Biosensors and Bioelectronics 26 (2011) 2323–2328

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Using silicon nanowire devices to detect adenosine triphosphate liberated from electrically stimulated HeLa cells

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ARTICLE INFO

Article history:
Received 2 August 2010
Received in revised form 1 October 2010
Accepted 2 October 2010
Available online 27 October 2010

Keywords:
Adenosine triphosphate
HeLa cell
Silicon nanowire field-effect transistor
Ionic strength

ABSTRACT

In this study, we used a biosensor chip featuring Abl tyrosine kinase-modified silicon nanowire field-effect transistors (SiNW-FETs) to detect adenosine triphosphate (ATP) liberated from HeLa cells that had been electrically stimulated. Cells that are cultured in high-ionic-strength media or buffer environments usually undermine the sensitivity and selectively of SiNW-FET-based sensors. Therefore, we first examined the performance of the biosensor chip incorporating the SiNW-FETs in both low- and high-ionic-strength buffer solutions. Next, we stimulated, using a sinusoidal wave (1.0 V, 50 Hz, 10 min), HeLa cells that had been cultured on a cell-culture chip featuring interdigitated electrodes. The extracellular ATP concentration increased by ca. 18.4-fold after electrical stimulation. Finally, we detected the presence of extracellular ATP after removing a small amount of buffer solution from the cell-cultured chip and introducing it into the biosensor chip.

1. Introduction

Thousands of complicated biological and chemical reactions occur in living cells, with most of them requiring an amount of energy for activation. In living cells, the universal unit of energy is provided by adenosine triphosphate (ATP). Extensive research has focused on the role of intracellular ATP as an energy source and its complex biochemical interactions with living cells (Hafner, 2000; Schneider et al., 1999). Several approaches have been developed previously for the detection of ATP. Firefly luciferase (Billard and DuBow, 1998) requires the presence of ATP to produce bioluminescence, allowing ATP detection at the picomolar level when using an enzyme-linked immunosorbent assay (ELISA). Although this approach features an appropriate detection range and limit, its drawbacks are that it is time-consuming and difficult to apply to real-time detection. An enzyme-modified electrode has also been demonstrated for the detection of ATP, with a detection limit on the nanomolar level. This technique was first fulfilled by co-immobilization of the enzymes onto the electrode (Kueng et al., 2004). The enzymatic reaction resulted in a current response that was proportional to the ATP concentration. The selective catalytic activity of an enzyme/ISFET (ion-sensitive field-effect transistor) system has also been adopted for the detection of ATP (Migita et al., 2007). The major drawbacks of enzyme/ISFET and enzyme/electrode sensors are their low sensitivities and slow responses. The use of field-effect transistors (FETs) based on nanowires (Cui et al., 2001; Wang et al., 2005b) or nanotubes (Star et al., 2006; Wang et al., 2007) as biosensors has also been studied extensively in biological research because of their sensitivity and their label-free and real-time detection capabilities. Wang et al. (2005b) reported the highly sensitive and label-free detection of ATP using Abl tyrosine kinase-modified silicon nanowire field-effect transistors (SiNW-FETs), with ATP binding to Abl distinguishable above the background noise at concentrations at least as low as 100 pM.

To date, most SiNW-FETs (Park et al., 2007) have been developed for the detection of pre-concentrated and purified target molecules in low-ionic-strength buffer environments; there has been little study of the use of SiNW-FET for in vitro or in vivo detection of molecules released from living cells (Wang et al., 2007). Lieber's group recently employed an array of SiNW-FETs to monitor the electrical signals from single neurons (Patolsky et al., 2006). They observed highly sensitive detection, stimulation, and inhibition of neuronal signal propagation between nanowires and axon junctions. Although a single-walled carbon nanotube transistor has been developed for the in situ detection of chromograni A (Wang et al., 2007), which is released from neurons, there are very few reports of the use of SiNW-FET or CNT-FET biosensors for the detection of chemical species released from cultured living cells.

