Nanoelectronic biosensing of dynamic cellular activities

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**NANYANG
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UNIVERSITY**

NANOELECTRONIC BIOSENSING OF
DYNAMIC CELLULAR ACTIVITIES

**NANOELECTRONIC BIOSENSING OF DYNAMIC
CELLULAR ACTIVITIES**

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SCHOOL OF CHEMICAL AND BIOMEDICAL ENGINEERING

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NANOELECTRONIC BIOSENSING OF DYNAMIC CELLULAR ACTIVITIES

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Table of Contents

Acknowledgements	i
Table of Content.....	ii
List of Figures.....	v
List of Abbreviations	xi
Abstract.....	xiii
Chapter 1 Introduction	1
1.1 Nanoelectronic biosensing based on semiconducting nanostructured materials .	1
1.2 Biosensors based on SiNW- and SWNT- FETs	3
1.3 Detecting dynamic cell secretion of biomolecules	8
1.4 Detecting cellular bioelectricity resulting from ion channel activities	13
1.5 Research objectives and overview of thesis.....	16
Chapter 2 Nanowire Fabrication and Characterization	18
2.1 Introduction.....	18
2.2 Poly-silicon nanowire fabrication	19
2.3 Crystalline silicon nanowire fabrication	23
2.4 The experimental set-up and nanowire characterization	26
2.5 Results and discussion	29
2.6 Conclusions.....	35
2.7 Acknowledgements.....	36

Chapter 3	Ultrasensitive detection of adipocytokines with CMOS-compatible Silicon nanowire arrays	37
3.1	Introduction.....	37
3.2	Materials and Methods.....	39
3.2.1	Molecules and chemicals	39
3.2.2	SiNW functionalization	39
3.2.3	Electrical measurement	40
3.2.4	Cell culture and differentiation	41
3.3	Results and discussion	42
3.4	Conclusions.....	50
3.5	Acknowledgements.....	51
Chapter 4	Nanoelectronic detection of triggered secretion of pro-inflammatory cytokines using CMOS compatible silicon nanowires	52
4.1	Introduction.....	52
4.2	Materials and Methods.....	54
4.2.1	Molecules and chemicals	54
4.2.2	SiNW functionalization	54
4.2.3	Electrical measurement	55
4.2.4	Cell culture.....	55
4.3	Results and discussion	56
4.4	Conclusions.....	63
4.5	Acknowledgements.....	64
Chapter 5	CMOS-compatible nanowire sensor arrays for detection of cellular bioelectricity	65
5.1	Introduction.....	65
5.2	Materials and Methods.....	66

5.2.1	Recording on nanowire chips.....	66
5.2.2	Cell isolation and culture	67
5.3	Results and discussion	68
5.4	Conclusions.....	74
5.5	Acknowledgements.....	75
Chapter 6 Non-invasive measurement of cellular bioelectricity based on carbon nanotube devices for high throughput drug screening.....		76
6.1	Introduction.....	76
6.2	Materials and Methods.....	77
6.2.1	Fabrication of SWNT-net device	77
6.2.2	Culturing HL-1 cells on SWNT-net device	77
6.2.3	Electrical recording	78
6.3	Results and discussion	78
6.4	Conclusions.....	92
6.5	Acknowledgements.....	92
Chapter 7 General Conclusions and Perspectives		93
7.1	General Conclusions	93
7.2	Perspectives.....	94
References		97
List of Publications		106

List of Figures

Figure 1.1	Multiplexed electrical detection of three cancer marker proteins. (a). Specific proteins detection by three SiNW devices in an array. The NW1, NW2 and NW3 were modified with mAbs for PSA, CEA and mucin-1, respectively. (b). Conductance versus time curve of three NWs in response to PSA, CEA and mucin-1. Black arrows indicate the points where the solution is injected followed sequentially as 1) 0.9 ng/ml PSA, 2) 1.4 pg/ml PSA, 3) 0.2 ng/ml CEA, 4) 2pg/ml CEA 5) 0.5ng/ml mucin-1, 6) 5pg/ml mucin-1.	5
Figure 1.2	Carbon nanotubes are modified with polymer layer (PEI and PEG).	7
Figure 1.3	(a) Schematic of p-type silicon nanowire sensing in response to T-cell activation. Prior to T-cell activation, a majority of the nanowire's silanol groups are deprotonated. After activation, the release of proton (H^+) increased protonation of the surface silanol groups and subsequently (b) leads to decrease in nanowire conductance. (c) Real time electrical measurement of extracellular acidification when B6 plerocytes were stimulated with anti-CD3.	10
Figure 1.4	a) Schematic of SWNT-net to detect ATP molecules after glutamate stimulation. b) Real-time electrical detection of ATP molecules. Upon injection of glutamate, transient current spikes were detected.	12
Figure 1.5	Schematic of SWNT FET modified with CgA antibody to detect CgA. (a) Glutamate stimulates neurons and increase of Ca^{2+} into the cytosol cause to release CgA. The CgA will then bind to CgA antibody functionalized on the SWNT FET. (b) Real-time electrical measurement of CgA. The neurons were stimulated by glutamate.	13
Figure 1.6	Nanowire recording from axon. a) Optical image of the axon grew on 50 SiNWs. The SiNW is uniformly spaced at 10 nm. b) Transient nanowire current signal recorded by the 50 individual SiNWs.	15
Figure 2.1	Poly-silicon nanowire array fabrication processes	19
Figure 2.2	SEM micrograph of nanowire fabrication processes. (a) Patterning on polysilicon followed with fin etching and oxidation with targeted fin width about 30 to 50 nm. (b) Contact open process opens a small hole at both ends of fin structures to create contact of metal with silicon. (c) Metal deposition followed by etching to form extended metal contact pad. (d) Passivation followed by HF etching release nanowire from oxide layer and have silicon nanowire width in 30 to 50 nm.	21

Figure 2.3	Fabricated polysilicon nanowire chip. (a) Optical image of a nanowire chip with two independent nanowire sensing regions. The chip is integrated with micro fluidic channel. (b) Micrograph taken by the scanning electron microscope showing that well aligned nanowire is separated at 2 μm interval and each wire is individually connected to metal lines with silicon nitride passivation. The insert illustrates the rectangular cross-section of a nanowire by TEM imaging.	23
Figure 2.4	Crystal nanowire array fabrication processes	24
Figure 2.5	(a) Optical image of a <i>crystalline</i> silicon nanowire chip with 40 clusters of 5 silicon nanowire sensors each (b) Zoom – in SEM image shows a cluster with 5 wires. (c) TEM cross-section of 5 wires in a cluster (d) TEM of a rectangular nanowire.	26
Figure 2.6	The schematic diagram of electrical testing set up. (a) Microscope and measurement platform are housed in a faraday cage and supported by anti-vibration table. Data is measured using axon patch clamp amplifier and analyzed by computer. (b) The SiNW chip is put on the stage and each individual nanowire is addressed under the microscope. (c) The zoom-in optical image (red rectangular in b) to illustrate two probe tips contact the metal pads.	28
Figure 2.7	Schematic of nanowire device measurement. While a voltage bias (V_{ds}) is applied across a nanowire, its drain-source current (I_{ds}) can be continuously monitored. Front gate voltage is applied through Ag/AgCl electrode immersed in the saline solution. Back-gate voltage is applied to bottom of silicon substrate.	29
Figure 2.8	Electrical characterization of silicon nanowire. (a) Nanowires conductance in an array is narrowly distributed. The Solid curve is a Gaussian fitting. (b) Typical I_{ds} - V_{ds} curve of individual silicon nanowire for varying back-gate voltage, V_g from -10 V to 10 V in 2 V steps (indicated by the dark arrow). The characteristic shows n-type FET behavior. (c) Measured conductance versus front-gate voltage of a nanowire swept from -1 V to +1 V at V_{SD} , 400 mV. The trans-conductance is 30 nS ($V_g = 0\text{V}$).	31
Figure 2.9	A normalized current power spectrum from a silicon nanowire biased at 400 mV. At low frequencies, $1/f$ noise dominates. The dark fitting curve indicated nanowire has $1/f$ noise amplitude A (4.4×10^{-9}) and exponent β (1.1) for frequency up to 70 Hz. The dashed horizontal line marks the thermal noise estimated from nanowire conductance and feedback resistor in the amplifier.	33
Figure 2.10	Nanowire current recorded in the bath solution for 30 minutes.	34

