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Direct, Electronic MicroRNA Detection for the Rapid Determination of Differential Expression Profiles**

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MicroRNAs (miRNAs) are an emerging class of diagnostic markers that can signify the presence of disease and be used to predict its course.[1,2] Indeed, miRNAs are now known to be involved in tumor metastasis,[3] stem-cell differentiation and renewal,[4] and viral replication.[5] The analysis of the intracellular levels of miRNAs is challenging, however, because their short lengths, low abundances, and high levels of sequence similarity present obstacles in the use of conventional analytical methods. Hybridization-based approaches (e.g. microarray analyses) are attractive for microRNA profiling because of the potential for extensive multiplexing and the discrimination of closely related sequences; however, such methodology requires large quantities (micrograms) of starting material.[6–8] The lack of sensitivity of existing array-based methods is related to the type of readout used: typically fluorescence signals emitted from an RNA-conjugated fluorophore. Very low levels of signal derived from low-abundance sequences are extremely difficult to detect without sophisticated optics. Impressive progress in this area has been made with the development of novel methods for the ultrasensitive detection of miRNA hybridization on array surfaces;[9,10] however, the methods available involve many steps and have not yet been validated with biological samples. We describe herein a new approach to ultrasensitive, direct, hybridization-based microRNA profiling using a multiplexed electronic chip and electrocatalytic readout. The very high sensitivity of this method enables the direct analysis of small samples (nanograms of total RNA) within 30 minutes. The power of this method is demonstrated by the identification of specific microRNA sequences that are overexpressed in human head and neck cancer cells relative to normal epithelial cells.

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We endeavored to develop a new method for microRNA profiling that would feature the convenience of array-based analysis, but would augment the power of such multiplexing with the exceptional sensitivity required to assay small biological samples for low-abundance microRNAs. We pursued an approach based on electronic readout and prepared a multiplexed chip that featured an electrode pattern generated by photolithography (Figure 1a). The chip was made by depositing a pattern of gold on the surface of a silicon wafer to provide a multiplexed set of leads and external contacts. A layer of SiO_2 was deposited on top of the gold to passivate the metal; then, in the final fabrication step, apertures of 500 nm in diameter were opened at the end of each lead to expose gold. To generate protruding microelectrodes, palladium was electrodeposited in the apertures (Figure 1b). The electrodeposition step can be engineered to produce highly nanostructured microelectrodes (NMEs; Figure 1b). Previous studies have indicated that nanostructured sensing elements

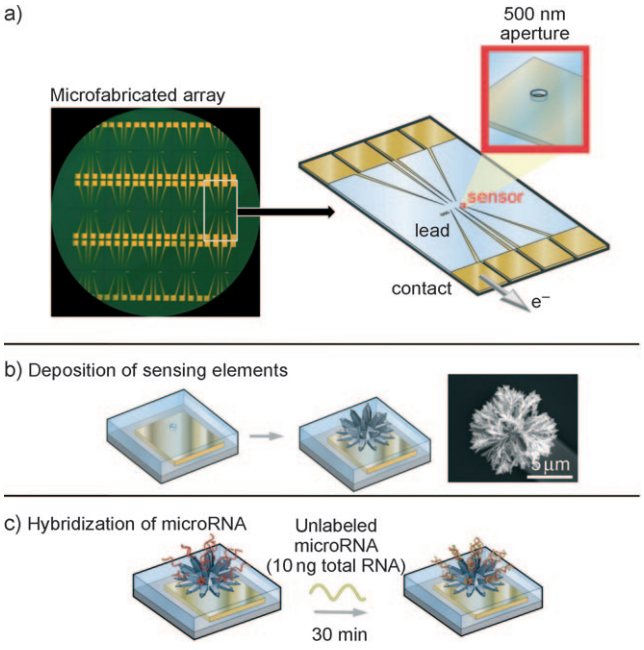

Figure 1. Electronic microRNA detection with nanostructured microelectrode (NME) chips. a) Photograph (left) showing microfabricated chips that feature 500 nm openings for the electrochemical deposition of NMEs, and illustration (right) of the chip structure. b) Schematic illustration of the generation of sensing elements by palladium electrodeposition. A scanning electron microscope image of a deposited nanostructured microelectrode is shown on the right. c) Hybridization of unlabeled microRNA in samples containing 10 ng of total RNA to a probe-modified chip. After 30 min, hybridization can be read out electrochemically by using an electrocatalytic Ru^{III}/Fe^{III} reporter system.

are more sensitive than bulk materials towards biomolecular analytes and that they facilitate surface-complexation reactions;[11,12] thus, the introduction of nanoroughness should be advantageous.