In this study, we prepared a system comprising a cell-culture chip and a sensor chip for the detection of ATP released from living cells. The cell-culture chip was developed for both the cell culturing and electrical stimulation of living cells. The HeLa cells were cultured in the medium on the cell culture chip. After cell

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doi:10.1016/j.bios.2010.10.003

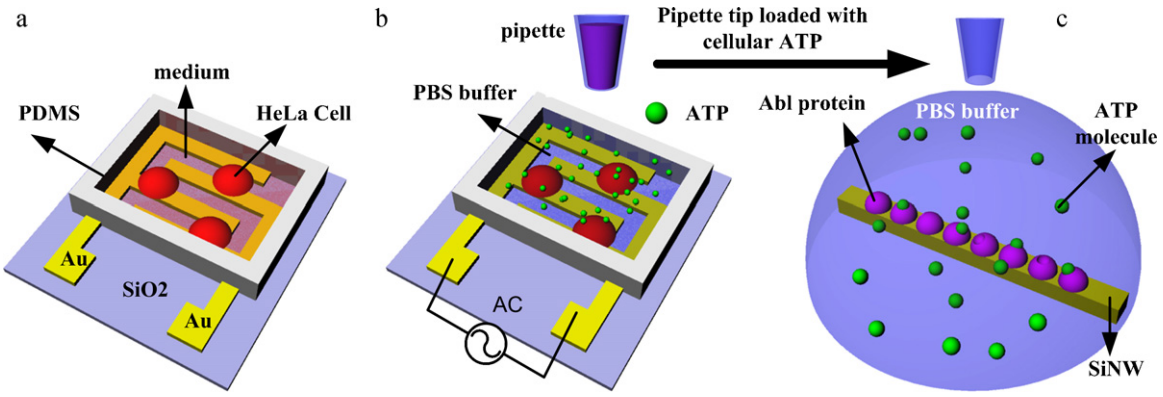

Fig. 1. Schematic representation of (a) cultured HeLa cells on a cell-culture chip, (b) electrical stimulation activating HeLa cells to release ATP, and (c) cellular ATP detected by an Abl-modified SiNW.

attachment (Fig. 1a), the cell culture environment was replaced by phosphate-buffered saline (PBS). Electrical stress caused the HeLa cells to liberate ATP into the PBS solution (Fig. 1b). We then used a pipette tip to remove a portion of the PBS solution, now incorporating the extracellular ATP, from the cell-culture chip and loaded it onto the biosensor chip featuring SiNW-FETs as sensors (Fig. 1c). Taking advantage of the high sensitively and selectivity of the Abl-modified SiNW-FET biosensor, we could detect the extracellular ATP released from the HeLa cells in the cell-culture chip, without the need for pre-concentration or and purification.

2. Experimental

2.1. Chemicals

Deionized water (>18 MΩ cm) was obtained from a purification system. All reagents employed in this study were of reagent grade and used as received. Sylgard 184 base (A) and curing agent (B) were purchased from Dow Corning (Mialand, MI). Abl tyrosine kinase was purchased from New England BioLabs (Beverly, MA). SU-8 photoresists (SU-8 2005 and 2050) were purchased from Microchem (Newton, MA). Dulbecco's modified Eagle's medium (low glucose, DMEM), fetal bovine serum (FBS), 0.05% trypsin/ethylenediaminetetraacetic acid (EDTA) solution, and PBS were purchased from GibcoTM Invitrogen Co. (Carlsbad, CA).

