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The fabrication, characterization and application of aptamer-functionalized Si-nanowire FET biosensors

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Abstract

An aptamer-functionalized silicon-nanowire (Si-NW) field effect transistor (FET) biosensor was successfully fabricated, characterized and applied to real-time electrical detection of binding with the target protein for biomedical applications. Surface modifications were carried out using 3-aminopropyl diethoxysilane and succinic anhydride to introduce amine and carboxyl groups onto Si substrates. Anti-thrombin aptamers with 5′-end amine groups were chemically grafted onto the surface-modified Si substrates through amide bond formation. Atomic force microscopic (AFM) analyses confirmed the successful immobilization of anti-thrombin aptamers on Si-NWs and their binding with thrombin samples. The anti-thrombin aptamers bound to Si-NWs through the linker appeared to have a mean height of approx. 4 nm and the thrombin/aptamer complex to have a mean height of approx. 8 nm. Fluorescence micrographs visualized the FITC-labeled thrombin after binding to anti-thrombin aptamers immobilized on Si-NWs. Furthermore, the anti-thrombin Si-NW FET biosensor was successfully applied to the real-time detection of electronic signals during and after binding with a thrombin sample at a concentration of approx. 330 pmol l^{-1} and the thrombin in blood samples.

(Some figures in this article are in colour only in the electronic version)

1. Introduction

Aptamers are oligonucleotides which can specifically bind to certain targets such as amino acids, drugs, proteins or other biomolecules. They are derived from a specific technique called systematic evolution of ligands by exponential enrichment (SELEX) [1–3]. Aptamers have many advantages such as stability at room temperature, no immuno-genicity or toxicity, versatile chemical modification of end groups, and so on. In addition, aptamers can be produced by a scalable chemical process with a low production cost and are clinically validated with a relatively short development period. They have not only better binding affinity and specificity to specific antigens with K_d values in a nanomolar range but also higher stability than antibodies [4]. Because of the advantages described above, aptamers have been widely investigated for various applications to diagnostics and therapeutics [5–7]. Aptamer-based electrochemical detection of platelet-derived growth factor in blood serum was reported to have a high specificity up to a picomolar range [6].

As is well known, a biosensor is an analytical device which converts a biological response into an electronic signal with a huge potential for many kinds of biomedical applications. Among various biosensors, silicon-nanowire (Si-NW) systems have attracted a lot of research interest because Si-NWs have very high surface-to-volume ratio and extreme sensitivity of the carrier mobility to variations in the electric field at their surface, making possible ultra-sensitive label-free detection of biomolecules [8]. In these semiconductors, the dopant type and concentration can be controlled precisely, which enables the sensitivity to be tuned in the absence of an external gate [9, 10]. The small size semiconductor NW can be used for sensitive, label-free, real-time detection of

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various chemical and biological molecules. For example, a PNA-functionalized Si-NW array was reported to detect DNA hybridization with a detection limit of 10 fmol [8] and biotin-modified Si-NWs to detect streptavidin down to at least a picomolar concentration range [10]. The term, aptasensor, stands for aptamer biosensor.

In this work, an anti-thrombin aptamer-functionalized p-type Si-NW field effect transistor (FET) was successfully fabricated on SiO_2/degenerated Si substrates, characterized by atomic force and fluorescence microscopy (AFM and FM) and applied to the detection of electronic signals during and after binding with thrombin samples. The Si substrate was surface-modified with 3-aminopropyl diethoxysilane (3-APDES) and succinic anhydride to introduce the following amine and carboxyl functional groups. The anti-thrombin aptamer specifically recognizes thrombin in human platelet-rich plasma and shows a dose-dependent inhibition of thrombin-induced platelet aggregation. The amine group was introduced to the end of the anti-thrombin aptamer with a sequence of 5′-NH_2-d(GGTTGGTGTGGTTGG)-3′. The aptamers could be successfully immobilized on an Si substrate through amide bond formation. After AFM and FM analyses of surface-immobilized anti-thrombin aptamers on Si substrates and their binding with thrombin, the anti-thrombin Si-NW FET aptasensor was applied for the real-time detection of current change at a constant bias voltage during and after binding with a thrombin sample at a concentration of approx. 330 pmol l^{-1} and the thrombin in blood samples.