Figure 2.11	Electrical characterizations of crystalline silicon nanowires. (a) Nanowires conductance in an array is narrowly distributed. The conductance is measured from 90 nanowires in an array. The Solid curve is a Gaussian fitting. (b) Measured conductance versus front-gate voltage at V_{SD} , 200 mV. The conductance of the n-type SiNW increased dramatically from 29.2 nS to 238.0 nS as V_g swept from -1 to +1 V.	35
Figure 3.1	Phase-contrast TEM images of 3T3-L1 cells before (a) and after (b) differentiation into adipocytes (the white scale bars correspond to 100 μ m). The inset in (b) shows the red staining of the fat droplets in adipocytes.	42
Figure 3.2	a) Schematic illustration of a nanowire chip functionalized with leptin antibodies and resistin antibodies in two separated sensing regions. b) The change in nanowire conductance relative to the conductance of the bare nanowire (1. reference), ΔG , resulting from each functionalization step (2, 3, and 4)..	44
Figure 3.3	Change in nanowire conductance versus the concentration of leptin (a) and resistin (b). Each data point was averaged from the measurements of 30 nanowires on 3 different chips functionalized with both leptin and resistin antibodies. The error bars indicate the standard deviations. The fittings (solid lines) indicate that the change in nanowire conductance is logarithmically proportional to the adipocytokine concentration.	46
Figure 3.4	The silicon nanowire modified with leptin antibodies (a) or resistin antibodies (b) did not response to bovine serum albumin (BSA) (100 μ g/ml or 1.4 μ M in PBS) and bovine serum (10% v/v in DMEM medium). The black line represents the nanowire conductance measured in 1 mM PBS without pre-treatment. The red line represents the nanowire conductance measured in 1 mM PBS after 30-minutes incubation with BSA solution. The blue line represents the nanowire conductance measured in 1 mM PBS after 30-minutes incubation with serum-containing medium.	47
Figure 3.5	Kinetics of leptin (a) and resistin (b) secretion from 3T3-L1 adipocytes are shown. Two dually functionalized nanowire chips were used to analyze the aliquots of culture medium collected from a flask of cell culture over a time period of 4.5 hours with (red triangles) and without (black circles) GPI stimulation, respectively. Each data point was averaged from the measurements on 5 nanowires on the same chip. The error bars represent the standard deviations. Leptin and resistin concentrations were determined based on the changes in nanowire conductance and the calibration curves presented in Fig. 3. Each data set is fitted by a sigmoidal function: $C_{sat}/(1+\exp((t_{half} - t)/\tau))$ to obtain the saturation	48

concentration (C_{sat}), half-time (t_{half}), and rate constant (τ). For basal leptin secretion, $C_{\text{sat}} = 2.54$ pM, $t_{\text{half}} = 166.4$ minutes, $\tau = 33.5$ minutes. For GPI stimulated leptin secretion, $C_{\text{sat}} = 7.98$ pM, $t_{\text{half}} = 204.5$ minutes, $\tau = 34.7$ minutes. For basal resistin secretion, $C_{\text{sat}} = 3.09$ pM, $t_{\text{half}} = 169.6$ minutes, $\tau = 20.6$ minutes. For GPI stimulated resistin secretion, $C_{\text{sat}} = 0.59$ pM, $t_{\text{half}} = 149.3$ minutes, $\tau = 17.8$ minutes.

- Figure 4.1** (a) Schematic illustration of SiNW chip dually functionalized with antibodies against TNF-alpha or IL-6. (b) The percentage change in SiNW conductance ($\Delta G/G_0$) before (1. reference) and after each functionalization step (2, 3, and 4). **57**
- Figure 4.2** Detection of TNF-alpha (a) and IL - 6 (b) with antibody functionalized SiNW chips. Perfusion of TNF-alpha or IL-6 containing buffer solution caused increase or decrease of the nanowire conductance in concentration dependent manner (left panel). Perfusion of buffer solution without cytokine or with serum supplemented cell culture medium which contains a large variety of proteins and other biomolecules did not alter the nanowire conductance. The percentage change of nanowire conductance is linearly proportional to the logarithmic concentration of the added cytokines (right panel). Each data point (mean +/- SEM) is the average from 10 SiNWs in 3 chips. **59**
- Figure 4.3** Time course of cytokine secretion measured from the macrophage culture medium (a) and blood serum from neonatal rats (b) collected at different time points after macrophages were stimulated with 100 ng/ml LPS or after intraperitoneal injected 50 μ g LPS to rat. The concentrations of TNF-alpha (middle) or IL-6 (bottom) in the culture medium or serum were calculated using the calibration curve presented in Figure 4.2. Each data point (mean +/- SEM) is the average from 10 SiNWs in 3 chips. **61**
- Figure 5.1** ECG recording from an intact beating heart. a) A beating rat heart was placed in the recording chamber on top of a wirebonded nanowire chip. b) Nanowire current was monitored at a voltage bias of 400 mV. In the left panel, a train of current spikes that was in concert with the heart beating is illustrated. A typical transient current event is shown in the right panel. c) Voltage signals recorded by an impaling nanopipette electrode into the heart. They are similar to the nanowire responses. **69**
- Figure 5.2** Nanowire recordings on individual cardiomyocytes. a) The cardiomyocytes were isolated from the hearts of neonatal rats and cultured on the nanowire chip. Two nanowires (I and II) are indicated by dark lines. b) The upper trace depicts the recorded nanowire current from nanowire-I. The lower trace is the recording **71**

from nanowire-II which was covered by a contracting myocyte. It showed a train of transient current events. c) A typical episode of the nanowire response from nanowire-II, involving one upstroke current followed by a downward one, is shown on the left. For comparison, an intracellularly recorded action potential using a nanopipette is depicted on the right.

Figure 5.3	Nanowire recordings from aortic smooth muscle cells. a) Rat aortic smooth muscle cells (A7r5) grew confluent on the nanowire chip. Nanowire sensing area is indicated by the dashed square. b) Transient nanowire current signals were induced by membrane depolarizing high K^+ solution. A typical current spike is highlighted in a smaller time scale.	72
Figure 6.1	SWNT-net device. (a) Illustration of SWNT-net device configured as liquid-gated field-effect transistor, and relation of source-to-drain conductance versus gate voltage applied to the bath solution from a typical device. $V_{ds} = 10$ mV. (The leak current measured is less than 1 nA) (b) SEM image of part of the SWNT-net ring as indicated by a square in a (not to scale). c. , d. and e. Zoom-in SEM images of different regions on SWNT-net as indicated by squares in b .	79
Figure 6.2	(a) Phase-contrast image of cardiomyocytes grown on the SWNT-net to confluence after 4-days culturing. (b) Illustration of simultaneous extracellular recording using a SWNT-net device and intracellular recording using an impaling nanopipette of bioelectrical signals (action potentials) from cardiomyocytes grown on top of the SWNT-net. (c) Spontaneous and periodic action potentials recorded intracellularly by the nanopipette (top) and simultaneous current responses of SWNT-net (bottom).	81
Figure 6.3	Four types of signal recorded by SWNT-net.	82
Figure 6.4	Action potentials (top traces) of HL-1 cell and SWNT-net responses (middle and bottom traces) before (red) and after (blue) application of TTX (a) or verapamil (b).	84
Figure 6.5	Action potentials (top traces) of HL-1 cell and SWNT-net responses (middle and bottom traces) before (red) and after (blue) application of TTX (a) or verapamil (b) or Cch (c).	85
Figure 6.6	Action potentials (top traces) of HL-1 cell and SWNT-net responses (middle and bottom traces) before (red) and after (blue) application of TTX (a) or verapamil (b) or propranolol (c).	86
Figure 6.7	Action potentials (top traces) of HL-1 cell and SWNT-net responses (middle and bottom traces) before (red) and after (blue) application of TTX (a) or verapamil (b).	87

Figure 6.8

ECG signals detected by SWNT-net FET. (a) SWNT-net response (left) to ECG signal produced by a beating rat heart at different orientations (i, ii, and iii). In the cartoons shown at right, A refers to atrium and V refers to ventricle. In the experiments denoted as iii, a nanopipette impaled into a ventricle was used to detect ECG of the ventricle simultaneously. The ECG signal recorded by the nanopipette coincides with the major spike in the SWNT-net signal. (b) and (c) The frequency and the features of the SWNT-net signal are sensitive to verapamil (b) and TTX (c).