2.2. Fabrication of SiNW-FET biosensors

The SiNW-FET biosensors were fabricated from an SOI (silicon on insulator) wafer having a buried oxide thickness (BOX) of 150 nm and a 50-nm-thick top-silicon film in the ⟨1 0 0⟩ orientation. The thickness of the top silicon layer was further reduced to 30 nm using dry oxidation and wet etching technology. The SiNW-FET biosensor was fabricated using e-beam lithography and subsequent dry etching. The samples were capped with a 10-nm-thick screening oxide layer before being doped through boron ion implantation at an energy of 10 keV; the dopants were then activated at 950 °C for 30 min in nitrogen ambient to ensure their uniform diffusion over the entire SiNW. Contact holes were patterned and Au/Ti metallization was performed and then the devices were sintered at 300 °C in nitrogen ambient for 60 min. To avoid short circuiting or leakage during measurement in the solution environment, SU8-2005 photoresist was patterned to protect the source and drain electrodes.

2.3. Surface modification of SiNW-FET biosensors

Abl tyrosine kinase was covalently coupled to the SiNW surface using a modification of Lieber's method (Wang et al., 2005b); Fig. S1a presents an idealized representation. To clean the surface of the biosensor chip, UV/ozone exposure was performed for 10 min using a commercial ozone cleaning system (UV-1, All Real). The cleaned biosensor chip was then immersed for 30 min in a solution of 1% (v/v) 3-(trimethoxysilyl)propyl aldehyde in EtOH/H_2O (95:5, v/v). The biosensor chip was washed with EtOH, dried, and then heated at 120 °C for 30 min. The aldehyde-presenting chip was placed in a solution of Abl tyrosine kinase (5 μg/mL) containing 4 mM sodium cyanoborohydride; unreacted aldehyde groups were quenched with 15 mM Tris buffer (pH 7.5) for 10 min.

2.4. Atomic force microscopy characterization

The immobilization process (surface morphology) was characterized at its various stages using atomic force microscopy (AFM). A commercial AFM instrument (JSPM-5200, JEOL) was adopted for this morphological analysis using a silicon probe (NSG 11, NT-MDT) having a nominal spring constant of 2.5 N m^{-1}. The atomic force microscope was operated in the tapping mode to minimize mechanical damage of the chemically modified surfaces.

2.5. Characteristics of SiNW-FET biosensors

The surface morphologies and dimensions of the SiNWs were examined using a field-emission scanning electron microscope (JSM-6700, JEOL). The electrical characteristics of the SiNW-FET biosensors were measured at room temperature using a semiconductor parameter analyzer (HP4156C, Agilent), performed on a probe station (VFTTP4, Lakeshore). SiNW-FET biosensors were also characterized in terms of their performance in low- and high-ionic-strength buffer environments.

2.6. Fabrication of cell-culture chip

The cell-culture chip fabricated in this study featured gold finger-like electrode structures and a cell culture well prepared from polydimethylsiloxane (PDMS). The chip substrate was a silicon wafer having a thermal oxide thickness of 3000 Å. The gold finger-like electrodes were fabricated using a standard photolithography and lift-off process; the gap between electrodes was ca. 100 μm. A Sylgard 184 A/curing agent mixture (10:1) was poured over a stainless-steel mold and cured until solidified; the PDMS well was then removed from a mold. After cleaning, the PDMS well (well size: 5 mm × 5 mm × 2 mm) was bonded to the surface of the cell-culture chip.

2.7. Cell culture and seeding onto the substrates

HeLa cells were cultured with DMEM containing 10% FBS solution. The HeLa cells were trypsinized in 0.05% trypsin/EDTA solution onto a culture flask, which was then cultured at 37 °C in a humidified chamber containing 5% CO_2. Prior to seeding the HeLa cells, the cell-culture chip was sterilized. To cultivate HeLa cells on the chip, the HeLa cells were peeled from a culture flask resuspended in the medium, and then thoroughly mixed by pipetting to disperse the cells. The HeLa cells were trypsinized in 0.05% trypsin/EDTA solution and seeded onto the chip in the medium at a concentration of 5×10^5 cells/mL. The cell was then incubated for 48 h prior to electrical stimulation.

