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ORIGINAL ARTICLE

Highly sensitive, label-free and real-time detection of alpha-fetoprotein using a silicon nanowire biosensor

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Abstract

Alpha-fetoprotein (AFP) is a tumor-associated fetal protein that can be expressed in large amounts in adult tumor cells, serving as a useful clinical tumor-marker. Silicon nanowire (SiNW) biosensors have emerged as a powerful tool in detecting protein biomarkers, due to their ultrahigh sensitivity, real-time response and label-free detection. We fabricated a SiNW-based field-effect transistor (FET) according to "top-down" methodology. First, anti-AFP antibodies were immobilized onto the surface of the SiNW-FET. A polydimethylsiloxane (PDMS) microchannel was then integrated to the modified SiNW-FET. Various concentrations of AFP were then pumped through the sensing area. We observed a current change that corresponded to binding of AFP onto the surface of our anti-AFP functionalized SiNW-FET biosensor. Concentrations of AFP as low as 0.1 ng/mL were detected. The results implicate our SiNW biosensor as an effective AFP biomarker detector with promising potential in clinical applications.

Key Words: *Biosensors, point-of-care testing, proteins and enzymes, nanotechnology, microscopy*

Introduction

Alpha-fetoprotein (AFP) is a tumor-associated fetal protein (termed an oncofetal protein) synthesized in the fetal yolk sac at 9 weeks gestation, and later in the fetal liver and the gastrointestinal tract. It cannot be found in the serum of healthy adults [1]. Certain tumors, however, produce elevated AFP levels, including endodermal and mixed mesodermal/endodermal tumors, hepatomas, hepatoblastomas, teratomas, as well as pancreatic, gastric and yolk sac tumors [2]. Therefore, AFP concentration levels in the serum are important for early diagnosis of these diseases. In pregnant women, fetal AFP levels have also been used to detect birth defects and neonatal disorders [3]. The upper limit of normal AFP level in the healthy adult is defined as 25 ng/mL [4].

As conventional techniques in detecting protein markers, which include enzyme-linked immunosorbent assay (ELISA) [5], radioimmunoassay (RIA) [6], and chemiluminescent enzyme immunoassay (CLEIA) [7], are very sensitive and selective, they are still suffering from time-consuming and laborious work, and some ELISA tests are not sufficiently sensitive for the detection of low level marker concentrations which exist in the early stages of the disease [8]. There also exist such problems like the wasted disposal of radiolabels in RIA, and the increased detection time and complexity of the sensor fabrication in the methods that are based on labeling technology. Currently, some methods used for AFP detection have achieved satisfactory performance. Wang et al. successfully detected AFP concentrations as low as 100 pg/mL with surface-enhanced Raman scattering-based immunoassay [9]. K. Li et al. reported an optical microfiber biosensor with a limit of detection for AFP of 0.2 ng/mL in PBS and 2 ng/mL in bovine serum [10]. In another study, W. Li et al. combined localized surface Plasmon resonance (LSPR) with immunoassay and the limit of detection of this substrate was 24 ng/mL [11]. Terada et al. even achieved a limit of detection for AFP as low as 15.4 pg/mL by using the modified fluorescent magnetic beads-based rapid immunoassay [12]. However, most of

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these methods are still not showing the ability of label-free and real-time detection of AFP.

The development of research on nanomaterials and biosensors make the silicon nanowire (SiNW)-based field-effect transistors (FETs) a superior selection. Namely, the SiNW-FETs enable ultrasensitive detection of agents (detection limit down to fM) that cause electric field changes and other perturbations, recognizing a broad range of biological and chemical species [13]. Samples can be unlabeled and detection requires minimal volumes. Other attractive properties include ultrahigh sensitivity, direct electrical readout and multiplexed detection [14–16]. Fabricated by 'top-down' or 'bottom-up' techniques, a SiNW-based FET device is composed of a semiconductor channel and source, drain and gate electrodes, all of which are located on a Si wafer with an insulating surface layer [17]. Through the semiconductive channel, the source and drain electrodes communicate with each other, and the gate electrode is employed to modulate the conductance of channel via an applied electrical potential. The silicon oxide coating on silicon nanowires can be easily linked to target receptor groups. Thus the SiNW-FET can be functionalized by immobilizing receptor molecules on its surface and selectively bind specific target analytes. When modulating the source-drain current, the change of charge carriers coursed by target-receptor binding will be observed through a semiconductor device analyzer. For a p-type nanowire device, when the sensor device is exposed to macromolecules in solution with a net negative (or positive) charge, specific binding will lead to an increase (or decrease) in the surface negative charge, and an increase (or decrease) in conductance [18]. Thanks to the excellent features, the SiNW-FETs have been proven to be ultrasensitive and selective sensors in numerous biosensing applications, including the detection of proteins [19], cancer markers [20], DNA [21], RNA [22], virus [23] and other biochemical species [13,24,25].