89

List of Abbreviations

APTES	(3-Aminopropyl) triethoxy-silane
BSA	bovine serum albumin
CEA	carcinoembryonic antigen
CMOS	Complementary Metal Oxide Semiconductor
CNTs	carbon nanotubes
DA	Dopamine
ECG	electrocardiogram
ECM	extracellular matrix
ELISA	enzyme-linked immunosorbent assay
FETs	field effect transistors
GPI	glucose pyruvate insulin
HDP	high density plasma
HF	hydrogen fluoride
IgE	immunoglobulin E
IL-1	interleukin-1
IL-6	interleukin-6
LOD	limit of detection
LBP	lipopolysaccharide binding protein
LPS	lipopolysaccharides
mAbs	monoclonal antibodies
mAChR	muscarinic acetylcholine receptor

MEAs	microelectrode arrays
mRNA	messenger Ribonucleic acid
PBS	phosphate buffered saline
RIA	radio-immunoassay
RMS	root mean square
SEM	scanning electron microscopy
SiNWs	silicon nanowires
SiNW-FET	silicon nanowire field effect transistor
SOI	silicon on insulator
SWNTs	Single walled carbon nanotubes
SWNT-net	single walled carbon nanotube network
TEM	transmission electron microscopy
TNF-α	tumor necrosis factor - alpha
TTX	tetrodotoxin
USG	undoped silicon glass

Summary

Detecting dynamic cellular activities such as exocytosis and bioelectricity is important in the field of molecular biology and medicine. Comparing to conventional biochemical assay, biophysical measurements, and optical methods, electronic biosensing based on nanomaterials have shown promising platform in resolving rapid biological events with ultra sensitivity, high spatial and temporal resolution. Here, we use silicon nanowire (SiNW) and single walled carbon nanotube (SWNT) configured as field effect transistor to detect dynamic cellular activities, in particular the time course of cytokines secreted from immune cells or adipose cells and bioelectrical activities of ion channels from electrogenic cells.

In this thesis, perfectly aligned arrays of long SiNWs were fabricated using top-down CMOS (complementary metal oxide semiconductor) compatible fabrication techniques and standardized in large-scale production of microelectronics. We demonstrate that these nanowire devices are able to detect cytokines secreted from cells with femtomolar sensitivity, high specificity, wide detection range, and ability for parallel multiplexed monitoring. The detection platform provide a novel tool to reveal the poorly understood signaling mechanisms of the molecules as well as their relevance in secretion related disease.

We also demonstrate that single walled carbon nanotubes network (SWNT-net) can be easily fabricated without necessity of micro/nano fabrication approach. Nanoelectronic sensor based SiNWs and SWNT-net can be used to non-invasively detect cellular bioelectrical signals with high-throughput, high sensitivity, ease of use and the capability

of long term continuous real time monitoring. Furthermore, nanoelectronic sensors also enable detection of electrocardiogram (ECG) signals from a beating rat heart. Taken together, we show that our nano devices can detect bioelectricity not only at cell level but also at tissue level. This method promise applications in both fundamental research and high-throughput drug screening targeting on ion channels.

Chapter 1

Introduction

1.1 Nanoelectronic biosensing based on semiconducting nanostructured materials

The advent of semiconducting nanostructured materials has opened up new avenues in the field of biosensors, due to their remarkable structural, electrical, and physiochemical properties. Their molecular dimension allows intimate interaction with biomolecules or biostructures, and permits the fabrication of miniaturized biosensors with small sensing elements for localized detection. Their unique physiochemical properties enable creation of versatile nano-bio-interfaces. And as the charge carriers largely or solely flow on their surface, biomolecules or biological processes that come into close interaction with nanomaterials induce significant conductance change. This renders them particularly suitable for electronic biosensing. The nanostructured materials such as single-walled carbon nanotube (SWNT) [1-3], nanowires [4-10], graphene [11-15] nanopores [16], and nanogaps [17-19] have been employed to develop various nanoelectronic biosensors.

Electronic sensing based on nanomaterials provides many superior advantages as compared to the conventional biochemical assay, biophysical measurements, and optical methods. Owing to their molecular dimension as well as their novel sensing mechanisms, nanoelectronic biosensors exhibit exquisite sensitivity. For examples, single virus detection and single gas molecule detection have been achieved using silicon nanowire sensors [20] and graphene sensors [21], respectively. In addition, it is suitable for the

development of miniaturized lab-on-a-chip systems and integrates it with electronic readouts. Furthermore, it provides simple, non-invasive, label-free, high-throughput detection with high temporal resolution. Being the rapid sensing modality, it is particularly ideal for detection of dynamic biological processes.

Among the variety of nanomaterials, silicon- and carbon-based semiconducting nanomaterials are perhaps most favorable to serve as the sensing elements in nanoelectronic biosensors. Silicon is the building block of modern electronics. Lithographic techniques standardized in the large-scale production of microelectronics can be used to fabricate miniaturized silicon-based nanoelectronic biosensors with potential of mass production. On the other hand, as carbon forms the backbone of biomolecules for all life on earth, many biomolecules can interact with carbon nanomaterials via *pi-pi* interactions and these materials often exhibit biocompatibility. In this thesis, we have utilized both silicon nanowires and carbon nanotubes for biosensing. These two one-dimensional materials share some common merits such as high field-effect properties. On the other hand, they provide different surface chemistry for different applications. For the silicon nanowire devices, we used single silicon nanowire as the sensing element which provides spatially resolved detection and its properties can be altered by the type of dopant used. For carbon nanotube devices, we used carbon nanotube network as the sensing element. In contrast to nanowire chips which require sophisticated fabrication techniques and processes, carbon nanotube devices that we used can be readily fabricated on bench-tops at low cost. And carbon nanotube network can be fabricated on flexible substrates to conformally attach to the curved target, such as, an organ.

Various SiNW-FETs and SWNT-FETs have been successfully employed as biosensors [1, 3, 22, 23]. However, most of these sensors are merely restricted to static detection of the presence of biological species. Here, we have used these nanoelectronic sensors to electrically detect various dynamic cellular activities, specifically, secretion of biomolecules and bioelectricity due to ion channel activities. These two processes are fundamental and ubiquitous, and underlie many critical diseases such as cardiovascular diseases, hormone disorders, neurodegenerative diseases, etc. We envision that the techniques developed here are instrumental not only to high throughput functional studies in biology but also to high throughput drug screening.

1.2 Biosensors based on SiNW- and SWNT- FETs

Both SiNWs and SWNTs are 1D semiconducting materials in which their tunable charge carriers transport due to quantum confinement have allowed them to transduce electrical signal upon biomolecules interaction, operating analogous to a field effect transistor (FET). Compared to conventional FETs where the conducting channel is buried underneath a dielectric insulating layer, the SiNW or SWNT in a nanoelectronic sensor is in direct contact with the sensing environment. Biomolecules may directly interact with these nanomaterials or bind with the recognition elements pre-conjugated on the surface of nanomaterials. The interaction or binding may cause change of nanomaterial conductance due to change of the local dielectric environment, or electrostatic gating (field effect) imposed by the surface charge of biomolecules or charge transfer between the molecules and nanomaterials. The former is likely due to change in gate efficiency

coupling, where capacitance of the gate is reduced because of the low permittivity of the biomolecules relative to the electrolyte. Interacting molecules may inhibit the binding of ion in the electrolyte to come closer to the sensor surface, thereby reduce the electrical conductance. Electrostatic gating or charge transfer is an effect caused by absorption of charged species that induce a screening mechanism in the nanomaterials. Donor of electron charge from biomolecules cause shift in a transistor transfer characteristic. Conversely, withdrawal of electron from sensor will cause adverse effect.

SiNW- and SWNT- FETs have been utilized as various biomolecular sensors. Zheng et al have demonstrated multiplexed detection of three cancer markers proteins, PSA, carcinoembryonic antigen, and mucin-1, in undiluted serum at femtomolar concentration (Figure 1.1) [23]. Compared to conventional biochemical assays (e.g., ELISA), this SiNW FET sensor offers label-free, high sensitivity and multiplexing detection. Similarly, Kim et al describe the real time label free detection of PSA in phosphate buffered solution down to 30 aM concentration using antibody-modified *n*-type SiNWs [7]. As expected, binding of negatively charged PSA (isoelectric point of 6.9) with its antibody at pH 7.6 buffer solutions decreased the nanowire conductance. Conversely, binding of positively charged PSA with its antibody at pH 6.0 solution increased the nanowire conductance. These observations confirm that charged PSA can electrostatically gate the nanowire conductance. As another example, detection of immunoglobulin G and immunoglobulin A at 100 fM using antibody modified SiNW have been elucidated by Stern et al [4].

Figure 1.1 Multiplexed electrical detection of three cancer marker proteins. (a). Specific proteins detection by three SiNW devices in an array. The NW1, NW2 and NW3 were modified with mAbs for PSA, CEA and mucin-1, respectively. (b). Conductance versus time curve of three NWs in response to PSA, CEA and mucin-1. Black arrows indicate the points where the solution is injected followed sequentially as 1) 0.9 ng/ml PSA, 2) 1.4 pg/ml PSA, 3) 0.2 ng/ml CEA, 4) 2pg/ml CEA 5) 0.5ng/ml mucin-1, 6) 5pg/ml mucin-1[23].