2.8. Assaying ATP released from the HeLa cells

The assay of an ATP dilution series in a plate with a constant luciferase concentration was performed according to the protocol described by the manufacturer (ATP bioluminescence assay Kit HS II, Roche Diagnostics GmbH, Mannheim, Germany). ATP dilutions in a volume of 50 μL were assayed with luciferase regent (50 μL) in microplates on a luminometer (Multiskan Ascent, Thermo Labsystems, Waltham, MA). The light signal was integrated for 10 s after a delay of 1 s.

The HeLa cells cultured in the stimulated cell chip were incubated for at least 48 h prior to stimulation. After 48 h, the chip was washed three times with PBS buffer and then fresh PBS buffer (100 μL) was gently added into the PDMS well. The HeLa cells were stimulated for 10 min using a function generator (DS345, Stanford Research Systems) that generated a sine-wave waveform (amplitude: 1 V; frequency: 50 Hz). After electrical stimulation, a portion (50 μL) of the PBS buffer was collected from the top of the well without touching the HeLa cells. Luciferase reagent (50 μL) and dilution buffer (50 μL) were added to this PBS buffer sample and the bioluminescence was measured according to the protocol described above.

3. Results and discussion

3.1. AFM characterization of the sample surface

We used AFM to characterize the sample surface morphology after both chemical modification and protein immobilization. Fig. S1b and c displays AFM images recorded before and after silanization, respectively. The UV/ozone-treated sample had a smooth surface (roughness: 0.21 nm; as shown in Fig. S1b). The aldehyde-terminated sample exhibited a relatively coarse morphology, mainly due to intermolecular random cross-links into the multilayer (Wang et al., 2005a). Because the silicon surface modified by UV/ozone was homogeneous and smooth, the observed circles were aggregates of aldehyde–silane molecules, whose heights were ca. 1.4–4.5 nm. We observed many aggregates, ranging from 20 to 80 nm in diameter, on these multilayer-covered surfaces. The coupling of Abl tyrosine kinase to the aldehyde-terminated surface resulted through the formation of covalent bonds between the aldehyde groups and primary amino groups, such as those of lysine residues, of the surface of Abl tyrosine kinase. Fig. S1d reveals the presence of many small grains on the aldehyde-terminated surface, suggesting the successful immobilization of Abl.

3.2. Characterization of the SiNW-FET devices

As a demonstration, we fabricated the prototype SiNW-FET biosensor chip displayed in Fig. 2a. Fig. 2b displays a typical SiNW (width: 50–100 nm; length: 10 μm) employed in this device. The electrical conductances of SiNWs having widths of 50 and 100 nm were each measured at a bias of 0.1 V through two-terminal measurements in air (Fig. 2c); their mean values were ca. 312.5 ± 19.5 and 568.5 ± 32.9 nS, respectively.

We used a liquid gate to measure the sensitivities of the fabricated 50- and 100-nm SiNW-FET biosensors toward liquid

