Chapter 9

Silicon Nanowire Biosensor for Ultrasensitive and Label-Free Direct Detection of miRNAs

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Abstract

MicroRNA (miRNA), a large and growing class of 18–24-nucleotide long, noncoding RNA molecules in all known animal and plant genomes, is a key player in gene regulation. The functions of miRNA are yet to be understood with respect to how and where it is produced and the changes within an organism associated with variations in miRNA expression level. The expression profiles serve as molecular diagnostics for diseases and new targets in drug discovery. Consequently, highly sensitive and selective detection of miRNA is playing a significant role in understanding miRNA functions. Existing major methods of detecting miRNA are dependent on hybridization, in which a target miRNA molecule is hybridized to a complementary probe molecule. Recently developed detection methods introduce nanomaterials to the hybridized duplex to enhance the sensitivity. However, all of them are indirect, involving labeling or conjugating process. To overcome the above-mentioned issues, we have demonstrated a highly sensitive and label-free direct detection method for miRNA by using peptide nucleic acids (PNAs)-functionalized silicon nanowires (SiNWs) biosensor. The sensor is capable of detecting target miRNA as low as 1 fM (10^{-15} M), as well as identifying fully matched versus mismatched miRNA sequences. More importantly, the SiNW biosensor enables miRNA detection in total RNA extracted from HeLa cells. The developed detection method shows potential applications in label-free, early detection of miRNA as a biomarker in cancer diagnostics with very high sensitivity and good specificity.

Key words: Silicon nanowire, Biosensor, Label-free detection, Hybridization, Peptide nucleic acid, MicroRNA

1. Introduction

MicroRNAs (miRNAs) are noncoding RNA molecules ranging in size from 18 to 24 nucleotides, which regulate gene expression in both plants and animals. They play an important role in developmental and cell biology, including stem cell differentiation and development. In addition, miRNAs are clinically important biomarkers for many cancer types because miRNA expression can be

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correlated with cancer type and stage. It is therefore critical to develop robust detection methods for miRNAs with high sensitivity, selectivity, as well as simplicity (1).

Owing to its small size, detection of miRNA is challenging. Many methods for detection of miRNA have been developed including northern blotting (2, 3), microarray (4, 5), polymerase chain reaction (PCR) (6), surface plasmon resonance (SPR) (7), as well as a variety of nanoparticles-based (8), conducting polymer nanowires-based (9), and bioluminescence-based techniques (10). Northern blot allows gene expression quantification and miRNA size determination. However, the low sensitivity and time-consuming laborious procedures of northern blot make it difficult for routine miRNA analysis (2, 3). Nucleic acid assays combine amplification by polymerase chain reaction (PCR) and detection using fluorophores as labels (4, 5). Microarray-based detection methods also require labeling to visualize the hybridization event (6). To improve detection sensitivity of miRNA, a SPR-based method is capable of detecting miRNA in total RNA samples down to femtomolar concentrations (7). Moreover, some conjugates like nanoparticles (8), enzymes (10) are introduced to the hybridized duplex, which enhances the signals. The sensitivity is thus improved by using these strategies. Very recently, a nanogapped microelectrode-based biosensor array has been fabricated for ultrasensitive electrical detection of miRNAs (9).

To date, all reported methodologies require labeling and conjugating steps and are thus time-consuming and indirect. Therefore, there is a demand for miRNA detection methods that are sensitive, direct, simple, rapid, and label-free for measurement of miRNA directly from a cellular extract. Direct and label-free electrical readout systems provide an extremely attractive sensing modality, which is widely applicable for miRNA detection. Silicon nanowires (SiNWs) are ultrasensitive biosensors that are capable of detecting nucleic acids (11–17) and proteins (18–20). In this chapter, we describe a new method of directly detecting miRNA excluding labeling and conjugating processes using peptide nucleic acids (PNAs)-functionalized silicon nanowires (SiNWs) biosensor with high sensitivity and good specificity. As PNA does not have an anionic phosphate backbone, the hybridization between PNA and miRNA eliminates repulsion, resulting in increased melting temperature and subsequently enhanced hybridization efficiency. The SiNW biosensors functionalized with PNA are capable of discriminating between single base differences, as in single nucleotide polymorphisms (SNPs). Furthermore, miRNA in total RNA extracted from cancer cell lines are detectable by the developed SiNW biosensors. Due to high surface-to-volume ratio that the nanowire dimensions confer, the sensitivity is greatly enhanced and the detection limit can be lowered to femtomolar concentrations.