2. Materials and methods

2.1. Materials

Fluorescein isothiocyanate (FITC), 3-aminopropyl diethoxysilane (3-APDES), succinic anhydride (SA), sodium carbonate (SC), phosphate buffered saline (PBS) tablet, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC), *N*-hydroxysulfosuccinimide (sulfo-NHS), gold colloid and alcian blue were purchased from Sigma-Aldrich Co. (St Louis, MO). Poly(chlorotrifluoroethane) (PCTFE) was purchased from SPL Life Science Co. (Pocheon, Korea) and the silicon wafer was from Silicon Materials Inc. (Pittsburgh, PA). Silane gas (SiH_4) was obtained from Takachiho Chemical Industrial Co. (Tokyo, Japan). Human α-thrombin was obtained from Enzyme Research Laboratories (South Bend, IN). All reagents were used without further purification.

2.2. Preparation of Si nanowires

P-type silicon nanowires (Si-NWs) were prepared as reported elsewhere [11–13]. Single-crystalline Si-NWs were grown by the Au-catalyst-assisted chemical vapor process. Au catalysts of nanometer scale were prepared by the dispersion of colloidal Au particles of 10–50 nm in diameter on SiO_2/Si(100) and subsequently loaded in a quartz tube furnace (KVTCVD-83, Korea Vacuum Technology, Korea). Then crystal growth was carried out under a constant flow of SiH_4 precursors at 490 °C. The prepared Si-NWs were stored at room temperature before use.

2.3. Surface modification of Si substrates

Surface modifications of Si wafers and Si-NWs were carried out according to the protocols of Gao *et al* [8] and Hong *et al* [14]. Si wafers (1 cm × 1 cm) were immersed in 2 wt% 3-APDES solution under an inert environment for 3 h. The surface-modified wafers were immersed in ethanol, ethanol/acetone (1:1 (v/v)) and acetone in a sequential manner. They were sonicated for 1 min at each washing step. Then, the aminosilylated Si wafer pieces were immersed in vials containing 1 wt% SA solution in 100 mM SC buffer to convert amine groups to carboxyl groups on Si wafers. Each vial was incubated at room temperature for 4 h. The Si wafer pieces were taken out from the solution, harshly washed and rinsed with enough deionized water. The carboxylated Si wafer pieces were dried in a vacuum chamber (30–40 mTorr). Si-NWs were also surface-modified using the same protocol.

2.4. Surface immobilization of anti-thrombin aptamer

An anti-thrombin DNA aptamer with an amine terminal group of 5′-NH_2-d(GGTTGGTGTGGTTGG)-3′ was synthesized using an oligonucleotide synthesizer (Mer Maid 12, Plano, TX) as reported elsewhere [15]. The anti-thrombin aptamer (0.138 μmol/vial; 3 vials) was dissolved in phosphate buffer (PB, 200 mmol, pH 8) to prepare the aptamer solution at a concentration of 20 μmol. The carboxylated substrates were immersed in PB (10 ml) solution and surface-activated by the addition of EDC (3.0 mg) and sulfo-NHS (1.7 mg). Then, the aptamer solution (500 μl) was added and incubated at room temperature. The resulting substrates were washed extensively with PB at pH = 8 and water in a sequential manner to remove and deactivate the remaining EDC and sulfo-NHS [16]. They were sonicated for 3 min at each washing step. Finally, the substrates were air-dried and stored in clean vials for several hours.

2.5. Atomic force microscopic analysis of anti-thrombin aptamer

Atomic force microscopic (AFM, Multimode 3500, Digital Instrument, NY) analysis was carried out in a tapping mode to confirm the surface immobilization of aptamers on the Si substrates. The size of aptamers on Si-NWs was measured from the AFM cross-sectional image. In addition, after incubation with thrombin and blood samples, the size of thrombin/aptamer complexes on the Si-NWs was also measured from AFM cross-sectional images. The average sizes of bare Si-NWs, the aptamer bound on Si-NWs and the aptamer/thrombin complex on Si-NWs were statistically analyzed using more than 10 samples.