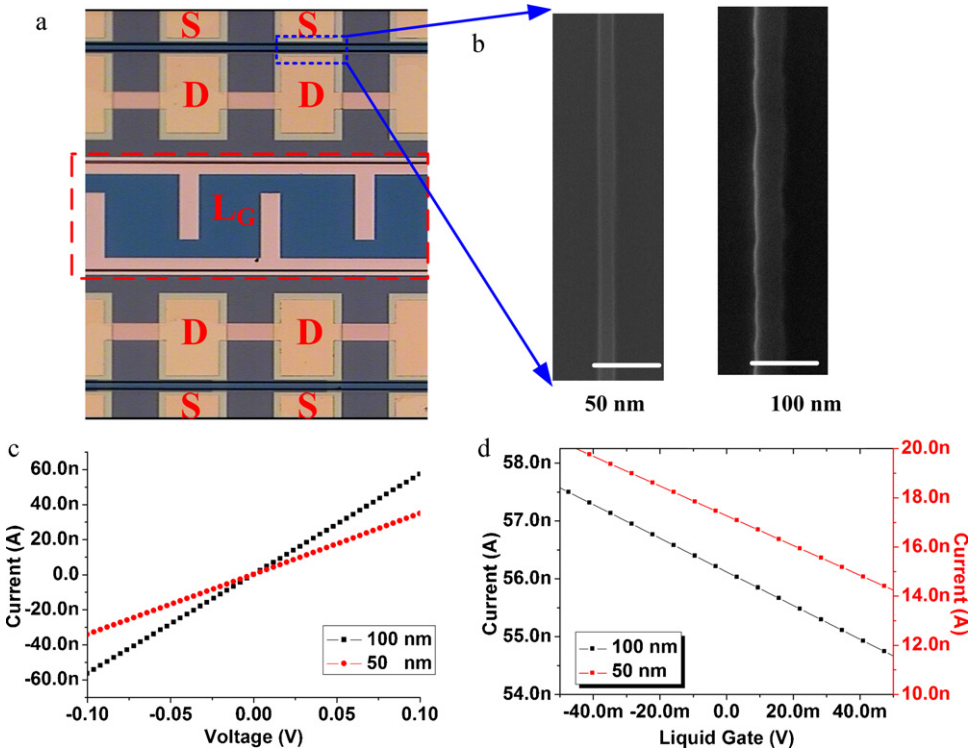

Fig. 2. (a) Optical image of a portion of the SiNW-FET biosensor chip (S, source; D, drain; L_G, liquid gate) and (b) SEM images of SiNWs having different widths (scale bar: 200 nm). Plots of (c) I_{DS} versus V_{DS} and (d) I_{DS} versus L_G for SiNW-FETs, measured in buffer solution at room temperature.

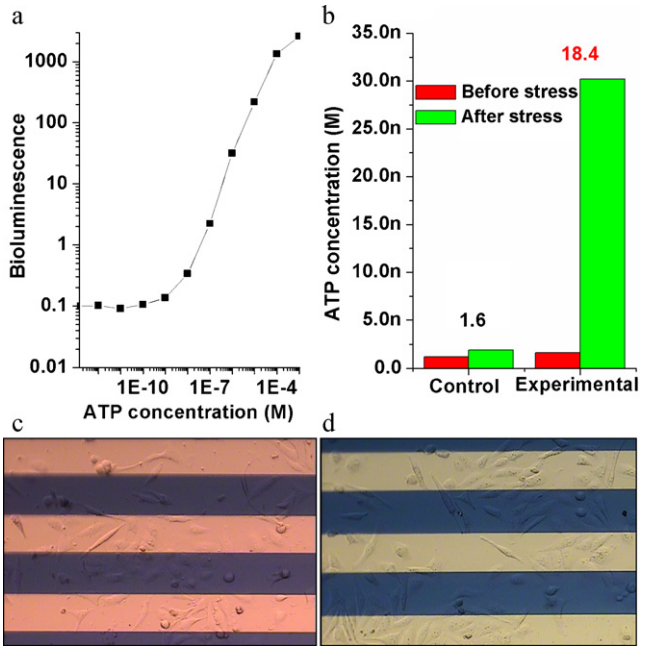

Fig. 3. (a) Luminescence plotted with respect to the ATP concentration. (b) Levels of ATP released from HeLa cells in PBS buffer. (c and d) Photographs of HeLa cells cultured on the chip (c) before and (d) after applying a sine-wave waveform at 1 V and 50 Hz for 10 min; the brighter patterns are the gold electrodes.