SiNW- FETs also have been used for the detection of DNA hybridization. Hahm et al reported that peptide nucleic acid (PNA) receptors modified on SiNW devices allow to detect PNA-DNA hybridization down to 10 fM concentrations [24]. This is expected because binding of negatively charged DNA with PNA increases the negative surface charge density around the *p*-type nanowire, and consequently induces holes accumulation (positive charge carriers) and increase of the nanowire current. This sensor is able to differentiate wild type gene of the cystic fibrosis transmembrane receptor from the $\Delta F508$ mutation. In another report, 12-mer probe oligonucleotide modified *p*-type nanowire is

able to detect the target ssDNA molecules with a detection limit of 25 pM [25]. In addition, SiNW- FETs have also been used for the detection of single virus [20], neurotransmitter [6], and small molecules [5].

SWNT- FETs have also been widely used in biosensing applications. Many molecules can interact with the SWNTs through hydrophobic or *pi-pi* interaction. To fetch and specifically detect certain molecule in a complex solution, recognition elements need to be functionalized onto SWNTs. Dai and co-workers reported that bio-recognition elements conjugated to polyethylene oxide functionalized SWNT enable specific protein detection [26]. First, repelling agents to biomolecules, Tween-20, were applied to the nanotubes. This step modified nanotube surface with polyethylene oxide (PEO) chain. Bio-recognition elements were then covalently linked to the PEO-functionalized tube. They used this approach for specific detection of mAbs to the human autoantigen U1A. Alternatively, Star et al used other biomolecule-repelling agents, poly(ethylene imine) (PEI) and poly(ethylene glycol) PEG, to noncovalently functionalize the nanotube [27]. The polymer coated devices were then biotinylated by incubate them in a 15 mM DMF solution of biotin-N-hydroxysuccinimide ester. SWNT-FETs functionalized as such were used to specifically detect binding of streptavidin which introduces scattering sites on the nanotube and subsequently reduces the nanotube conductance (Figure 1.2) [27].

Figure 1.2 Carbon nanotubes are modified with polymer layer (PEI and PEG) [27].

SWNTs can be non-covalently functionalized with linker molecules for subsequent covalent functionalization of probe proteins. For example, Besteman and co-workers immobilized glucose oxidase (GOD) onto SWNT via a linker molecule (1-pyrenebutanoic acid succinimidyl ester), which on one end firmly attaches to the nanotube sidewalls through *pi-pi* interaction with a pyrene group and on the other end covalently reacts with the amino group on the enzyme by an amide bond [3]. With addition of glucose, the conductance of GOD-functionalized SWNT device increased, probably due to the products of the catalytic reaction or conformational and charge state changes of GOD.

Another important application of SWNT-FET is the detection of DNA hybridization. Single strand DNAs (ssDNAs) could wrap around SWNT via hydrophobic interaction

and/or *pi-pi* stacking between nucleobases and nanotube sidewalls, which induced electrical doping of SWNT [28]. After the complementary DNAs were incubated with the ssDNAs-immobilized SWNT device, hybridizations specifically occurred and caused negative-shift of the conductance curve. SWNT-FET has also been applied for detection of pathogenic bacteria by functionalized with aptamers [29] or antibodies [30].

The application of SiNW and SWNT biosensor discussed so far, however are only used in static detection of biological targets. There are only a few examples of using SiNW- and SWNT-FETs to detect dynamic biological processes, which will be briefly reviewed in the following sections.

1.3 Detecting dynamic cell secretion of biomolecules

Regulated cell secretion (exocytosis) is a dynamic process by which secretory vesicles discharge their contents to the extracellular environment when they are fused with cell membrane [31]. This highly regulated process is mediated by a plethora of proteins and is essential to many cellular functions. For examples, neurotransmitters released from the presynaptic neuron allow the transmission of signals between neurons [32]. Hormone secretion (insulin, glucagon, somatostatin, and so on) from endocrine cells regulate many aspects of metabolism [33]. The malfunction of exocytosis process can lead to neurological disease [34], hormone disorders [35] and so on. Therefore, developing experimental methods for study exocytosis is important to understand this fundamental and ubiquitous cell function and to drug discovery targeting on this process.

Regulated cell secretion is conventionally analyzed by biochemical assays. This method is limited to very low sensitivity and poor temporal resolution. Furthermore, it only provides averaged ensemble measurements from a population of cells. Alternatively, electrophysiological method, membrane capacitance measurement, can detect exocytosis at a single cell level with high temporal resolution and sensitivity. However, there is limitation associated with the membrane capacitance measurement. For example, it is invasive (involving disruption of cell membrane), thus, not suitable for long-term recording. The electrochemical detection using microfiber electrode can also be used to monitor exocytosis [36-39]. However, this method is only applicable to oxidizable molecules released from cells. Furthermore, the small sensing electrode is only limited for detection of substances from small region on the cell surface. Most notably, both electrophysiological and electrochemical methods are not possible for high-throughput detection due to several requirements such as high skills, expensive equipments and tedious procedures.

SiNWs and SWNTs based nanoelectronic sensors may provide alternative novel platforms to resolve the dynamic cell secretion of biomolecules. SiNW-FETs were used to detect antigen-specific T-cell immune response [40]. When activated by anti-CD3, T-cells release acidic metabolites to cause extracellular acidification (Figure 1.3). As SiNW surface exhibits silanol groups (hydroxyl groups bound to silicon) in solution, acidification at the narrow gap between the SiNW and the cell membrane leads to decrease of negative charges, and thus decrease of the *p*-type nanowire conductance. The immuno-response of a small number of T-cells (~210 cells) can be detected.

Figure 1.3 (a) Schematic of p-type silicon nanowire sensing in response to T-cell activation. Prior to T-cell activation, a majority of the nanowire's silanol groups are deprotonated. After activation, the release of proton (H^+) increased protonation of the surface silanol groups and subsequently (b) leads to decrease in nanowire conductance. (c) Real time electrical measurement of extracellular acidification when B6 plasmacytoma cells were stimulated with anti-CD3 [40].

Nanoelectronic transistors based on single-walled carbon nanotube network (SWNT-net) field effect transistor have recently been used to detect the dynamic release of biological molecules from cells. SWNT-net FETs have been coupled with living neuroglial astrocytes to detect the ATP biomolecules release [41]. In this studies, neurotransmitter glutamate molecules applied in the solution can bind to target receptors on the membrane of astrocytes, whereby Ca^{2+} influx is elicited and the vesicular secretion of ATP molecules is triggered (Figure 1.4). Upon stimulation, transient current spike from SWNT-net were observed. This is because ATP molecules are negatively charge and their released at the vicinity of the SWNT impose *p*-doping effect. Thus, the nanotube conductance shows increasing as depicted in Figure 1.4b. One single spike consists of an initial rapid rising phase of ~ms and a long-lasting decaying phase of ~s, likely corresponding to release of ATP from single astrocytes. The results indicated this SWNT-net platform could detect not only the local ATP release from living astrocytes, but also the kinetics of single cell secretion.

Figure 1.4 a) Schematic of SWCNT-net to detect ATP molecules after glutamate stimulation. b) Real-time electrical detection of ATP molecules. Upon injection of glutamate, transient current spikes were detected [41].

However, the detection in this example relies on that the target biomolecules can interact directly with the sensing nanomaterials and alter their conductance. The specificity of detection relies on the specific biological contexts. In many cases, specific detection requires functionalization of recognition elements. Wang et al have showed that glutamate stimulated chromogranin A (CgA) released from living cortical neurons can be specifically detected by immunoglobulinG (IgG) antibody functionalized FETs based on single SWNT (Figure 1.5), with a low detection limit of 1 nM [42]. The negative charges of CgA cause increase in channel conductance of *p*-type SWNT.

Figure 1.5 Schematic of SWNT FET modified with CgA antibody to detect CgA. (a) Glutamate stimulates neurons and increase of Ca^{2+} into the cytosol cause to release CgA. The CgA will then bind to CgA antibody functionalized on the SWNT FET. (b) Real-time electrical measurement of CgA. The neurons were stimulated by glutamate [42].

1.4 Detecting cellular bioelectricity resulting from ion channel activities

Electrogenic cells such as muscle and neuron cells are able to generate electrical signals which are commonly found in the form as action potentials (transient increase of membrane potential with millisecond time scale and amplitude of ~ 100 mV). The action potentials are produced by protein ion channels embedded within the plasma membrane. Bioelectricities are essential to many biological functions, such as controlling muscle contraction and communication between neuron [43]. Dysfunction of ion channels is responsible for many diseases including neurological disease [44-48], cardiovascular diseases [49, 50], and so on [35]. Hence, ion channels are the primary drug targets.