In this work, we fabricated a SiNW-FET microfluidic biosensor for AFP detection. Our biosensor was coated with anti-AFP. Upon AFP binding, changes to the electrical current allowed a quantitative determination of AFP concentrations. It is of note that our target biomolecule was seldom adsorbed onto PDMS sidewalls, which would have prevented the SiNW biosensor from attaining a high sensitivity [26]. The silicon nanowire biosensors in this study performed as highly sensitive, real-time responsive and effective label-free tools in detecting alpha-fetoprotein.

Materials and methods

Materials and reagents

Silicon-on-insulator wafers (SOI; 6 in., p-type (100), ρ: 10–20 Ω cm) were purchased from Shanghai Simgui Technology Co., Ltd. (Shanghai, China). Polydimethylsiloxane (PDMS) was acquired from Dow Corning Co (Michigan, USA). Tetramethylammonium hydroxide (TMAH; 25% (w/w), 99.9999%) solution was obtained from Alfa-Aesar (Shanghai, China). All the other reagents and protein samples including 3-aminopropyl-triethoxysilane (APTES), glutaraldehyde (Glu), phosphate buffered saline (PBS, pH 7.4), bovine serum albumin (BSA), fluorescein isothiocyanate (FITC)-labeled anti-glutathione-s-transferase(GST) antibody, AFP antigens, AFP monoclonal antibody and IgG antigens were obtained from Sigma-Aldrich and used as received without further purification. Anti-IgG labeled Au nanoparticles (10 nm) were purchased from Shanghai Sangon Biotech (Shanghai, China). All solutions were prepared with deionized water (DI; $R \geq 18.2$ MΩ cm) generated from a Millipore system.

Fabrication of the SiNW-FET

The SiNW-FET was fabricated by 'top-down' methodology as previously described [27]. First, the top layer of SOI wafer was thinned to a thickness of approximately 50 nm using thermal oxidation. For the fabrication of the SiO_2 mask, 20 nm of a SiO_2 thin film was deposited on the SOI wafer using plasma-enhanced chemical vapor deposition (PECVD). The critical dimension of the SiO_2 pattern was designed to be 700 nm by employing Nikon stepper lithography (Nikon NSR 1755i B). The wafer was then transferred into 100 mL 25% TMAH solution for wet etching at 70°C for around 23.5 min to acquire the <100 nm SiNW. For the definition of source, drain and back gate contacts, a Ni/Au (20 nm/100 nm) bilayer was deposited onto the wafer. The ohmic contact was then obtained by annealing the wafer at 400°C using rapid thermal annealing. In order to avoid biosensor interference and unnecessary functionalization of non-sensing areas on the surface, the passivation layer composed of a SiO_2/SiN_x (200 nm/200 nm) bilayer was finally introduced using PECVD, followed by lithography to open the SiNW detection window (375 μm long, 5.77 μm wide), reaction ion etching to remove the SiN_x layer of SiNW region and buffered oxide etching to remove SiO_2 layer. The schematic diagram of the SiNW-FET biosensor is shown in Figure 1.