environments. The SiNW-FET exhibits a larger transconductance in response to the sweep of liquid gate, indicating more sensitivity toward molecular absorption on its SiNW surface. Individual SiNW-FET devices were characterized in aqueous buffer solution (10 μM PBS, pH 7.4); a liquid gate potential was applied to a reference electrode with respect to the ground, while a small bias voltage (a large liquid bias voltage might have resulted in electrochemical reaction) was applied over the source and drain to monitor the conductance of the SiNW-FET. The source–drain current (I_{DS}) of the SiNW-FET device plotted with respect to the liquid gate voltage (V_{LG}) revealed behavior similar to that of a p-type FET. The most important parameter in evaluating FET performance is the transconductance ($g_m = \partial I_{DS}/\partial V_{LG}|V_{DS}$). In the linear region, transconductance is a measure of the current response with respect to the liquid gate voltage. The mean values of transconductance of the SiNW-FETs incorporating 50- and 100-nm-wide SiNWs were 60.5 ± 5.2 and 29.1 ± 6 nS, respectively (Fig. 2d). Thus, the FET-based sensor with the higher transconductance was sensitive in response to additional surface charge or potential change. The transconductance is highly correlated to the surface-to-volume ratio (Elfstrom et al., 2007).

3.3. Electrical stimulation of HeLa cells

Luciferase (Pellegatti et al., 2005) is typically used to assay ATP concentrations in cell-free supernatants after cell or tissue stimulation. To calibrate the concentration of ATP released from the HeLa cells after electrical stimulation, we employed luciferase to construct a calibration curve. In this study, the working range of the ATP bioluminescence Assay Kit HS II was between 10^{-3} and 10^{-9} M ATP (Fig. 3a). After cell culturing, we stimulated the HeLa cells for 10 min using a sine-wave pulse amplitude of 1 V with a frequency of 50 Hz. This electrical stimulation resulted in an 18.4-fold enhancement in ATP release from the HeLa cells (Fig. 3b). In a control experiment, the HeLa cells were maintained for 10 min under the same conditions as the stimulated cells, except for the absence of electrical stimulation; in the absence of electrical stimulation, the cell-cultured solution exhibited no evident change in its bioluminescence signal. It has been reported that high voltage stressing not only induces changes in the cellular membrane structure and permeability but also results in its breakdown (Kumagai et al., 2004). Fig. 3c and d presents typical optical microscope images of the HeLa cells before and after electrical stimulation. We observe no significant differences in the morphologies of these two sets of HeLa cells, possibly indicating that the cellular membranes of the HeLa cells were not damaged upon electrical stimulation. Our results do confirm, however, that electrical activation of HeLa cells results in the release of ATP into PBS buffer solution.

3.4. Sensing ATP in a solution environment

Although the performance of a SiNW-FET biosensor is dependent on the pH and the concentration of the buffer, protein detection using SiNW-FET biosensors is typically performed in serum or tissue culture media at high ionic strength, at which SiNW-FET biosensors lose their inherent sensitivity and selectivity (Cheng et al., 2006). This situation arises because a variation in charge induced beyond the Debye length cannot be detected by a SiNW-FET biosensor because it is shielded by counter ions in the solution environment (Schoning and Poghossian, 2002). As a result, only potential changes that occur within distances on the order of the Debye length can be detected. Therefore, placing targets close to the surface of the SiNW and optimizing the Debye length can dramatically improve the performance of SiNW-FET biosensors.

We compared the performance of our SiNW-FET biosensor in buffers of high (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, and 1.4 mM KH_2PO_4) and low (1.5 μM HEPES buffer at pH 7.5 containing 1 μM $MgCl_2$ and 1 μM EGTA) ionic strength. The Debye screening length (λ_D), which is expressed in Eq. (1) (Stern et al., 2007), was ca. 211.5 nm for the low-ionic-strength buffer and ca. 0.7 nm for the high-ionic-strength buffer solution:

$$\lambda_D = \frac{2}{\sqrt{4\pi l_B \sum_i \rho_i Z_i^2}} \quad (1)$$

where l_B is the Bjerrum length (0.7 nm), $\sum_i$ is the sum over all ion species in the buffer solution, and ρ and z are the density and valence, respectively.