To test the electronic chip for sensitivity and specificity in microRNA detection, Pd NMEs modified with PNA probes were exposed to total RNA for hybridization (Figure 1c). Complexation was assayed with a redox reporter system[13–15] previously shown to exhibit femtomolar sensitivity when used in conjunction with nanostructured electrodes and PNA probes.[13] This reporter system relies on the accumulation of Ru^{III} when nucleic acids hybridize at an electrode surface. The signals observed with this reporter are amplified by the inclusion of ferricyanide, which can regenerate Ru^{III} chemically after its electrochemical reduction. Titrations of the microRNA sequence miR-21 showed detectable signal changes relative to noncomplementary control sequences when solutions containing as little as 10 aM of the target were exposed to the chip-based NMEs (Figure 2). This concentration corresponds to approximately 10 molecules per microliter of sample. The very high level of sensitivity is accompanied by a limited dynamic range of only 10^2; however, for the detection of microRNAs this tradeoff is merited given the low abundance of these sequences. A small dynamic range may necessitate multiple analyses of unknown samples at varied dilutions. Nonetheless, 5–10-fold changes in the concentration of sequences in the regime in which the sensor response is linear can be detected readily.

Two crucial additional sensing criteria are specifically required for microRNA detection. First, closely related sequences—different by as few as one base—must be accurately distinguished. Second, sequence appendages, such as those found in mature and precursor microRNAs, must be discriminated. We sought to challenge our system with each of these requirements. We investigated first the specificity of the assay for mature microRNA sequences by analyzing signal changes observed when the chip was exposed to solutions containing either the full-length, double-stranded, precursor form of miR-21, or the significantly shorter, single-stranded, mature miR-21 sequence. The signal for the hairpin precursor structure approached background levels, whereas a robust signal change was observed for mature miR-21 (Figure 3a, right).

We evaluated the sensitivity of the detection approach to point mutations by monitoring the response of probe-modified sensing elements to two closely related sequences, miR-26a and miR-26b. Probes complementary to each sequence were arrayed on the chip, and the responses to the complementary sequences were monitored (Figure 3a, left). The signal observed when miR-26a was introduced was approximately four times as high for the fully matched miR-26a probe as for the mismatched miR-26b probe; similarly, the signal observed when miR-26b was introduced was approximately 4.5 times as high for the fully matched miR-26b probe as for its mismatched counterpart. These results indicate that this multiplexed chip can successfully discriminate closely related microRNA sequences, but also that parallel interrogation of arrayed probes would be necessary to distinguish closely related sequences.

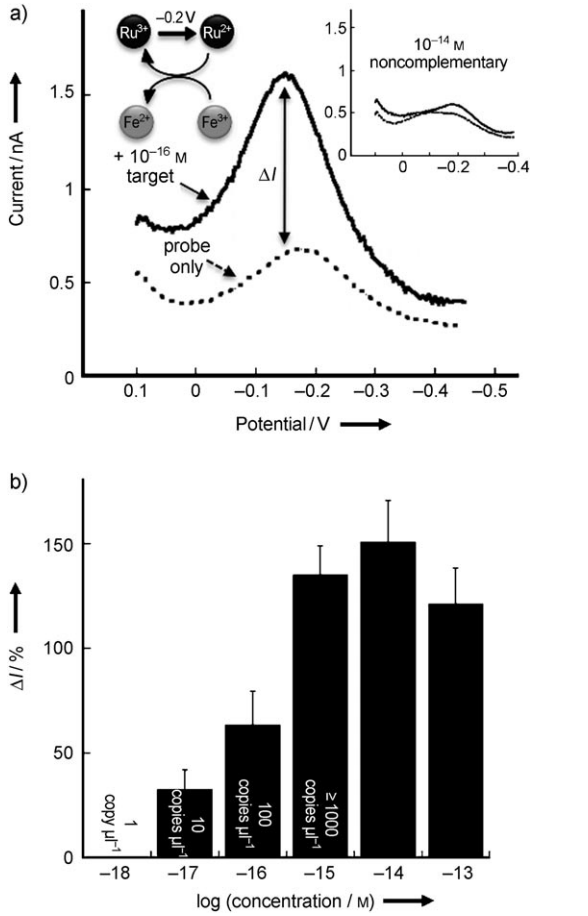

Figure 2. Electrocatalytic microRNA detection: readout and sensitivity. a) Differential pulse voltammetry (DPV) showing the signal increase observed after incubation of the complementary target (miR-21, 100 aM) with a probe-modified NME for 30 min. The signal observed with a noncomplementary target at a 100 times higher concentration is shown in the inset. b) Determination of the sensitivity and detection limit for electronic miR-21 detection.