2. Materials

2.1. Reagents

1. Silicon-on-insulator (SOI) wafers with 145 nm buried oxide layer are used.
2. Piranha solution (see Note 1).
3. Buffered hydrofluoric (HF) acid (see Note 2).
4. 3-aminopropyltriethoxysilane (99%, Sigma-Aldrich).
5. Glutaraldehyde (25% in H_2O, Sigma-Aldrich).
6. PNA probes are synthesized by Eurogentec (Herstal, Belgium).
7. MiRNAs are synthesized by 1st BASE Oligos (Singapore).
8. 20×SSC buffer (1st BASE Oligos, Singapore).
9. Trifluoroacetic acid (TFA, Sigma-Aldrich).
10. Diethyl pyrocarbonate (DEPC, 99%, Aldrich).
11. TRIzol reagent (Invitrogen, Carlsbad, CA).
12. YM-50 Montage spin column (Millipore Corp., Billerica, MA).
13. RNaseZap (Ambion, TX).

2.2. Equipment

1. Oxide growth (Furnace, SEMCO).
2. DUV lithography (Clean Track 8 and NIKKON Scanner, TEL).
3. Fin etch (RIE, Precision 5000 Mark II, Applied Materials).
4. Ion implant (E500 HP Implanter, VARIAN).
5. Implant activation (RTP Systems AST 3000, STEAG).
6. Metal deposition (Endura HP PVD, Applied Materials).
7. Metal etch (Centura Metal Etcher, Applied Materials).
8. Oxide deposition (Centura PECVD System, Applied Materials).
9. Dry oxide etch (Oxide Etcher, TEL).
10. Probe station (Alessi REL-6100, Cascade Microtech).

2.3. DEPC-Treatment of Solutions

1. Add 0.1% (v/v) (DEPC) to water/buffer solutions involved in the experiment (see Note 3).
2. Store them overnight.
3. DEPC must then be completely destroyed by autoclaving (see Note 4).

2.4. Preparation of 2% 3-Aminopropyltriethoxysilane Solution

1. Prepare 10 ml of ethanol/H_2O/3-aminopropyltriethoxysilane (98.5:0.5:1 (v/v)) by mixing 9.85 ml of ethanol, 50 µl of ultrapure H_2O, and 100 µl of 3-aminopropyltriethoxysilane (see Note 5).
2. Do not use the mixture if it turns turbid.

3. The freshly prepared solution is used immediately.

2.5. Preparation of Glutaraldehyde Solution

1. Prepare 10 ml of 2.5% glutaraldehyde by dissolving 1 ml of 25% glutaraldehyde water solution in 9 ml of D.I. water.
2. Use the solution immediately.

2.6. PNA Preparation

1. The PNA sequence is N-AACCACACAACCTACTAC-CTCA-C.
2. Dissolve in a calculated amount of D.I. water including 0.1% TFA based on the PNA amount provided in the certificate as stock (see Note 6).
3. Buffer preparation: dilute stock 20×SSC buffer to 1×SSC buffer, store at room temperature.
4. Dilute PNA to 10 μM in the 1×SSC buffer.
5. Store in aliquots at –20°C.