2.6. Fluorescence microscopic analysis of the aptamer/thrombin complex

Anti-thrombin aptamers, immobilized on the surface of Si-NWs, were dispersed on a PCTFE surface and used for the analysis. The aptamer bound Si-NWs were wetted with PBS, where human whole blood (10 μl) was dropped. After that,

the blood was washed out with an excess amount of PBS solution and the anti-thrombin aptamer/thrombin complex was FITC labeled for the analysis with a fluorescence microscope (FM, Axioplan 2, Carl Zeiss, Germany). The FITC labeling was carried out in 100 mM SC buffer to maximize the FITC fluorescence [16]. As a control, Si-NWs dispersed on a PCTFE substrate were also treated with the same protocol and observed with an FM and an optical microscope (OM, DM2500P, Leica, Germany).

2.7. Fabrication of Si-NW field effect transistors

The p-type Si-NW field effect transistors (FETs) were fabricated on SiO_2/degenerated Si substrates. After defining the outer contact pads and interconnects by photolithography and Ni/Au (10/50 nm) evaporation, the ethanol solution containing Si-NWs was deposited on the oxide surface. Then, e-beam lithography, subsequent Ni/Au (30/150 nm) evaporation and lift-off techniques were employed to define electrodes on the Si-NWs. The channel length between the source and drain electrodes was approx. 1.5 μm. A nanometer pattern generation system (NPGS) equipped with a scanning electron microscope (SEM, Tescan, Czech Republic) was used for e-beam lithography [17].

2.8. Real-time electrical detection of thrombin

After covalent immobilization of anti-thrombin DNA aptamers on the Si-NW channels, we measured the real-time current of Si-NW FET functionalized with anti-thrombin DNA aptamers during the injections of thrombin and blood samples at a constant bias voltage (V_b). In order to minimize any environmental effect, we used a home-made Teflon electrochemical cell with a Pt probe tip as a reference electrode, which was designed to expose Si-NW FETs only to the injected samples [18]. All measurements were performed in ambient air at room temperature. First, acetate buffer (200 μl) as a control solution was dropped onto the aptamer-modified Si-NW FETs through the electrochemical cell while V_b was maintained at a value of 100 mV. We waited for about 30 min to settle the fluctuating conductance of FETs to a constant value after each injection of the buffer. To prepare thrombin samples, each 1 μl of thrombin solution (1.73 μmol l^{-1}) and 4 μl of blood sample was diluted in acetate buffer (pH 5.4, 250 μl), respectively. When the devices began to maintain a steady current, 10 μl of thrombin or blood sample was injected into the electrochemical cell containing the acetate buffer (200 μl). The resulting thrombin concentration was approx. 330 pmol l^{-1}. A control experiment using Si-NW FETs without the aptamers was also performed using the same protocol. The real-time electrical detection was carried out in triplicate.

3. Results and discussion

3.1. Surface immobilization of aptamers on Si substrates

Aptamers have been widely investigated for applications to diagnostics [6, 7]. The platelet-derived growth factor in blood serum was reported to be detected by aptasensors with a high sensitivity up to a picomolar range [7]. As a novel detection component, Si-NW systems have been used for ultra-sensitive label-free detection of biomolecules by monitoring the change in conductance or electrical properties [8]. In this work, an anti-thrombin aptamer-functionalized Si-NW FET was successfully fabricated, characterized and applied for the detection of electronic signals during and after binding with thrombin samples. Figure 1 shows an optical microscope image of aptamer-functionalized Si-NWs between Au electrodes in the FET biosensor. The magnified image in the circle shows the sequence of anti-thrombin aptamers immobilized on the Si-NW FET through amide bond formation. The aminosilylation on the Si surface was carried out using 3-APDES. Then, succinic anhydride was used as a spacer for the following carboxylation of amine groups on the surface. Anti-thrombin DNA aptamers were successfully immobilized on Si substrates through amide bond formation between amine groups ($-NH_2$) of the aptamers and activated carboxyl groups (–COOH) with EDC and sulfo-NHS on the surface. After immobilization of anti-thrombin aptamers, Si substrates were deactivated completely to exclude the possible conjugation with proteins in the sample solution.