The derivation of a "fingerprint" of microRNA expression from cell lines representing a particular tumor type relative to microRNA expression in normal cells has previously been shown to be a powerful approach for the identification of microRNAs that can serve as biomarkers of disease in patients.[16,17] Having confirmed the specificity and sensitivity of the chip towards microRNA targets, we then tested it by using RNA samples extracted from normal human cells and RNA samples derived from human head and neck squamous cancer cells grown in culture. For example, total RNA extracted from the human hypopharyngeal squamous cancer FaDu cell line and a normal oral epithelial cell line was titrated onto a nanostructured microelectrode displaying a probe complementary to miR-205 (Figure 3b). A positive signal was observed with as little as 5 ng of RNA derived from the FaDu cells, whereas no signal change was observed for normal epithelial cells with up to 20 ng of RNA. This result indicates that the signal response corresponds to a unique marker present at significantly higher levels in the cancer cell lines.

We profiled two different microRNAs, miR-21 and miR-205, in a panel of total RNA samples. We also monitored a

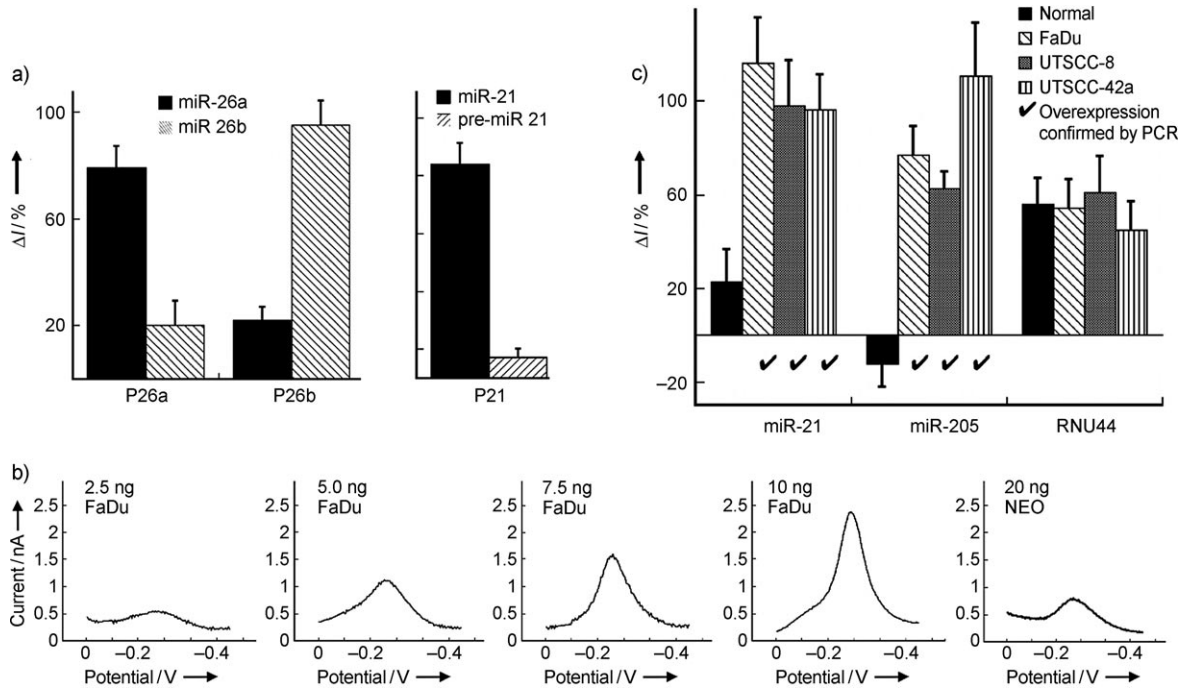

Figure 3. Validation of the chip-based miRNA-detection method and application to expression profiling. a) Left: The specificity of the assay toward small variations in the target sequence was analyzed by monitoring cross-hybridization of miR-26a (1 fM) and miR-26b to the corresponding probes P26a and P26b. Right: The discrimination of mature and precursor miR-21 was verified by the analysis of miR-21 (1 fM) and in vitro transcribed pre-miR-21 with the probe P21, which is specific for the mature microRNA. See Table S1 in the Supporting Information for all sequences used. b) Response of a chip-based miR-21 sensor to FaDu total RNA. An increase in the peak current was observed as the amount of total RNA was increased from 2.5 to 10 ng. No response was observed when normal oral epithelial (NEO) cellular RNA (up to 20 ng) was incubated with the sensor. c) Analysis of miR-21 and miR-205 expression levels in normal oral epithelial cells and three head and neck cancer cell lines (FaDu, UTSCC-8, and UTSCC-42a). RNU44 was used as the endogenous control owing to its stable expression across all these cell lines. A solution containing 1 ng μL^{-1} of total RNA was used for miR-21 and miR-205 analysis; for RNU44 detection, the concentration of total RNA was 0.5 ng μL^{-1}. These experiments were performed in a multiplexed format with arrayed probes analyzed in parallel on a single chip. The background signals have been subtracted to give the results shown. Error bars represent the standard error in data collected at more than five sensors.