Electrophysiological technique (patch clamp) or fluorescence methods based on voltage-sensitive are currently used to measure the change of transmembrane potential due to the ion channel activities. Although patch-clamp technique is able to detect single ion channel electrical activities [51-53], it is invasive and thus does not allow recording beyond ~10 mins. And it is very low throughput due to tedious procedures and delicate equipments required. Only one cell can be recorded at a time with low success rate. Voltage sensitive dyes can be used to probe the rapid changes in membrane potential [54-56]. However, the toxicity of the dyes to the cells upon illumination makes it unsuitable for long term recordings. And the availability and sensitivity of these dyes largely limit their applications.

Bioelectricity can be recorded extracellularly (thus non-invasively) at multi-sites in a cell network using microfabricated microelectrode arrays (MEAs) [57-63] or conventional field effect transistors (FETs) [64-69]. But MEAs and conventional FETs are limited to low sensitivity and is not capable to detect signal from small cellular structures such as neurites (<100 nm in diameter) due to their micro-sized sensing elements. Therefore, nanoelectronic biosensors using SiNWs and SWNTs as the sensing elements may provide new opportunities because of their small size and exceptional electrical properties.

SiNW FETs to detect cellular electrical activity was first demonstrated by Lieber's group [70]. The devices were fabricated using bottom-up approach. Specifically, short *p*- or *n*-type SiNWs were pre-synthesized followed by non-trivial assembly onto fabricated metal electrode arrays. The living neurons isolated from rat brain were cultured on top of the SiNWs array. Each individual neuron was directed to grow across multiple nanowires

with the help of polylysine patterning on the nanowire surface. The action potentials of neurons were detected as transient increases of SiNW current (Figure 1.6). This is because trans-membrane ionic current flows at the narrow gap between neuron and SiNW alter the surface potential. As a consequence, it changes the SiNW conductance. It is also important to note that the small active junction of device ($0.02 \mu\text{m}^2$) enables localized detection of action potential and at very high spatial resolution. Furthermore, the authors also demonstrated action potential propagation along a long axon was successfully resolved with the array format of the SiNW sensors. In addition, SiNW FETs can also electrically inhibit the propagation of nerve impulses (action potentials). In another study, Liber's group also using SiNW FETs fabricated on flexible polymer substrates to couple with embryonic chicken heart and detect its bioelectricity [71].

Figure 1.6 Nanowire recording from axon. a) Optical image of the axon grew on 50 SiNWs. The SiNW is uniformly spaced at 10 mm. b) Transient nanowire current signal recorded by the 50 individual SiNWs [70].

Carbon nanotube networks have been coupled with living cells. In recent studies, carbon nanotube substrates have been shown can enhance cell attachment, growth and differentiation [72-75]. Furthermore, neurons grown on a conductive carbon nanotube network exhibited higher efficacy of neural signal transmission [76]. This is attributed to tight contact of carbon nanotubes with the cell membrane, which creates electrical shortcuts between the proximal and distal compartments of neuron. Besides, it has been shown that electrical stimulation via SWNT-net can impose neuronal action potential [77]. Despite the favorable electrical interface between nanotubes and excitable cells, carbon nanotubes have not been utilized to detect bioelectricity.

1.5 Research objectives and overview of thesis

The goal of this thesis is to fabricate nanoelectronic sensor based on SiNWs and SWNTs and demonstrate their ability to detect cellular activities, specifically, dynamic secretion of biomolecules and bioelectricity. We also aim to provide proof-of-concept demonstrations that such nanoelectronic approach is instrumental to fundamental research in cell biology and in high throughput drug discovery.

Perfectly aligned SiNW array are fabricated using top-down approach compatible to CMOS (Complementary Metal Oxide Silicon) technology (Chapter 2). The SiNW FETs exhibit prominent field-effect, therefore, are ideal for detecting biological signals in solution.

In chapter 3 and 4, we demonstrate that SiNWs can be readily functionalized with antibodies as recognition elements; and the functionalized SiNW FETs are able to detect adipokines secreted by the adipose cells and pro-inflammatory cytokines secreted from immune macrophages in a complex biological solutions (culture medium or blood serum) with a simple and label-free electronic detection scheme, femtomolar sensitivity, high specificity, wide detection range, and ability for parallel detection of multiple targets. The secretion dynamics of these molecules were revealed, for the first time in some cases. In addition, we discovered for the first time that insulin differentially regulates the secretion of leptin and resistin.

In Chapter 5 and 6, we demonstrate that nanoelectronic sensor fabricated using SiNWs and SWNTs can non-invasively detect bioelectricity at cell level or tissue level (beating heart). Furthermore, we show that drugs targeting on specific ion channels can be readily assessed based on SWNT-FET responses. Finally, general conclusions and perspectives for nanoelectronic biosensing will be outlined in chapter 7.

Chapter 2

Nanowire Fabrication and Characterization

2.1 Introduction

One-dimensional silicon nanowires (SiNWs) configured as field effect transistors (FET) have the potential to function as highly sensitive sensors for the label free detection of various biomolecules [20, 23]. In most of these studies, the SiNW devices are made by “bottom-up” processes which are largely restricted to research laboratories due to low yield, poor scalability, and poor integrability. To avoid these limitations, we employed a reproducible, scalable and CMOS compatible ‘top-down’ method to produce uniform and aligned long silicon nanowires involving deep ultraviolet lithography and self-limiting oxidation [78, 79]. In the self limiting oxidation, patterned silicon is oxidized in dry O₂ at high temperature and a highly stressed oxide layer is formed around the nanowire. The stress generated during oxidation prevented the wires from oxidizing away completely thus Si core diameter of the SiNWs can be self-limited by it. Here we report hundreds of perfectly aligned nanowires can be fabricated in one chip using our approach. The cross-sectional dimension of silicon nanowires can be readily controlled to different sizes, ranging from a hundred to less than ten nanometers, while its length is fabricated to 100 μm with precise control. We employed the “top-down” approach to fabricate two types of silicon nanowire arrays: *poly-silicon* nanowires and *crystalline* silicon nanowires. Both of them exhibit *n*-type field effect characteristic and are extremely sensitive to minute electrochemical perturbations.

2.2 Poly-silicon nanowire fabrication

Figure 2.1 Poly-silicon nanowire array fabrication processes

Poly-silicon nanowire array fabrication processes are depicted in Figure 2.1. In brief, the silicon nanowires in array format were realized on 8-inch diameter silicon wafers using CMOS compatible processes. 120 nm thick poly-silicon film was deposited on 100 nm thick silicon-oxide which serves both as the etching stop-layer as well as the back gate dielectric. The poly-silicon layer was doped with *n*-type impurities (phosphorous) using an ion implanter with the implant dosage of 4×10^{13} ions/cm² and the energy of 30 KeV. Ion implantation is a material engineering process by which ions of a material can be implanted into another solid and thus change the physical properties of the solid. Subsequently, dopants were activated by rapid thermal annealing at 1000 °C. Photoresist strips with critical dimensions of 70 – 80 nm were patterned onto the polysilicon layer

using deep ultraviolet lithography followed by resist trimming. Then the resist pattern was transferred to polysilicon using reactive ion etching. To further reduce the dimension, the resulting silicon wires were oxidized at 900 °C to realize 30 to 50 nm silicon nanowires depending on the oxidation time. In this stress limiting oxidation process, a large normal stress is generated back towards the Si-SiO₂ interface and the stress generated during oxidation prevented the wires from oxidizing away completely [80]. The contact-pad structures at the ends of the nanowires were heavily and selectively doped (dosage of 5×10^{15} ions/cm²) through silicon dioxide patterning. The metal interconnections were prepared through standard aluminum metallization on silicon contact pads and subsequent alloying to achieve Ohmic contacts. The device was later passivated by 200 nm thick silicon nitride layer. The reasons of conduct passivation are to enhance biocompatibility of silicon nanowire chip and achieve single active area for collection of signal. It is also helps solving the problem of metal corrosion in the process of wire release from the silicon oxide. Finally, metal pads and silicon nanowires were exposed through lithography and etching processes. The completions of this process realize silicon nanowire width in 30 to 50 nm. Figure 2.2 depicts the SEM images of nanowire arrays in each step of chip fabrication processes.

Figure 2.2 SEM micrograph of nanowire fabrication processes. (a) Patterning on poly-silicon followed with fin etching and oxidation with targeted fin width about 30 to 50 nm. (b) Contact open process opens a small hole at both ends of find structures to create contact of metal with silicon. (c) Metal deposition followed by etching to form extended metal contact pad. (d) Passivation followed by HF etching release nanowire from oxide layer and have silicon nanowire width in 30 to 50 nm.