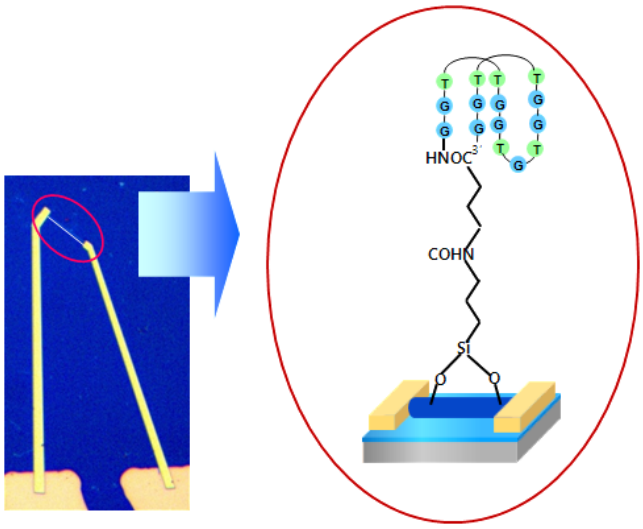


Figure 1. An optical microscope image of anti-thrombin DNA aptamer-functionalized silicon nanowires (Si-NWs) between Au electrodes in the field effect transistor (FET) biosensor with a magnified schematic representation for the Si-NW FET aptasensor.

3.2. AFM imaging of aptamers immobilized on Si substrates

Before Si-NW tests with a cylindrical morphology, an Si wafer was chemically modified for aptamer immobilization on the flat surface and analyzed by AFM to visualize the surface modification with the aptamer. Figure 2 shows 3D AFM height images before and after the anti-thrombin DNA aptamer binding to the Si wafer. In contrast to figure 2(A), part (B) shows many small peaks on the Si wafer confirming the successful immobilization of anti-thrombin DNA aptamers on the Si wafer. Aptamers appeared to be bound uniformly throughout the surface of the Si wafer. Based on the results

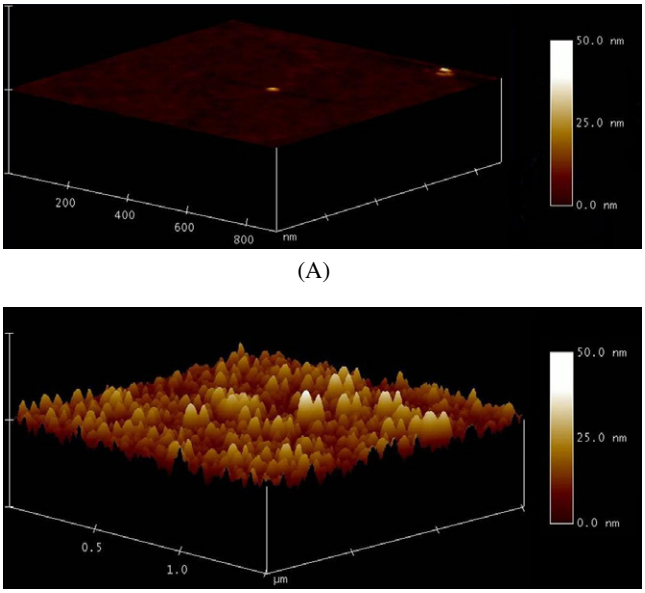


Figure 2. 3D atomic force microscopic (AFM) height images of Si wafer (A) before and (B) after surface immobilization of anti-thrombin DNA aptamers.

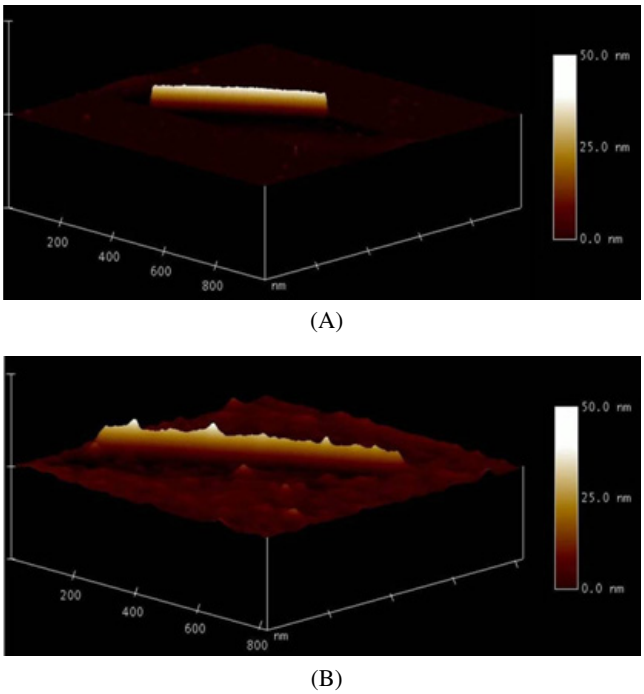


Figure 3. 3D atomic force microscopic (AFM) height images of Si-NW (A) before and (B) after surface immobilization of anti-thrombin DNA aptamers.