2.7. Preparation of miRNA Oligos

1. Let-7b is a complementary target, whose sequence is 5′-UGAGGUAGUAGGUUGUGUGGUU-3′.
2. Let-7c is a one-base mismatched target, whose sequence is 5′-UGAGGUAGUAGGUUGUAUGGUU-3′.
3. Control is a non-complementary target, whose sequence is 5′-AUGCAUGCAUGCAUGCAUGCAA-3′.
4. Dissolve in a calculated amount of D.I. water based on the miRNA amount provided in the certificate as stock.
5. Buffer preparation: dilute stock 20×SSC buffer to 0.01×SSC buffer, store at room temperature.
6. Dilute miRNA to various concentrations in the 0.01×SSC buffer.
7. Store in aliquots at –20°C.

2.8. Extraction of Total RNA from Hela Cells

1. Total RNA from human HeLa cells is extracted using TRIzol reagent according to the manufacturer's recommended protocol.
2. MiRNAs in the total RNA are enriched using an YM-50 Montage spin column.
3. RNA concentration is determined by UV-vis spectrophotometry.
4. Store in aliquots at –80°C.

3. Methods

SiNW sensors are typical FET-based devices, which contains source, drain, and gate electrodes. Figure 1 shows a schematic

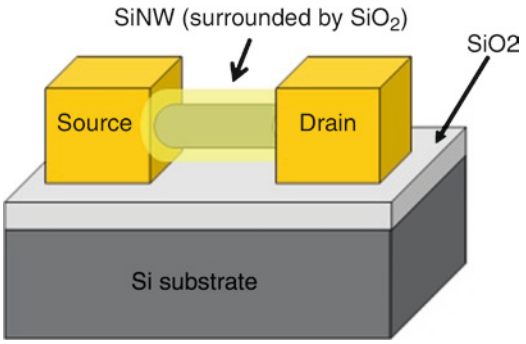

Fig. 1. Schematic of a single SiNW biosensor consisting of source (S) and drain (D) contacts and an individual SiNW surrounded by SiO_2 (reproduced from ref. (17) with permission from Elsevier Science).

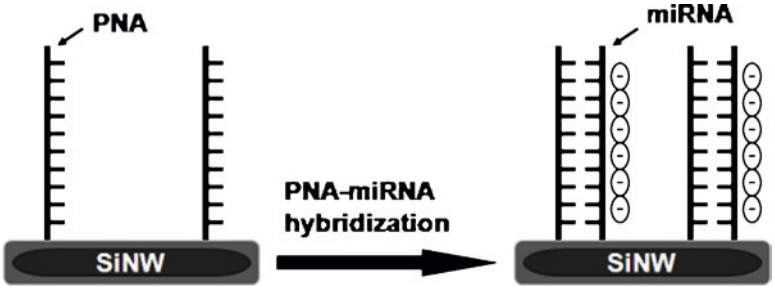

Fig. 2. Schematic illustration of the label-free direct hybridization assay developed for ultrasensitive detection of miRNA (reproduced from ref. (17) with permission from Elsevier Science).

diagram of a typical SiNW biosensor. The sensing mechanism by SiNW can be understood in terms of the change in charge density which induces a change in electric field at the SiNW surface. For example, binding of biomolecules with negative charge to the surface of *n*-type FET leads to an increase in device resistance.

Ultrasensitive, direct, and label-free detection for microRNA plays an important role. Figure 2 illustrates the working principle of the SiNW biosensor for detection of miRNA. PNA is covalently immobilized on the electrically addressable SiNW surface via conventional silane chemistry. Electrical biosensing by SiNW is based on change in resistance of the SiNWs due to depletion of charge carriers in its "bulk" when negatively charged miRNA originating from the phosphate groups on the miRNA backbone are bound to the PNA-functionalized surface via hybridization. When the targeted species, miRNAs complementary to the immobilized PNA, are present, resistance change occurs, whereas when they are noncomplementary, resistance change is minimal. As each wire is provided with independent metal contacts, resistance

can be individually measured. The miRNAs for hybridization were let-7b (complementary), let-7c (one-base mismatched), and control (noncomplementary).