Figure 2.3a depicts the optical image of *poly-silicon* nanowire chip, which has two independent SiNW sensing regions. Each sensing regions has a parallel array of 100 nanowires and each wire is 100 μm in length. As depicted in the zoom-in scanning electron microscopy (SEM) image (Figure 2.3b), nanowires are uniformly separated at 2 μm intervals and electrically addressable by individual metal contact lines with silicon nitride passivation. A typical transmission electron microscopy (TEM) image of a slice of cross-sectioned nanowire taken using focused ion beam (FIB) (inset in Figure 2.3b) indicates a rectangular cross-section with approximately 30 nm in width and 40 nm in height. The rounded top edge achieved by removing ~ 50 nm of silicon dioxide in diluted hydrofluoric acid. This is necessary to avoid sharp-edge effects in nanowire properties and non-specific interactions with biomolecules. The foot at the base of the wire provides necessary mechanical strength for the nanowires to survive through the solution-based processes.

Figure 2.3 Fabricated polysilicon nanowire chip. (a) Optical image of a nanowire chip with two independent nanowire sensing regions. The chip is integrated with micro fluidic channel. (b) Micrograph taken by the scanning electron microscope showing that well aligned nanowire is separated at 2 μm interval and each wire is individually connected to metal lines with silicon nitride passivation. The insert illustrates the rectangular cross-section of a nanowire by TEM imaging.

2.3 Crystalline silicon nanowire fabrication

The array of single *crystal* silicon nanowires were fabricated on silicon-on-insulator (SOI) wafers based on the CMOS compatible top-down fabrication processes derived and improved from fabricated *poly-silicon* nanowire. Figure 2.4 depicted the fabrication processes.

Figure 2.4 Crystal nanowire array fabrication processes

8 inch silicon on insulator (SOI) wafers with top silicon layer of thickness 70 nm and buried silicon-oxide layer 145 nm thick were used. The *n*-type impurities (phosphorus) were doped onto the silicon layer using ion implanter and achieve a phosphorous ion concentration of $\sim 1 \times 10^{18} \text{ cm}^{-3}$. The dopants were activated by thermal annealing at 1000 °C for 10 seconds. Deep ultraviolet lithography was used to define the photoresist strips of $\sim 160 \text{ nm}$ wide onto the silicon layer. It is further trim down to reach a dimension of $\sim 90 \text{ nm}$ by resist trimming process. Then, the resist pattern was transferred to silicon layer by reactive ion etching. The patterns silicon layer is oxidized at 900 °C in self limiting oxidation processes and achieve critical wire dimension $\sim 40 \text{ nm}$ followed by subsequent etching away of silicon dioxide. The silicon contact-pad structures at the ends of the

nanowires were selectively and heavily doped with phosphorous ions to a concentration $>5 \times 10^{20} \text{ cm}^{-3}$ followed by aluminum metallization and alloying to establish Ohmic contact with metal interconnections. The metal is etched away followed by silicon nitride (200 nm) passivation and deposition of high density plasma (HDP) silicon dioxide (1500 nm) layers. The double passivation layers of silicon nitride and the thick dense silicon dioxide greatly reduce the undesired leak current and provide reliable protection during long-time wet-etching. Finally, the metal pads and silicon nanowires were exposed through the lithography and etching processes.

Figure 2.5a depicts the optical image of *crystalline* silicon nanowire chip, which consists of two SiNW arrays. Each array has 90 wires, each wire is 100 μm long, grouped in cluster (5 wires each), separated uniformly at 200 μm . Such layout is suitable for multiplexed sensing as cluster may serve as one sensing unit for a particular molecular target. The zoom-in micrograph (Figure 2.5b) taken by scanning electron microscope showing one of the nanowire clusters, in which the nanowires are uniformly separated at 2 μm interval. A typical TEM (transmission electron microscopy) image of a thin slice of nanowire cross-sectioned by FIB (Focused ion beam) indicates a cross-section approximately 45 nm in width and 60 nm in height (Figure 2.5d). We are able to address each individual nanowire under the microscope and locate the probe tips on the metal pads to electrically connect the wire.

Figure 2.5 (a) Optical image of a *crystalline* silicon nanowire chip with 40 clusters of 5 silicon nanowire sensors each (b) Zoom – in SEM image shows a cluster with 5 wires. (c) TEM cross-section of 5 wires in a cluster (d) TEM of a rectangular nanowire.

2.4 The experimental set-up and nanowire characterization

Electrical characterization of SiNW arrays is essential for monitoring device properties and capabilities used for biosensing applications. Electrical characterization of SiNW arrays were carried out before surface modification and cell culturing. These tests include I-V curve measurement to find out the conductance of nanowire, back-gate and front gate measurement to show that the sensitivity of the silicon nanowire device is tunable and conductance of SiNW is greatly influenced by field (charge) effect.

The electrical properties of SiNW arrays is measured using Axon patch clamp amplifier (Multiclamp 700B, Axon instrument) or HEKA patch clamp amplifier (HEKA

Elektronik, Lambrecht, Germany). The typical measurement set up is shown in Figure 2.6a. The measurement system composed of microscope (Olympus BX51W1), patch clamp amplifier (Multiclamp 700B, Axon Instruments), low noise data acquisition systems (Digitadata 1440A, Axon Instruments) and computer. The SiNW chip is positioned on a microscope stage which is housed in a Faraday cage (Figure 2.6b). The Faraday cage is able to eliminate external static electrical fields from environment that might cause significant noise during recording. The whole measurement system is supported by the anti-vibration table (Technical Manufacturing Corporation, Peabody, MA, USA). The position of the wire is addressed under the microscope and probe tips are made contact to the metal pad with the help of 3 axis micromanipulator (Burleigh, Germany). Figure 2.6c highlighted two probe tips are contacting on the metal contact pad to electrically connect individual nanowire.

In brief, we first measure the nanowire conductance between two terminals, source (S) and drain (D) electrodes (Figure 2.7). Source drain voltage is applied from 0.0 V to +0.5 V and current response is filtered at 1 kHz, sampling rate is at 10 kHz. Then, we conduct back-gate or front-gate measurement by using second voltage channel from patch clamp amplifier. In the back-gate voltage measurement, voltage from +10V to -10V is applied at the bottom of the chip. For measurement of front-gate effect, small droplet of 1x PBS is located on nanowire sensitive area. Voltage from -1V to +1 V is applied through Ag/AgCl electrode that has immersed into the ionic solution. Source-drain voltage, V_{SD} is applied at constant 400 mV. The front-gate voltage will modulate the source drain current.

Figure 2.6 The schematic diagram of electrical testing set up. (a) Microscope and measurement platform are housed in a faraday cage and supported by anti-vibration table. Data is measured using axon patch clamp amplifier and analyzed by computer. (b) The SiNW chip is put on the stage and each individual nanowire is addressed under the microscope. (c) The zoom-in optical image (red rectangular in b) to illustrate two probe tips contact the metal pads.

Figure 2.7 Schematic of nanowire device measurement. While a voltage bias (V_{ds}) is applied across a nanowire, its drain-source current (I_{ds}) can be continuously monitored. Front gate voltage is applied through Ag/AgCl electrode immersed in the saline solution. Back-gate voltage is applied to bottom of silicon substrate.

2.5 Results and discussion

Electrical characterization verifies that SiNWs arrays are fabricated in high sensitivity and reproducibility. The process produces *poly-silicon* nanowires uniform in their sizes and properties. Consequently, their conductance is narrowly distributed (Figure 2.8a). Figure 2.8b depicts the linear current (I) versus voltage (V) behavior were observed for all the devices studied. These suggesting SiNW-metal contacts are Ohmic and implying that there do not have Schottky effect.

Our nanowire devices are highly responsive to the field modulations, as demonstrated in Figure 2.8b. Back-gate voltage measurement shows that sensitivity of the silicon

nanowire device is tunable by changing the back gate voltage. The reason is that back-gate effect can be established by applying a voltage from bottom of nanowire chip. In more detail, this electrical effect is caused by the silicon substrate act as a back-gate electrode and the middle thick silicon oxide layer act as an insulator to form the potential established by the back-gate voltage and therefore influences the conductance of nanowire. From the measurement result, nanowire conductance shows increase at positive back-gate voltage, V_g . This shows the typical behavior for *n*-type nanowire. This tunable conductance behavior by back-gate effect can be applied to practical use to boost up the sensitivity of nanowire. It is useful especially to detect low concentration of molecular solution, as applying back-gate effect, the sensitivity of nanowire will be boosted up and give significant feedback to detect low amount of molecule in solution.

Silicon nanowire is also electrically accessed through liquid gate voltage and its results are presented in Figure 2.8c. Front gate voltage (V_g) is swept from negative to positive, resulting increase of source-drain nanowire conductance. In the 1x PBS solution, applied a positive front gate voltage would attract negative ions (Cl^-) and expel positive ions (K^+ , Na^+ , Ca^{2+}) to the silicon nanowire surface to form a positive front-gate and the negative voltage from the probe will give the opposite results. Thus, distribution of ions in the solution is voltage controlled and influence charge effect on the silicon nanowire. The conclusion of this experiment is that a change in voltage of the liquid gate can produce a detectable change in source-drain current. This is an important electrical characteristic of silicon nanowire as showing its capability to detect and differentiate presence of field established by charges or other ions. It is crucial to apply silicon nanowire as biosensor to detect target molecule in the liquid by determining its charge value on surface. This field

effect property of SiNWs make them naturally very suitable for detection of cellular bioelectricity from living cells or detect biomolecules secreted from cells.