Fabrication of the PDMS-based microfluidic channel

The microfluidic channel patterns were first defined on a cleaned silicon wafer by photolithography using MA6/BA6 aligner (Suss Microtech, Germany); this was followed by inductively coupled plasma (ICP) etching (STS MPX HRM). Figure 2(A) shows a model schematic of the microfluidic channel. The mixture of PDMS prepolymer and its curing agent

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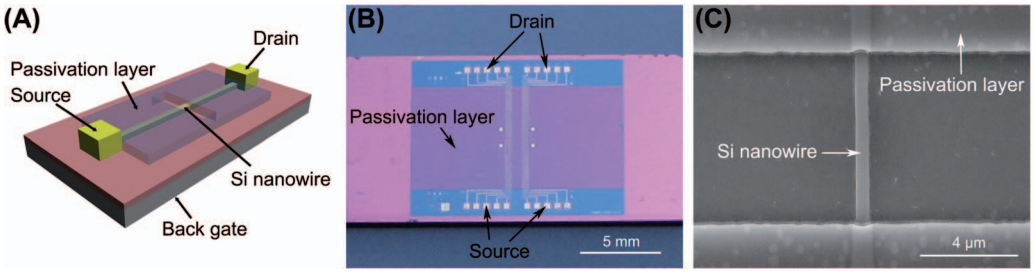

Figure 1. (A) Schematic diagram of the SiNW-FET biosensor. (B) Optical image of fabricated SiNWS-FETs. (C) Scanning electron microscopy image of one SiNW device. The width of SiNW was 509 nm, while the length was 21.15 μm and the thickness was 12nm (the scale bar is 4 μm.).

(mass ratio 10:1) was cast against the silicon mold containing the channel structure to yield polymer replicas. The mixture was degassed by vacuum pumping and then cured at 75°C for 40 min. The elastomeric replica containing the channel structure was then peeled away from the silicon mold. The PDMS microfluidic channel was acquired with a length of approximately 12 mm, width of approximately 0.6 mm and height of approximately 0.05 μm (Figure 2B).

Surface functionalization of the SiNW-FET

Anti-AFP antibodies were covalently linked to the SiNW surface using a four-step procedure (the process is shown in Figure 3). First, the surface was cleaned in oxygen plasma for 5 min to generate hydroxyl groups (OH-). Second, the OH- covered SiNW was promptly immersed in 2% (v/v) APTES anhydrous ethanol solution for 40 min. Unbound APTES molecules were then evaporated by heating at 120°C for 1 h. In this step, amine groups were grafted onto the surface of the SiNW in preparation for linking to glutaraldehyde molecules. Third, the SiNW was dipped in 2.5% (v/v) glutaraldehyde aqueous solution for 1 h to modify the surface with aldehyde groups before they can bind to amine groups from the anti-AFP antibody. It has been reported that polymerization of APTES on silicon surface can occur and leads to a heterogeneous surface [28], but this polymerization of APTES did not affect the biosensor measurement. Protein molecules immobilized covalently on the APTES-Glu surface has been reported to be stable [29] even when there is a double bond between the aldehyde group of Glu and the protein amine group. Finally, the SiNW was cleaned using

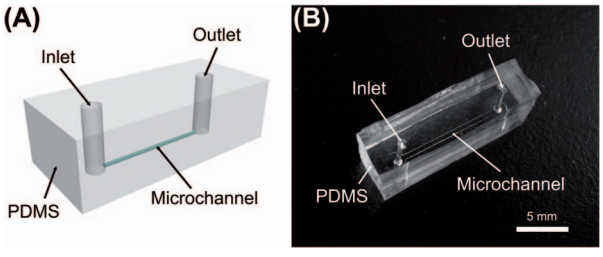

Figure 2. (A) Schematic diagram of the PDMS microfluidic channel. (B) Optical image of the PDMS microfluidic channel.

deionized (DI) water at room temperature (25 ± 2°C), before drops (4 μL per drop) of 1 mg/mL anti-AFP antibody in PBS buffer solution (pH 7.4) covered the sensing areas for 2 h. The anti-AFP functionalized SiNW biosensor was cleaned and stored at 4°C.