3.1. Fabrication of Silicon Nanowire Device

1. The silicon wafers are doped with *n*-type (phosphorous) impurities using an ion implanter where implant dose is varied from 1×10^{13} to 1×10^{15} cm^{-2} and energy from 30 to 50 keV.
2. The dopants are then activated in rapid thermal annealing furnace and nanowire-fins are patterned using standard DUV lithography in the array format.
3. Silicon is etched in reactive ion etcher and resulting fins (60–80 nm wide) are oxidized in O_2 at 900°C for 2–6 h to realize nanowire array.
4. The two ends of the nanowires are further doped to obtain *n*+ regions, followed by connecting to contact metal and alloying to realize Ohmic contacts.
5. The device is passivated by silicon nitride film except for the active nanowire sensor area and metal pads.
6. Etching in nanowire area is carried out using a combination of dry and wet release.
7. Figure 3 shows an optical image of a SiNW sensor chip having two portions of SiNW arrays and 100 SiNWs in each portion. The individual SiNW has a dimension of ~50 nm in diameter and a length of 100 μm.

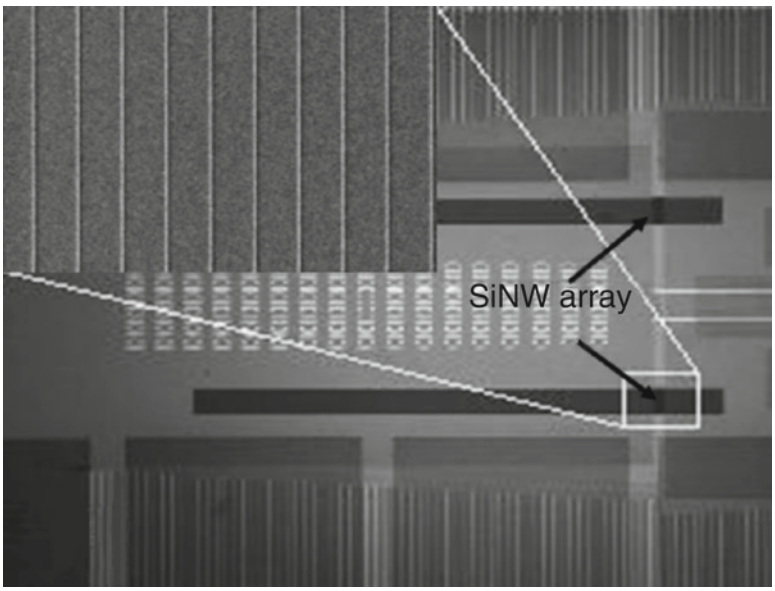

Fig. 3. Optical image of a SiNW sensor chip. Each chip consists of two portions of individually addressable SiNW arrays. Inset image shows high-resolution SEM image of ten nanowires in each portion. The individual SiNW has a dimension of ~50 nm in diameter and a length of 100 μm (reproduced from ref. (17) with permission from Elsevier Science).

3.2. Surface Functionalization of Silicon Nanowire

1. Immerse the chips in solution of 2% 3-aminopropyltriethoxysilane for 2 h.
2. Wash the chips with absolute ethanol for three times and blow-dry.
3. Immerse the chips in 2.5% glutaraldehyde in water for 1 h.
4. Wash the chips with water and blow-dry.
5. The chips are used immediately.

3.3. Immobilization of PNA on the Nanowire Surface

1. Apply 20 µl of 10 µM PNA in 1× SSC on the nanowire surface.
2. Place the chips in a humid atmosphere at room temperature overnight (see Note 7).
3. Wash the chips with the same buffer, rinse with water before measurement, and blow-dry.