control RNA molecule, RNU44. We used three different head and neck squamous cancer cell lines and compared the response of the microelectrode chip to these total RNA samples relative to that observed for RNA isolated from normal oral epithelial cells (Figure 3c). As expected, RNU44 levels remained constant in all four cell lines, as judged by the electrochemical response measured for each total RNA sample exposed to a sensing element modified with a complementary probe. However, miR-21 and miR-205 signals were both significantly elevated in the cancer cell lines. These microRNAs were judged to be present at more than 10^2-fold higher levels in the cancer cell lines than in the normal epithelial cells. The overexpression of these targets was confirmed by conventional quantitative PCR (see the Supporting Information). Both miR-21 and miR-205 have been observed previously to be elevated in primary human head and neck squamous carcinomas; thus, there is a significant potential for these micro-RNAs to serve as diagnostic biomarkers for this malignancy.[18,19]

In conclusion, the microRNA-detection chip described herein offers the sensitivity and specificity required for the analysis of these nucleic acid biomarkers, which are among the most challenging detection targets. The electronic readout of microRNA profiles offers a rapid, yet highly accurate, method to assay RNA samples directly for specific sequences, and the lack of labeling or amplification renders this approach extremely straightforward and efficient. These features are not attainable with other PCR or hybridization-based approaches.

Experimental Section

Materials: 6-Mercapto-1-hexanol (MCH, 97%), hexaamine ruthenium chloride (99.9+%), potassium ferricyanide (99%), and palladium(II) chloride (99.9+%) were purchased from Sigma–Aldrich Canada Ltd. (Oakville, ON); perchloric acid (70%), acetone (ACS grade), and isopropyl alcohol (IPA, ACS grade) were obtained from EMD (Gibbstown, NJ); thiolated PNA oligomers were obtained from Biosynthesis Inc. (Lewisville, TX; HPLC-purified grade). PNA probes had a Cys-Gly dipeptide at their N terminus; Gly acts as a spacer, whereas Cys provides a free thiol for immobilization on the electrode surface. Synthetic microRNAs (5′-end-phosphorylated and purified by HPLC) were obtained from Eurofins MWG Operon (Huntsville, AL). All PNA and RNA sequences are shown in Table S1 in the Supporting Information.

Chip fabrication: The chips were fabricated at the Canadian Photonics Fabrication Center. Three-inch silicon wafers were passivated with a thick layer of thermally grown silicon dioxide. A 350 nm gold layer was deposited on the chip by electron-beam-assisted gold evaporation. The gold film was patterned by standard photolithography and a lift-off process. A 500 nm layer of insulating silicon dioxide was deposited by chemical vapor deposition. Apertures of

500 nm in diameter were imprinted on the electrodes by standard photolithography, and 2×2 mm^2 bond pads were exposed by standard photolithography.