with the Si wafer, the surface immobilization of aptamers on Si-NWs was also carried out and analyzed by 3D AFM as shown in figure 3. Figure 3(A) shows a 3D AFM height image of bare Si-NWs and figure 3(B) shows that of aptamers bound to Si-NWs being immobilized at a certain interval. Anti-thrombin aptamers were thought to be bound all around the Si-NWs with a cylindrical morphology, which resulted in different heights in the 3D AFM image (figure 3(B)).

3.3. AFM and FM analyses of anti-thrombin aptamer/thrombin complex

In order to confirm more clearly that what were bound to Si substrates were anti-thrombin DNA aptamers, we assessed the binding capability of immobilized anti-thrombin aptamers with thrombin samples. As is well known, the anti-thrombin DNA aptamers can bind to the target protein of thrombin in blood samples with high affinity and specificity. The average sizes of bare Si-NWs, the aptamer bound on Si-NWs and the aptamer/thrombin complex on Si-NWs were statistically measured with the AFM cross-sectional images using more than 10 samples. As shown in figure 4, AFM analysis revealed that the aptamers bound to Si-NWs had a mean height of 4.15 ± 0.65 nm being immobilized at a certain interval. Considering the molecular size of the anti-thrombin aptamer (approx. 2.2 nm) [19] and linkers formed by 3-APDES, succinic anhydride and sulfo-NHS, the mean height of aptamers bound to the Si substrate was thought to be reasonable. Furthermore, AFM analysis revealed that the aptamer/thrombin complexes bound to Si-NWs, after incubation with thrombin samples, had a mean height of 8.23± 0.48 nm. The blood sample also resulted in the same mean height. Since the diameter of thrombin is known to be several nanometers [20, 21], the mean height of the thrombin/aptamer complexes with the linkers was also thought to be reasonable. Considering all the results in figures 2–4, AFM was thought to be a useful tool to visualize the immobilization of anti-thrombin aptamers on Si substrates of Si wafer and Si-NWs, and their binding with thrombin samples.

In addition, we could confirm the surface immobilization of anti-thrombin aptamers to Si-NWs by optical and fluorescent microscopic (OM and FM) analyses. The OM in figure 5(A) shows Si-NWs dispersed on a PCTFE substrate as a control. Figures 5(B) and (C) show the FM images of the control and the FITC-labeled thrombin bound to the aptamers which were immobilized onto the dispersed Si-NWs on PCTFE. The FM image of the control of Si-NWs without the aptamers clearly confirmed there were no remaining proteins in the blood sample non-specifically bound to Si-NWs after complete washing with PBS. In contrast, FITC-labeled thrombins bound to anti-thrombin DNA aptamers were observed randomly throughout the PCTFE surface, which might be attributed to the fact that Si-NWs were dispersed randomly as shown on the OM image. The FITC labeling would be used as a detection tool for an optical aptasensor system. All these results from AFM and FM analyses confirmed that what were bound on Si substrates were anti-thrombin DNA aptamers and their complexes with thrombin.

3.4. Real-time electrical detection of thrombin

The ability of aptamers to strongly bind to the target protein has been exploited for biosensor applications. For example, it has been previously reported to use carbon nanotubes (CNT) and