3.4. Sequence Specificity of the PNA-Functionalized SiNW Biosensor

1. Apply the furnished acrylic wells to the NW region after carefully peeling off the cover of double-side tape (see Notes 8 and 9).
2. The chip surfaces are decontaminated by RNaseZap, followed by a wash with 70% ethanol (see Note 10).
3. 20 µl of 0.01×SSC buffer solution is added to the well as buffer medium.
4. The SiNW resistances are measured with the probe station (see Note 11).
5. Resistances are measured at 0.1 V.
6. Rinse the chip with water after measurement.
7. Apply 20 µl of 1 nM miRNA in 0.01×SSC on the surface.
8. Place the chips in a humid atmosphere at room temperature for 1 h.
9. Wash the chips with the same buffer, rinse with water, and blow-dry.
10. 20 µl of 0.01×SSC buffer solution is added to the well as buffer medium.
11. Resistances are measured at 0.1 V again.
12. The resistance change of SiNWs before and after PNA-miRNA hybridization is recorded.
13. Data are analyzed as an average of the responses from 15 measurements.
14. The high sequence specificity is demonstrated by hybridizing the PNA-functionalized SiNW sensor to the three different types of targets (let-7b, let-7c, and control, respectively). An example of the results of the sequence specificity produced is shown in Fig. 4.

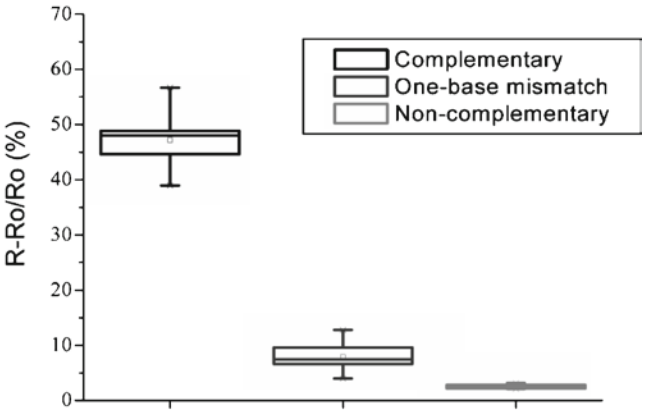

Fig. 4. Hybridization specificity demonstrated by response of the PNA-functionalized SiNW biosensors to fully complementary (let-7b), one-base mismatched (let-7c), and noncomplementary miRNA sequences. The specific resistance change was obtained from hybridization of let-7b to the SiNW device immobilized with PNA. As can be seen, a significant change (~47.2%) was observed when 1 nM let-7b was used, whereas only a negligible change was obtained when same concentration of noncomplementary miRNA was applied to the SiNW device. To evaluate the capability of the SiNW device for discrimination of single base mismatch in miRNAs, let-7c was tested at 1 nM. The increase in resistance for let-7c was ~7.9% which is much lower than that of let-7b. The high specificity suggests that the SiNW device allows for label-free discrimination between the fully matched and mismatched miRNAs, offering unique advantage over other technologies which require labeling and additional tags (reproduced from ref. (17) with permission from Elsevier Science).

3.5. Detection Response of the PNA-Functionalized SiNW Biosensor

1. Apply the acrylic wells to the sensor surface carefully after PNA immobilization.
2. The chip surfaces are decontaminated by RNaseZap, followed by a wash with 70% ethanol.
3. 20 µl of 0.01×SSC buffer solution is added to the well as buffer medium.
4. Resistances are measured at 0.1 V.
5. Rinse the chip with water after measurement.
6. Different concentrations of miRNA are applied to the PNA-functionalized SiNW sensor.
7. Various resistances are measured at 0.1 V.
8. The detection response of the PNA-functionalized SiNW biosensor is proportional to the concentrations of the complementary target miRNA. Examples of the response as a function of concentrations of miRNA are shown in Fig. 5.