Fabrication of nanostructured microelectrodes: Chips were cleaned by rinsing sequentially with acetone, IPA, and deionized water for at least 30 s and dried with a flow of nitrogen for approximately 2 min. Electrodeposition was performed at room temperature with a Bioanalytical Systems Epsilon potentiostat by using a three-electrode system with an Ag/AgCl reference electrode and a platinum-wire auxiliary electrode. Apertures (500 nm in diameter) on the fabricated electrodes were used as the working electrode and were contacted by using the exposed bond pads. A 2 mm portion of the chip was immersed in the plating bath containing palladium(II) chloride (5 mM) and perchloric acid (0.5 M) and incubated for approximately 5 min prior to electroplating. Pd NMEs were fabricated by using DC potential amperometry at an applied potential of −100 mV for 6 s.

Modification of NMEs with PNA probes: Single-stranded thiolated PNA probes were dissolved in a buffer solution (pH 7) containing sodium phosphate (25 mM) and sodium chloride (25 mM). MCH (10 mM) was then added to a final MCH concentration of 100 nM. This PNA probe solution was then deposited on the chip in a dark humidity chamber overnight at 4 °C. The probe-modified Pd NMEs were rinsed thoroughly with the above buffer solution before measurements. For multiplexed experiments, chips with eight individually addressable leads were used.

Target hybridization: Hybridization solutions were solutions of the target at various concentrations in sodium phosphate buffer (pH 7.0, 25 mM) containing NaCl (25 mM). Pd NMEs were incubated with 10 μL of the hybridization solution in a humidity chamber at 37 °C for 30 min. The chip was then cooled and washed thoroughly with the buffer before electrochemical analysis.

Electrochemical measurements: An electrochemical analyzer (BASi, West Lafayette, IN) was used for electrochemical measurements in an aqueous solution containing $[Ru(NH_3)_6]^{3+}$ (10 μM), $[Fe(CN)_6]^{3-}$ (4 mM), sodium phosphate (pH 7.0, 25 mM), and NaCl (25 mM). Cyclic voltammetry (CV) was conducted before and after the addition of the solution of the target at a scan rate of 100 mV s^{-1}. Differential pulse voltammetry (DPV) was performed with a potential step of 5 mV, a pulse amplitude of 50 mV, a pulse width of 50 ms, and a pulse period of 100 ms. Cyclic voltammetry signals before and after hybridization were collected with a scan rate of 100 mV s^{-1}. The limiting reductive current (I) was quantified by subtracting the background current at 0 mV from the cathodic current at −300 mV. Signal changes corresponding to hybridization were calculated according to the following equation: $\Delta I = 100(I_{ds} - I_{ss})/I_{ss}$ (ss = before hybridization, ds = after hybridization). The detection limit was calculated by determining the first concentration at which the signal, after subtraction of the background signal (noncomplementary ΔI), was two times higher than the standard deviation of a noncomplementary control sample at a concentration of 10 fM.

RNA extraction for PCR analyses and amplification protocol: Total RNA was extracted from cell lines with the mirVana kit (Ambion). The quality of samples was assessed by reverse transcription–PCR analysis of the endogenous control RNU44 by using the Applied Biosystems TaqMan microRNA Assay. This assay includes a reverse-transcription (RT) step with the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, CA) in which a stem-loop RT primer hybridizes specifically to an miR molecule and is then reverse transcribed with a MultiScribe reverse transcriptase. The reverse-transcription mix included stem–loop RT primers (50 nM), 1 × RT buffer, deoxynucleoside triphosphates (0.25 mM each), MultiScribe reverse transcriptase (3.33 U μL^{-1}), and an RNase inhibitor (0.25 U μl^{-1}). The reaction mixture (7.5 μL) was incubated in an Applied Biosystems 7900 Thermocycler for 30 min at 16 °C, 30 min at 42 °C, and 5 min at 85 °C, and was then held at 4 °C. The RT products were subsequently amplified with sequence-specific primers (hsa-miR-21 primer 4373090 and hsa-miR-205 primer 4373093 from Applied Biosystems) by using the Applied Biosystems 7900HT Real-Time PCR system. The PCR mix (10 μL) contained the RT product (0.67 μL), 1 × TaqMan Universal PCR Master Mix, the TaqMan probe (0.2 μM), the forward primer (1.5 μM), and the reverse primer (0.7 μM). The reaction mixtures were incubated in a 384-well plate at 95 °C for 10 min, and then subjected to 40 cycles of treatment at 95 °C for 15 s and 60 °C for 1 min.

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