Fig. 4 reveals that Abl-modified SiNW-FETs detected the extracellular ATP released from the HeLa cells. Fig. 4a presents the real-time recorded increases in the responses of the SiNW-FETs upon successive additions of ATP at increasing concentrations in the low-ionic-strength buffer solution. The binding of negatively charged ATP to the kinase catalytic domain increased the negative surface charge density and, thereby, increased the conductance of the SiNWs. We performed control experiments using a SiNW-FET prepared in the same way, except that Abl protein was not coupled to its surface. Non-specific ATP binding caused very small changes in conductance, nearly independent of the different concentrations of the added ATP solution. These experiments reveal that the conductance changes observed for the Abl-modified SiNW device resulted from specific binding of ATP to the Abl tyrosine kinase. These observations also suggest that our devices exhibited high sensitivity toward ATP, in good agreement with results reported by other groups (Wang et al., 2005b).

The binding of negatively charged ATP molecules to the Abl tyrosine kinase also resulted in increases in conductance of the SiNW-FETs in the high-ionic-strength buffer solution (Fig. 4b). The molecular weight of Abl tyrosine kinase is ca. 45 kDa; its size is ca. 3–4 nm. In contrast, the Debye screening length at the SiNW surface is ca. 0.7 nm. Thus, the high ionic strength of the buffer partially screens ATP's intrinsic charge when binding to Abl tyrosine kinase. Because the bound Abl protein on the SiNW surface was not

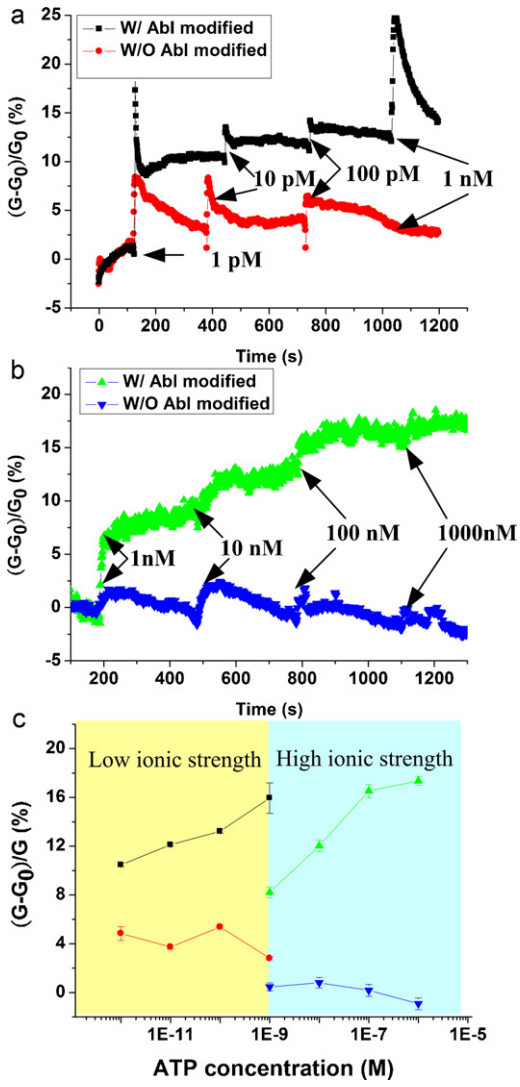

Fig. 4. SiNW-FETs as electronic biosensors for ATP in aqueous solutions of (a) low and (b) high ionic strength. Changes in response $[(G - G_0)/G_0]$ of the SiNW-FETs when monitoring ATP binding onto the Abl. (c) Changes in response plotted with respect to the ATP concentration for the Abl-modified and unmodified SiNW-FETs in buffers of low and high ionic strength. The conductance (G) was normalized with respect to the initial conductance G_0.

well aligned with the device surface, it is, however, possible that the distance between the ATP-binding sites of the tyrosine kinase domain and the SiNW surface was shorter than the Debye length. As a result, we found that ATP concentrations as low as ca. 1 nM could be detected by the Abl-modified SiNW-FET in the high-ionic-strength buffer. The almost negligible change in conductance of the unmodified SiNW-FET indicates that our design, with a value of λ_D of ca. 0.7 nm, effectively screens out the effect of unbound ATP on the sensor. Therefore, in the high-ionic-strength buffer, despite a slight degradation in the SiNW-FET biosensor's sensitivity, its unambiguous selectivity improved significantly (Fig. 4c).