Integration of the SiNW biosensor with the PDMS microfluidic channel

The PDMS microfluidic channel was cleaned by ultrasonic cleaning in anhydrous ethanol and DI respectively for 8 min. The channel was then dried with nitrogen and cleaned using oxygen plasma for 1 min, before being promptly mounted on the SiNWs at approximately 7 N/cm^2 pressure. A reversible seal between the PDMS microfluidic channel and functionalized SiNW biosensor formed (Figure 4). The integrated SiNW biosensor was ready for detection experiments.

Sensing measurement

The integrated SiNW biosensor was connected to a peristaltic pump and an Agilent B1500A semiconductor device analyzer (Agilent Technologies Inc., USA) before sensing measurements could begin. The pump ceaselessly transported the solution from sample vessels over the SiNW biosensor a speed of 17 μL/min. The measurements were recorded at room temperature (25 ± 2°C).

Results

Electrical characterizations of the SiNW-FET

The electrical characterization of the SiNW-FET used in our experiments exhibited an ambipolar transfer characteristic (Figure 5). Positive gate, negative gate, and strong gate voltage dependence with an on/off ratio of about 10^5 could be observed.

Surface functionalization of the modified SiNW-FET

Figure 6 depicts the scanning electron microscopy (SEM) images from a mimetic experiment designed

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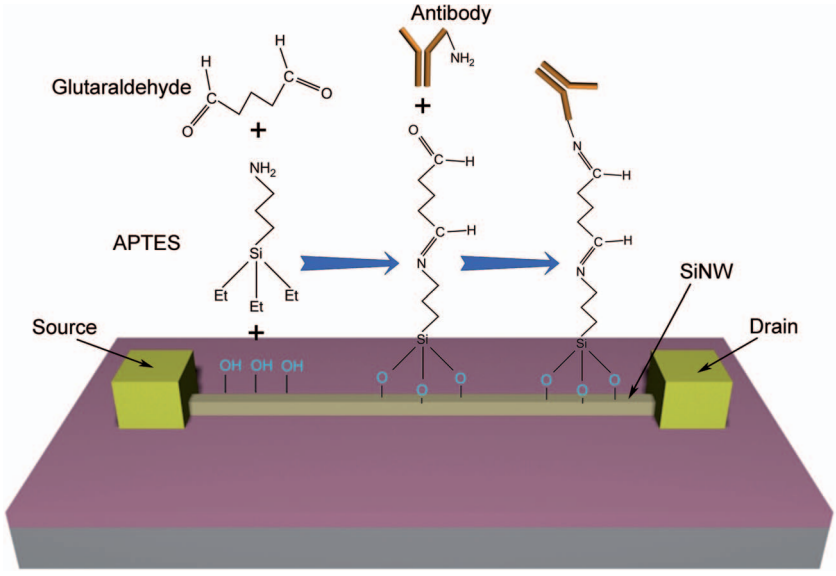

Figure 3. Schematic illustration of chemical covalent modification method for functionalization of SiNW surface.

to confirm the functionalization method. In Figure 6A, the Au nanoparticles labeled with anti-IgG were randomly distributed on the IgGs-modified surface of the SiNW at relatively high density. In stark contrast, Au nanoparticles labeled with anti-IgG were barely observed on the BSA-modified (Figure 6B) and unmodified (Figure 6C) SiNW surfaces.

Fluorescence images (Figure 6D and E) showed that green FITC-labeled anti-GST antibodies were well immobilized on the APTES-Glu modified surface of SiNW, and could hardly be observed on the unmodified surface.

Electrical detection of AFP antigens

A stable current (background curve, Figure 7A) was obtained when BSA solution and 0.1 × PBS solution was pumped through the sensing area of SiNW. Then, an increase in current (approximately 2 nA) was observed when the solution flowing through the SiNW changed from 0.1 × PBS to 0.1 ng/mL AFP solution (at approximately 65 s; Figure 7B). Figure 7C also shows an increase in current (approximately 10 nA) at about 50 s, when the solution changed from 0.1 × PBS to 1 ng/mL AFP. A calibration curve was finally plotted by calculating the biosensor response to different concentrations of AFP solutions (Figure 7D).