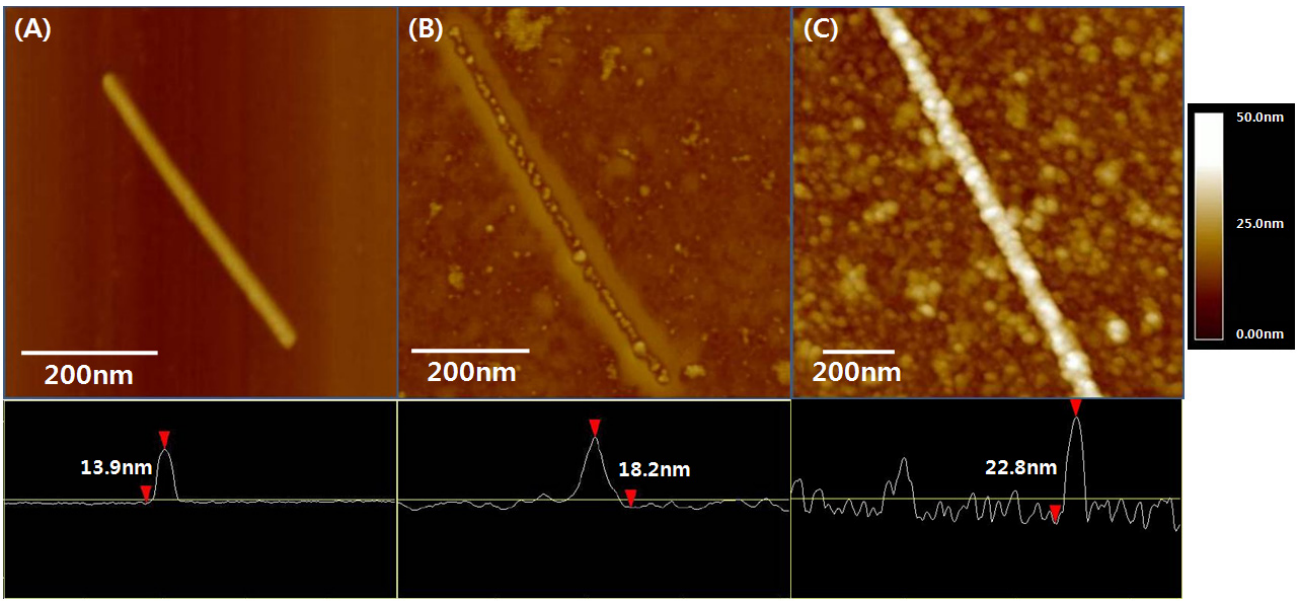
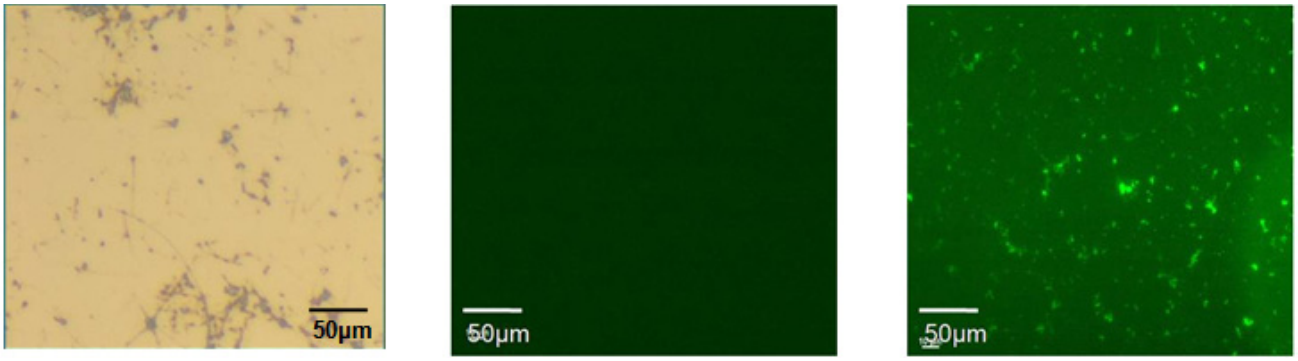


Figure 4. 2D atomic force microscopic (AFM) height and cross-sectional images for (A) bare silicon nanowires (Si-NWs), (B) anti-thrombin DNA aptamers on Si-NWs and (C) thrombin bound anti-thrombin DNA aptamers on Si-NWs.