Fig. 5 reveals a typical real-time conductance change of Abl-modified and unmodified SiNW-FETs on detection of the extracellular ATP released from the HeLa cells. The results were obtained when applying a 10 μL aliquot of PBS from the cell-culture chip, after electrical stimulation, onto Abl-modified and unmodified SiNW-FETs. Electrical stress of the HeLa cells caused a large amount of ATP to accumulate in the PBS. These negatively charged ATP species bound to Abl and, thereby, electrically modulated the SiNW conductance. We observed a 1.24 ± 0.04% increase in conductance

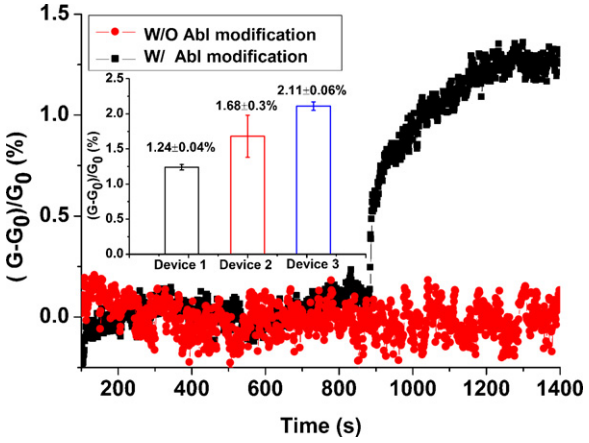

Fig. 5. Detection of extracellular ATP using Abl-modified and unmodified SiNW-FETs. (Inset) Conductance changes of three different Abl-modified SiNW-FETs on extracellular ATP detection. The SiNW-FETs were from same process wafer. The 10 PBS (10 μL) introduced into the sensor chips were from three different cell-culture chips (cultured with same cell density) after electrical stimulation.

of the Abl-modified device, but no significant change in conductance for the unmodified SiNW-FET. The inset of Fig. 5 presents the conductance change observed from three Abl-modified SiNW-FETs on extracellular ATP detection. Fig. S2 presents real-time conductance change of three Abl-modified SiNW-FETs on extracellular ATP detection. Usually, electrical stimulation not only triggers HeLa cells to release ATP into the extracellular environment; but also enhances the inflow of Ca^{2+} ions in exposed cells through the activation of ligand-gated calcium channels (Seegers et al., 2002). Therefore, the conductance of our SiNW-FETs might also be affected by fluctuations of the Ca^{2+} concentration in the sensing environment. Notably, however, in the absence of calmodulin surface modification, SiNWs are immune to changes in Ca^{2+} concentrations, as reported by Lieber's group (Cui et al., 2001).

4. Conclusions

Electrical stimulation at 1 V and 50 Hz for 10 min leads to an 18.4-fold increase in the release of ATP from HeLa cells into the extracellular matrix. We have found that SiNW-FETs (50 nm wide, 30 nm thick) can be used to monitor the levels of ATP at detection limits as low as 1 pM and 1 nM in low- and high-ionic-strength buffers, respectively. After removing an aliquot of the PBS solution containing extracellular ATP immediately after electrical stimulation from cell-culture chip and then placing it onto the SiNW-FET biosensor chip, we observed a 1.24 ± 0.04% increase in conductance of the Abl-modified SiNW device, but no such change for the unmodified device. Combining a cell-culture chip with a SiNW-FET biosensor chip allows the detection of extracellular molecules released from living cells.

Acknowledgments

We thank the National Science Council and the MOE-ATU Program in Taiwan for financial support.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2010.10.003.

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