Discussion

As exhibited in the ambipolar transfer characteristic (Figure 5), the positive gate voltage conduction indicated an accumulation mode with holes in the inversion layer, while the negative gate voltage used electrons as the conduction species [13,30]. We also observed strong gate voltage dependence with an on/off ratio of about 10^5. This electrical characterization indicated no electrical problems with the SiNW-FET and marked its suitability as a biosensor in our sensing measurements.

In the work of surface functionalization of the modified SiNW-FET, 2 mg/mL IgG antigens and BSA were respectively immobilized onto the

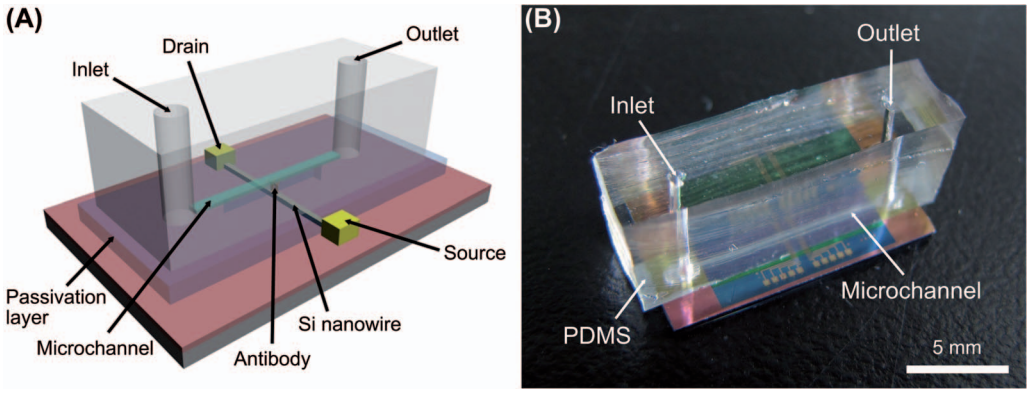

Figure 4. (A) Schematic diagram of the integrated SiNW biosensor. (B) Optical image of the integrated SiNW biosensor.

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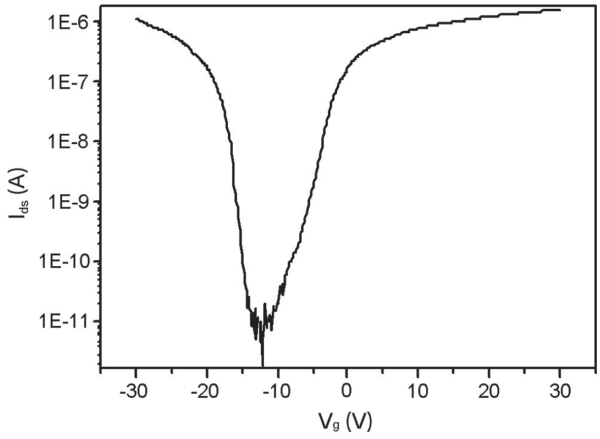

Figure 5. Ambipolar electrical characterization of the SiNW-FETS before the surface modification at a various back-gate voltage. V_g is the back-gate voltage while I_{ds} is the current between source and drain.

aldehyde-surface of the SiNW to provide amino groups. PBS (PH 7.4) with 2 mg/mL Au nanoparticles (10 nm) labeled with anti-IgG were then simultaneously dropped onto the surface of IgGs-modified, BSA-modified and unmodified SiNW at room temperature (25 ± 2°C) for 3 h. After washing with PBS and drying with nitrogen, three times, SEM was used to image the functionalization results. SEM images in Figure 6 demonstrated that proteins could be successfully immobilized onto the APTES-Glu-modified surface of the SiNW, thus this method could be further used in the immobilization of anti-AFP antibodies.

We also employed FITC-labeled anti-GST antibodies to verify the chemical covalent modification method. Figure 6 shows that when FITC-labeled anti-GST antibodies were pumped through the SiNW biosensor, they successfully bound to the glutaraldehyde (Figure 6D), while barely adhering to the unmodified surface (Figure 6E). The results again implied that the anti-AFP could be successfully immobilized onto the surface of the SiNW biosensor by using the surface modification method in this work and therefore capable of capturing AFP antigens.