(A) (B) (C)

Figure 5. (A) Optical micrograph (OM) of silicon nanowires (Si-NWs) without anti-thrombin aptamer as a control, and fluorescence micrographs (FM) of (B) the control and (C) FITC-labeled thrombins bound to anti-thrombin aptamers which were immobilized onto the dispersed Si-NWs on poly(chlorotrifluoroethane).

anti-thrombin DNA aptamer for aptasensor applications [22]. However, CNT is known to have some drawbacks such as considerable difficulties in controlling semiconducting properties during the growth and surface modification [23]. In contrast, the Si-NW has a good electroconductivity and the proper semiconducting properties, which can be controlled easily for electrical device applications. Figure 6 shows the current variation in time after injections of thrombin samples. Acetate buffer, as a control, did not cause the current change. However, the device showed an apparent drop in current upon the injection of thrombin with a concentration of approx. 330 pmol l^{-1}. The systematic decrease of current observed in the aptamer-functionalized FET biosensor might be ascribed to the molecular gating effect caused by the addition of thrombin as point charges. As is well known, FET is a kind of transistor depending on an electric field which controls the conductivity of charge carriers in a semiconductor material. The binding of biomolecules onto the surface of an Si-NW aptasensor causes accumulation and depletion of carriers in Si-NWs. Since the iso-electric point of thrombin is 7.0–7.6, the thrombin molecules are positively charged under the experimental condition of acetate buffer at pH = 5.4 [24]. These positively charged thrombin molecules can effectively screen the negative charges of anti-thrombin DNA aptamers and act as positive point charges on the gate dielectrics, resulting in the charge depletion in the Si-NW channel and the decrease in conductance of the p-type Si-NW aptasensor. The aptamer-functionalized Si-NW FET was characterized in detail and reported elsewhere [25]. When blood samples were injected, the current also changed significantly. The concentration of thrombin in the blood sample was thought to be higher than that of the thrombin sample (approx. 330 pmol l^{-1}) from the larger electronic signal change. As shown in the inset of figure 6, however, there were no measurable changes in current for a control without the aptamers except the fluctuations observed whenever a droplet

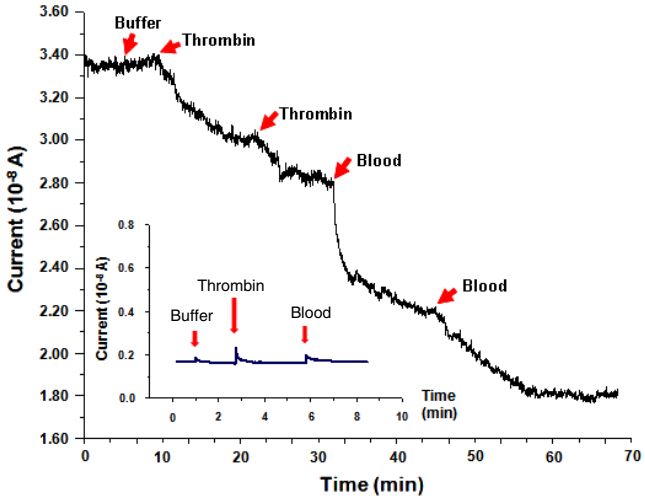


Figure 6. Real-time detection of electronic signals (current) from an anti-thrombin DNA aptamer-functionalized p-type silicon-nanowire (Si-NW) field effect transistor (FET) biosensor during and after binding with thrombin samples. Inset shows the response of an as-prepared Si-NW FET without the aptamers.

of the sample reached the device. All these results supported the possible application of an aptamer-functionalized Si-NW FET biosensor for the real-time detection of electronic signals during and after protein binding. The biomarkers in blood samples would be detected by the aptasensor and analyzed for the diagnosis of various diseases. The novel aptamer-functionalized Si-NW FET biosensor will be investigated further using target-protein-specific aptamers for the detection of various proteins (biomarkers) in the blood samples for clinical diagnostic applications.

4. Conclusions

Anti-thrombin DNA aptamers were successfully immobilized on Si substrates through the amide bond formation between amine groups of the aptamers and carboxyl groups on the surface. AFM analysis clearly visualized the molecular binding of anti-thrombin aptamers onto an Si wafer and Si nanowire. The aptamer bound to the Si substrate through the linker appeared to have a mean height of approx. 4.3 nm and the thrombin/aptamer complex to have a mean height of approx. 8 nm. FM analysis of the aptamer/thrombin complex together with the AFM analysis allowed us to confirm what were bound to Si substrates were anti-thrombin DNA aptamers. Based on these results, the anti-thrombin aptamer-functionalized Si-NW FET biosensor was successfully applied for the real-time detection of electronic signals during and after binding with a thrombin sample at a concentration of approx. 330 pmol l^{-1} and the blood sample.

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