Figure 2.8 Electrical characterization of silicon nanowire. (a) Nanowires conductance in an array is narrowly distributed. The Solid curve is a Gaussian fitting. (b) Typical I_{ds} - V_{ds} curve of individual silicon nanowire for varying back-gate voltage, V_g from -10 V to 10 V in 2 V steps (indicated by the dark arrow). The characteristic shows n-type FET behavior. (c) Measured conductance versus front-gate voltage of a nanowire swept from -1 V to +1 V at V_{SD} , 400 mV. The trans-conductance is 30 nS ($V_g = 0$ V).

We also measure the current noise spectral density using Axon patch clamp amplifier for band width frequency 2 kHz. The *poly-silicon* nanowire was biased at $V_{ds} = 400$ mV. Figure 2.9 depicts the normalized power spectrum (S_I / I^2). The results indicated that our nanowires has very low $1/f$ noise compared to carbon nanotube based FETs. The line fitting (A / f) at the low frequency range (<70 Hz) in the spectrum (solid line) gives the normalized $1/f$ noise amplitude of $A = 4.4 \times 10^{-9}$, which is 3 orders smaller than carbon nanotube based FETs [81, 82]. The theoretical thermal noise due to the nanowire conductance and the feedback resistor in the amplifier is calculated using the equation [83] $S_i = \frac{4k_B T}{R_m}$ where k_B is the Boltzmann constant (1.38×10^{-23} J/K), T is the absolute room temperature in Kelvin unit (300 K) and R_m is the resistance originated from nanowire resistor and amplifier feedback resistor (187 M Ω). The dashed line in Figure 2.9 indicated calculated theoretical thermal noise. The measured RMS current noise is 1.1 pA, not much higher than the estimated inevitable thermal noise of 0.5 pA. This suggested that $1/f$ noise, which is the dominant noise in other FET devices [81, 84], does not cause significant extra noise in SiNWs.

Figure 2.9 A normalized current power spectrum from a silicon nanowire biased at 400 mV. At low frequencies, $1/f$ noise dominates. The dark fitting curve indicated nanowire has $1/f$ noise amplitude A (4.4×10^{-9}) and exponent β (1.1) for frequency up to 70 Hz. The dashed horizontal line marks the thermal noise estimated from nanowire conductance and feedback resistor in the amplifier.

Nevertheless, it should be pointed out that when nanowires are immersed in the culture medium with cells for days, the nanowire noises usually increase likely due to the non-specifically absorbed proteins or other molecules which induce charge scattering. Appropriate passivation methods may be used to avoid this problem.

Conventional planar FETs has conductance channel buried under a gate oxide whereby electrochemical instability does occur on the oxide surface results in conductance drift with time. This unfavorable condition is caused by penetration of ionic species such as Na^+ , K^+ ions into the thin gate oxide [85]. In contrast, our nanowire conductance is stable in the electrolyte solution. Figure 2.10 depicts only a small drift (< 10 pA) in the 30 minutes recording and this drift is presumably due to evaporation

induced change in salt concentration. Thus, the results suggested that SiNWs show good long term stability in solution.

Figure 2.10 Nanowire current recorded in the bath solution for 30 minutes.

Our *crystalline* silicon nanowires are also produced in high sensitivity and reproducibility. In contrast to polysilicon nanowires whose conductance is significantly compromised by charge scattering effects, single *crystalline* SiNWs give higher conductivity therefore larger transconductance (current response) in response to a given field-effect (from the biomolecules). The crystalline SiNWs show 1.5 times higher transconductance compared to the poly-silicon nanowires. Figure 2.11a depicts the nanowire conductance distribution which gives an average conductance of 132.2 nS with a standard deviation of 11.1 nS. This indicates the uniformity of nanowire dimensions and properties realized in CMOS fabrication techniques. Figure 2.11b shows that our *crystalline* SiNWs manifest *n*-type field effect characteristics and are highly responsive to the gate voltage (V_g) applied, via an Ag/AgCl electrode, to the saline solution bathing the SiNW. Specifically, its conductance varies from 29.2 to 238.0 nS while V_g changes from -1 to +1 V. This demonstrates that *crystalline* SiNWs is desirable for electrical biosensing because its conductance is highly sensitive to electrochemical perturbations at nanowire surface or its immediate vicinity owing to the large surface-to-volume ratio.

Figure 2.11 Electrical characterizations of crystalline silicon nanowires. (a) Nanowires conductance in an array is narrowly distributed. The conductance is measured from 90 nanowires in an array. The Solid curve is a Gaussian fitting. (b) Measured conductance versus front-gate voltage at V_{SD} , 200 mV. The conductance of the n-type SiNW increased dramatically from 29.2 nS to 238.0 nS as V_g swept from -1 to +1 V.

2.6 Conclusions

In this chapter, perfectly aligned arrays of silicon nanowire fabricated using top-down approach that employed standard technique for semiconductor manufacturing in large scale production of microelectronics were presented. The electrical performance for each type of nanowire arrays was carefully discussed. The high density nanowires realized is also suitable for parallel multiplexed biomolecular sensing. Furthermore, our nanowire also shows extreme low noise and nanowire conductance is very stable when measured in fluidity environment.

2.7 Acknowledgements

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Chapter 3

Ultrasensitive detection of adipocytokines with CMOS-compatible silicon nanowire arrays

3.1 Introduction

Nanostructured materials such as nanoparticles [86], nanopores [7, 87], carbon nanotubes [41, 88], and nanowires [89], are bringing unprecedented opportunities to biology. Their nanoscale dimensions allow them to interact intimately with and spy on the nanoscopic world of biomolecules. One-dimensional silicon nanowires (SiNWs) configured as field-effect transistor (FET), in particular, have recently emerged as a novel class of nanoelectric biosensors for rapid and highly sensitive detection of various biospecies [22, 90] including DNAs [25], protein biomarkers [4, 23], virus [20] and toxin [91]. In the present work, perfectly aligned arrays of single crystalline silicon nanowires were fabricated using top-down CMOS (complementary metal–oxide–semiconductor) compatible fabrication techniques standardized in large-scale production of microelectronics. We demonstrate that these nanowire devices are able to detect adipocytokines secreted by the adipose cells using a simple detection scheme with femtomolar sensitivity, high specificity, wide detection range, and ability for parallel multiplexed monitoring. This development is of obvious scientific and clinical significance in terms of revealing the poorly understood signaling mechanisms of these newly recognized hormone molecules as well as their relevance in common diseases such as obesity, diabetes, cardiovascular disease, and so on [92-94].

Adipose tissue, which was previously regarded as a passive energy (fat) storage tissue, has recently been recognized as a highly active endocrine organ secreting a range of signaling molecules called adipocytokines (including leptin, resistin, adiponectin, TNF- α , etc.) [95]. These molecules have widespread effects on health due to their important regulatory roles in energy homeostasis [96], glucose and lipid metabolism [92, 96], immune response [97], neuronal functions [98], neuroendocrine functions [99], and bone formation [100]. But, the mechanisms that trigger secretion of these molecules and how they act collaboratively in regulating many physiological functions are still poorly understood. Here, we studied the secretion of leptin and resistin using the nanowire approach. Leptin, encoded by the *obese* gene, was discovered in 1994 as the first member of the growing family of adipocytokines [101]. This 16-kDa protein is secreted, in response to hyperinsulinemia after a meal or other stimuli, as a signal of nutritional status to the hypothalamus, whereby increasing insulin sensitivity, inhibiting food intake, enhancing energy expenditure and regulating body fat. Resistin (a 12.5-kDa adipocytokine discovered in 2001) is also regulated by nutritional status and is thought to cause insulin resistance in obesity related type 2 diabetes mellitus [102]. Although it is evident that leptin and resistin play distinct regulatory roles in metabolic activities, the interplays between their secretion and regulation are poorly studied [103].

Developing effective assay methods for adipocytokines would certainly provide a great impetus to understand the multifaceted physiological roles of these molecules and their implications in pathogenesis. Current analytical methods for adipocytokines include immunoblotting, immunohistochemistry, immunoprecipitation, enzyme-linked immunosorbent assay (ELISA), and radio-immunoassay (RIA). These methods, however,

are time-consuming and generally of low sensitivity, and require separation and/or concentration and/or labeling and/or signal amplification. Although RIA provides high sensitivity, the involvement of radioisotopes demands extra precautions and expensive facilities. Herein, we demonstrate that silicon nanowire based field-effect transistor (SiNW-FET) offers a novel alternative to detect adipocytokines secreted from adipocytes.