To block unspecified absorption sites on the PDMS sidewalls during sensing measurement, approximately 100 µL 0.05% BSA solution was pumped through the microchannel for 5 min. This was followed by approximately 100 µL of 0.1 × PBS solution, which flowed in the channel to obtain a stable current (background curve, Figure 7A). Then, directed by the microchannel, sample solutions at various AFP concentrations started to flow through the sensing area of the p-type SiNW biosensor. As anti-AFP antibodies recognized AFP epitopes, AFP molecules could selectively bind to the antibodies immobilized on the SiNW [31]. Sensing measurements were performed at gate voltage of 2.5 V. As the isoelectric point of the AFP antigen is 4.85 [30], such AFP concentration-related current changes (Figure 7B and C) could be explained because AFP molecules are negatively charged in 0.1 × PBS solution (PH 7.4). When negatively charged AFP molecules were captured by the receptor on the surface of the SiNWs, an accumulation of hole carriers caused an increase in current.

A logarithmic relationship was observed between the current change ratio and AFP concentration (C_{AFP}) from 0.1–10 ng/mL. The limit of detection (LOD) was measured to be 0.1 ng/mL (signal/noise ratio of 3). The calibration curve was fitted as $\Delta I/I_0 = 0.05320 + 0.03255 \times \lg(C_{AFP})$, in which I_0 stood for the background I_{ds}, ΔI for the I_{ds} change between background and upon addition of AFP. As was presented in the nanowire FET-type biosensors,

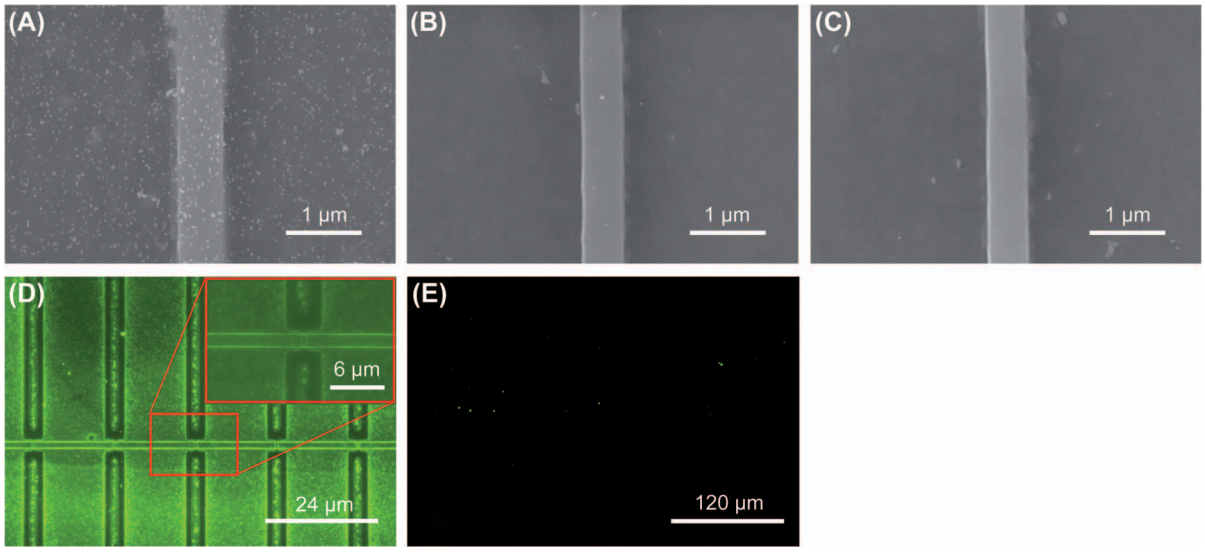

Figure 6. Surface functionalization of the modified SiNW-FETs. (A), (B) and (C) SEM image of IgGs-modified SiNW, BSA-modified SiNW and the unmodified SiNW with Au nanoparticles labelled anti-IgGs conjugates respectively. All the scale bars are 1 µm. (D) Fluorescence image of glutaraldehyde-modified SiNWs with FITC-labeled anti-GST antibodies (the scale bars are 6 µm and 24 µm). (E) Fluorescence image of unmodified SiNW with FITC-labeled anti-GST antibodies (the scale bar is 120 µm).