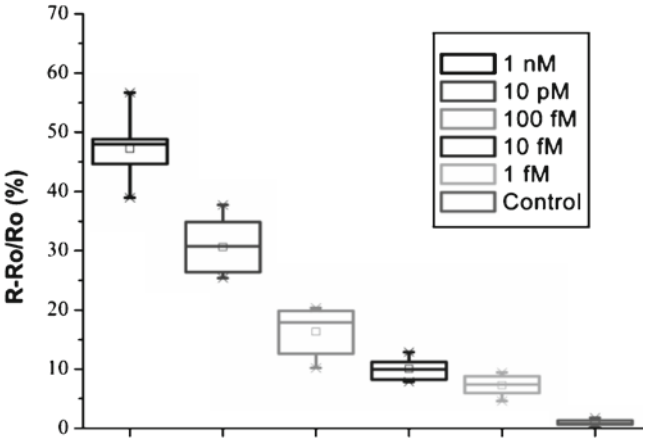

Fig. 5. Response of the PNA-functionalized SiNW biosensors to the complementary miRNA (let-7b) of varying concentrations. Different known concentrations of miRNA were tested with different groups of SiNWs. The resistance change before and after PNA-miRNA hybridization primarily depends on the amount of charge layer contributed by miRNA. The more the target miRNA molecules hybridized, the more negative charges accumulated on the SiNW surface, and thus the higher the resistance increase. As described above, an obvious resistance increase was obtained when 1 nM let-7b was hybridized to the PNA-functionalized SiNW device. It was observed that resistance change drops as a function of varying concentrations of let-7b. A 7.3% response was still observed while 1 fM let-7b was employed, which is distinguishable from the control signals. This indicates that ultralow concentrations of miRNA can effectively be detectable down to 1 fM with the device used in the work without labeling/tagging (reproduced from ref. (17) with permission from Elsevier Science).

3.6. Detection of miRNA in Hela Cells

1. Apply the acrylic wells to the sensor surface carefully after PNA immobilization.
2. The chip surfaces are decontaminated by RNaseZap, followed by a wash with 70% ethanol.
3. 20 µl of 0.01×SSC buffer solution is added to the well as buffer medium.
4. Resistances are measured at 0.1 V.
5. Rinse the chip with water after measurement.
6. Aliquots of the total RNA are diluted with 0.01×SSC and subsequently applied to a PNA-functionalized SiNW device for 1 h.
7. Resistances are measured at 0.1 V.
8. The results obtained with resistance change are normalized with respect to the total RNA.
9. The concentration of let-7b detectable in the total RNA extracted from Hela cells is determined.

4. Notes

1. Piranha solution is very dangerous, being both strongly acidic and a strong oxidizer. It must be handled extremely carefully.
2. HF is an extremely hazardous liquid and vapor and is highly corrosive to eyes and skin. Goggles and gloves must be used during operation.
3. Unless stated otherwise, all buffer solutions should be prepared in water.
4. All buffer solutions and water are treated with diethylpyrocarbonate by mixing 0.1% of diethylpyrocarbonate with the solutions and storing them overnight prior to autoclaving.
5. A glass petri dish must be used for this step. EtOH will dissolve plastic petri dishes and cause chips to adhere to the dish.
6. All the tips used in the experiment are sterilized prior to use.
7. Put a wet paper on the bottom of a Petri dish, place the SiNW chip on the top of the paper, and seal the dish with Parafilm.
8. Rectangular wells (inner size: ~0.8×0.4 cm^2) are cut by laser machine after double-side tape is fixed on the bottom of the acrylic plate. Ensure that wells are properly adhered to the surface of the NW chip to prevent any leakage of the solution.
9. Check for leakage with DI water after pasting the wells on the surface.
10. Treatment is conducted in a Class II Biohazard Safety Cabinet.
11. SiNW terminals: (1) Drain connected to voltage source; (2) Source connected to common ground (any one of the common pads); (3) Substrate connected to common ground.

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