47−53.
(9) Shi, R.; Chiang, V. L. *Biotechniques* **2005**, *39*, 519−525.
(10) Raymond, C. K.; Roberts, B. S.; Garrett-Engele, P.; Lim, L. P.; Johnson, J. M. *RNA* **2005**, *11*, 1737−1744.
(11) Chen, C. F.; Ridzon, D. A.; Broomer, A. J.; Zhou, Z.; Lee, D. H.; Nguyen, J. T.; Barbisin, M.; Xu, N. L.; Andersen, M. R.; Lao, K. Q.; Livak, K. J.; Guegler, K. J. *Nucleic Acids Res.* **2005**, *33*, e179.
(12) Lusi, E. A.; Passamano, M.; Guarascion, P.; Scarpa, A.; Schiavo, L. *Anal. Chem.* **2009**, *81*, 2819−2822.
(13) Poehlmann, C.; Sprinzl, M. *Anal. Chem.* **2010**, *82*, 4434−4440.
(14) Yin, H. S.; Zhou, Y. L.; Huang, H. X.; Meng, X. M.; Ai, S. Y. *Biosens. Bioelectron.* **2012**, *33*, 247−253.

(15) Dong, H. F.; Jin, S.; Ju, H. X.; Hao, K. H.; Xu, L. P.; Lu, H. T.; Zhang, X. J. *Anal. Chem.* **2012**, *84*, 8670−8674.
(16) Zheng, G.; Patolsky, F.; Cui, Y.; Wang, W.; Lieber, C. M. *Nat. Biotechnol.* **2005**, *23*, 1294−1301.
(17) Kroh, E. M.; Parkin, R. K.; Mitchell, P. S.; Tewari, M. *Methods* **2010**, *50*, 298−301.
(18) Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular Cloning, A Laboratory Manual*, 2nd ed.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 1989.
(19) Wang, J.; Rivas, G.; Luo, D.; Cai, X.; Valera, F. S.; Dontha, N. *Anal. Chem.* **1996**, *68*, 4365−4369.
(20) Shantanu, K.; Deepak, B. *Appl. Microbiol. Biotechnol.* **2006**, *71*, 575−586.
(21) Muyal, J. P.; Singh, S. K.; Fehrenbach, H. *Crit. Rev. Biotechnol.* **2008**, *28*, 239−251.
(22) Randles, J. E. B. *Discuss. Faraday Soc.* **1947**, *1*, 11−13.
(23) Gao, Z.; Bobacka, J.; Ivaska, A. *Electrochim. Acta* **1992**, *38*, 379−385.
(24) Sabatani, E.; Rubinstein, I. *J. Phys. Chem.* **1987**, *91*, 6663−6669.
(25) Popovich, N. D.; Wong, S. S.; Yen, B. K. H. *Anal. Chem.* **2002**, *74*, 3127−3133.
(26) Carter, C.; Brumbach, M.; Donley, C.; Hreha, R. D.; Marder, S. R.; Domercq, B.; Yoo, S. H.; Kippelen, B.; Armstrong, N. R. *J. Phys. Chem. B* **2006**, *110*, 25191−25202.
(27) Nair, P. R.; Alam, M. A. *Appl. Phys. Lett.* **2006**, *88*, 233120.
(28) Sassolas, A.; Leca-Bouvier, B. D.; Blum, L. J. *Chem. Rev.* **2008**, *108*, 109−139.
(29) Tansil, N. C.; Xie, H.; Xie., F. *Anal. Chem.* **2005**, *77*, 126−134.
(30) Gao, Z.; Yu, Y. H. *Biosens. Bioelectron.* **2007**, *22*, 933−949.
(31) Peng, Y.; Gao, Z. *Anal. Chem.* **2011**, *83*, 820−827.
(32) Allawi, H. T.; Dahlberg, J. E.; Olson, S.; Lund, E.; Olson, M.; Ma, W. P.; Takova, T.; Neri, B. P.; Lyamichev, V. I. *RNA* **2004**, *10*, 1153−1161.
(33) Nelson, P. T.; Baldwin, D. A.; Scearce, L. M.; Oberholtzer, J. C.; Tobias, J. W.; Mourelatos, Z. *Nat. Methods* **2004**, *2*, 155−161.
(34) Liu, R. H.; Nguyen, T.; Schwarzkopf, K.; Fuji, H. S.; Petrova, A.; Siuda, T.; Peyvan, K.; Bizak, M.; Danley, D.; McShea, A. *Anal. Chem.* **2006**, *78*, 1980−1986.
(35) Rothberg, J. M.; et al. *Nature* **2011**, *475*, 348−352.