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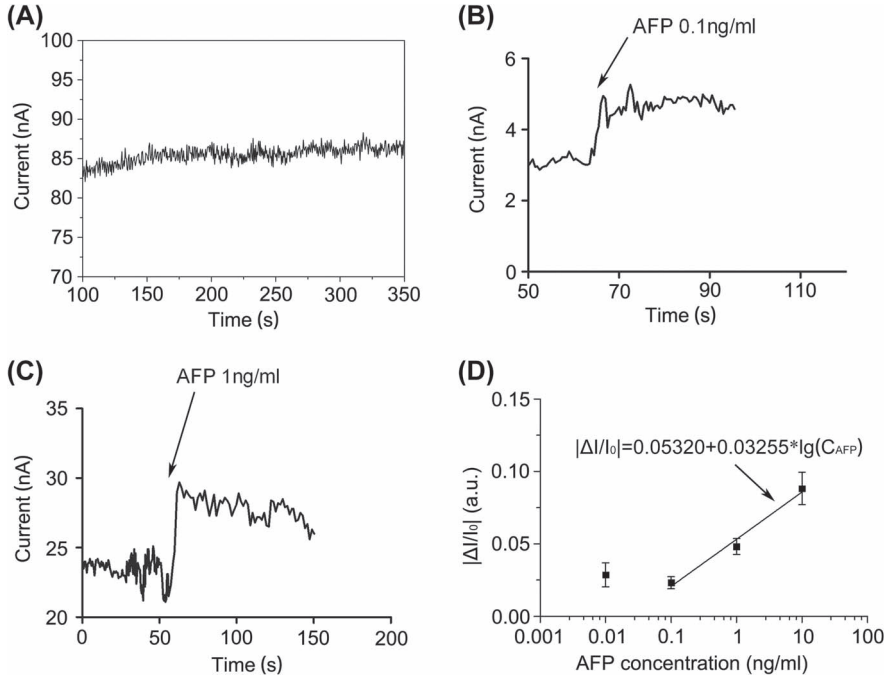

Figure 7. Time-current curves when different solutions flowed through the microchannel. (A) Background curve of 0.05% BSA solution and blank 0.1 × PBS solution in the microchannel. (B) and (C) Curves of the addition of 0.1 ng/ml and 1 ng/ml AFP solution. (D) The logarithm of AFP solutions concentrations versus the current change ratio. Error bars ($p < 0.01$) in the figure were obtained after repeated measurements of different concentrations of AFP solutions.

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response of the biosensor showed logarithmic dependence on the target protein concentrations [32]. Results of the electrical detection of AFP antigens demonstrated that the SiNW biosensor in this work could detect AFP molecules at a concentration as low as 0.1 ng/mL, which was comparable with the other label-free sensing systems recently reported in the literature (50 ng/mL [33]; 2 ng/mL [34]; 10 ng/mL [35]).

When the solution in the microchannel changed from 0.05% BSA to 0.1 × PBS, barely any current change was observed. Therefore, we can conclude that the receptors on the surface of our SiNWs were selectively capturing target AFP molecules, causing the current change. Meanwhile, BSA molecules relatively blocked the unspecified absorption sites on the PDMS sidewalls and decreased unspecified absorption interference.

It has been reported that the shift of thermodynamic equilibrium could lead to the dissociation of antigen-antibody complexes, which indicated that the NW sensor may be used repeatedly [31].

Conclusions

We fabricated a p-type SiNW biosenor functionalized with anti-AFP receptors and an integrated PDMS microchannel, which resulted in outstanding biosensing detection of AFP antigens. The time-current curves at different AFP concentrations (from 0.1–1 ng/mL) confirmed both selective and ultrahigh sensitive AFP detection. These results prove that our silicon nanowire biosensor is a highly sensitive, real-time responsive and effective label-free tool in detecting alpha-fetoprotein. Such capabilities have promising potential within clinical settings.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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