3.2 Materials and Methods

3.2.1 Molecules and chemicals

Polyclonal antibodies against murine leptin and murine resistin were purchased from Santa Cruz Biotechnology (USA). Murine resistin was from Biovision (USA). Murine Leptin, (3-Aminopropyl) triethoxy-silane (APTES), ethanolamine, glutaraldehyde and other chemicals were obtained from Sigma Aldrich (USA). The isoelectric points of leptin antibody and resistin antibody were determined by zeta potential measurement using Zetasizer (Malvern, UK).

3.2.2 SiNW functionalization

The SiNW chips were first soaked in 0.5 % hydro-fluoric acid solution for 7 minutes and rinse with copious amount of water. The nanowires were then left in ambient for 2 days to allow growth of a thin native SiO₂ layer that enables silanization. After cleaning with diH₂O and absolute ethanol, SiNW chips were immersed in 2% APTES (in absolute ethanol) for 30 minutes. They were then rinsed thoroughly with absolute ethanol and incubated in an oven at 150°C for an hour. The silanized SiNW chips were subsequently

dipped in 5% glutaraldehyde (in diH₂O) at room temperature for 2 hours followed by overnight incubation in the leptin or resistin antibody solution (2 µg/ml in 10 mM sodium bicarbonate, pH 9.0) at 4°C. After diH₂O rinsing, the SiNWs were treated in 1M ethanolamine for 30 minutes. The SiNW chips were finally ready for use after washing with copious amount of diH₂O and blowing dry with nitrogen. The SiNW array was separated into two sensing regions by silicone rubber. The two regions were functionalized differently for parallel detection of leptin and resistin.

3.2.3 Electrical measurement

Nanowire conductance was monitored using a patch-clamp amplifier (Multiclamp 700B, Axon Instruments) with a low-filter of 1 kHz, sampling frequency of 4 kHz, and bias voltage (V_{ds}) of 200 mV. A small volume (100 µl) of adipocytokine-containing sample solution (leptin or resistin in PBS, or 3T3-L1 adipocyte culture medium) was applied onto a functionalized nanowire chip for 30 minutes incubation at 4 °C followed by thorough diH₂O rinsing. During the recording, nanowires are immersed in 1 mM PBS solution titrated to pH 7.0. This low ionic strength increases the Debye length of charge screening to ~2.5 nm, thus enhancing the field-effect introduced by the biomolecules. The Debye length (a distance from the wire surface at which changes in charge can be detected) is estimated using the equation: $\lambda_D = 0.32 (I)^{-0.5}$, whereby I is the ionic strength of the buffer solution used for sensing. For 0.1x PBS solution, the ionic concentration is 18 mM.

3.2.4 Cell culture and differentiation

Before converting into adipocytes, 3T3-L1 are cultured undifferentiated in DMEM medium (Gibco, USA) supplemented with 10% (vol/vol) heat-inactivated bovine calf serum (Gibco) and 1% penicillin-streptomycin (Gibco) at 37°C in a humidified atmosphere of 5% CO₂ / 95% air. Two days after the 3T3-L1 fibroblasts reached confluency (referred as day 0), the cells were exposed to the differentiation medium containing fetal bovine serum (10% v/v), insulin (10 µg/ml), dexamethasone (1 µM), and sobutyl-1-methylxanthine (0.5 µM), for 48 h [104]. Cells were then maintained in the post-differentiation medium containing fetal bovine serum (10% v/v) and insulin (10 µg/ml). Differentiation can be confirmed by the morphology change of the cells and the appearance of fat droplets (lipids) inside the cells. The fat droplets were stained according to the following protocol. Cells were washed two times with phosphate-buffered saline and fixed in 10% solution of formaldehyde in aqueous phosphate buffer for 1 hour. Then, the cells were washed with 60% isopropanol, and stained with Oil Red *O* solution (in 60% isopropanol) for 10 minutes followed by repeated washing with distilled water to remove excess stain. Cells on day 10, which were sufficiently differentiated, were used for the experiments. Figure 3.1 depicts the successful differentiation 3T3-L1 fibroblast cell into adipocytes.

Figure 3.1 Phase-contrast TEM images of 3T3-L1 cells before (a) and after (b) differentiation into adipocytes (the white scale bars correspond to 100 μm). The inset in (b) shows the red staining of the fat droplets in adipocytes.

3.3 Results and discussion

Upon binding of charged biomolecules, the current that passes through a voltage-biased nanowire varies because of the electrostatic influence from the biomolecules. The specificity of such electronic transduction usually relies on the mediation of certain molecular recognition elements (e.g. antibodies) that are grafted onto the nanowire in order to selectively fish out the target molecules from the solution onto the nanowire surface. We crosslinked the antibodies against leptin or resistin onto SiNWs through a sequence of functionalization steps (Figure 3.2). SiNWs were first silanized with (3-Aminopropyl)triethoxy-silane (APTES). As expected, the positively charged amino groups imparted onto the SiNW surface caused an increase in the nanowire conductance because of its *n*-type field-effect characteristics (Figure 3.2). Subsequently, glutaraldehyde was conjugated covalently to the amino-modified SiNWs via one of its

aldehyde groups. The positively charged amino groups on SiNWs were quenched due to this reaction, leading to a reduction in SiNW conductance. Finally, the antibodies against leptin or resistin were immobilized onto the SiNWs by the covalent reaction between the amino groups on the antibodies and the free aldehyde groups from glutaraldehyde. As anticipated, conjugation of negatively charged leptin antibody and resistin antibody (whose isoelectric points are pH 4.1 and pH 4.4, respectively, as determined by zeta potential measurement) both decreased the nanowire conductance. These measurements indicate clearly that each functional step is successful and nanowire conductance can be sensitively modulated by the electrostatic influence from the attached molecules. To avoid interference from unwanted binding of other proteins or molecules, the remaining free aldehyde groups on the SiNWs were passivated with ethanolamine.

Figure 3.2 a) Schematic illustration of a nanowire chip functionalized with leptin antibodies and resistin antibodies in two separated sensing regions. b) The change in nanowire conductance relative to the conductance of the bare nanowire (1. reference), ΔG , resulting from each functionalization step (2, 3, and 4).

Physiologically, leptin is negatively charged as its isoelectric point is pH 5.7. When leptin molecules bind to a leptin-antibody functionalized SiNWs, decrease in nanowire conductance should be observed. The amplitude of such decrease should depend on the number of leptin-antibody pairs on the nanowire at equilibrium, which is determined by the leptin concentration. As demonstrated in Figure 3.3a, this is indeed the case. The nanowires produced consistent responses (current decrease) to leptin concentration as low as 10 fM (or 160 fg/ml). The responding amplitude is logarithmically proportional to the leptin concentration until it saturates at the leptin concentration of ~500 pM. And the detection can be realized over a wide range of concentrations that exceeds 4 orders of magnitude. In contrast to leptin, binding of resistin molecules to a resistin-antibody functionalized nanowire enhanced nanowire conductance because resistin is positively charged due to its high isoelectric point (pH 7.7). As demonstrated in Figure 3.3b, wide detection range (4 orders in concentration) and even higher sensitivity were achieved for resistin. The increase in nanowire conductance is logarithmically proportional to the resistin concentration below the concentration of ~500 pM. Even at the concentration as low as 10 fM (or 101 fg/ml) of resistin, nanowire response (220.0 pA), is about 10 times larger than the measurement variation (26.5 pA, $n = 30$ nanowires) and 100 times larger than the nanowire noise (2.3 pA). Despite that the isoelectric point of resistin is closer to the physiological pH compared to that of leptin, resistin gave rise to larger response probably because it carries more net charge or because its charge group is physically closer to nanowire when it binds with its antibody. The detection sensitivity for both leptin and resistin is comparable to the most sensitive RIA assay.

Figure 3.3 Change in nanowire conductance versus the concentration of leptin (a) and resistin (b). Each data point was averaged from the measurements of 30 nanowires on 3 different chips functionalized with both leptin and resistin antibodies. The error bars indicate the standard deviations. The fittings (solid lines) indicate that the change in nanowire conductance is logarithmically proportional to the adipocytokine concentration.

In order to quantify any background effect due to the nonspecific binding of proteins to the nanowires, a control experiment is carried out with device prepared in the same way as described in section 3.2.2. We investigated nanowire response to high concentrated proteins, bovine serum albumin (BSA) (100 $\mu\text{g/ml}$ or 1.4 μM in PBS) and bovine serum (10% v/v in DMEM medium). The Figure 3.4 shows negligible change in nanowire conductance. The results suggest that our nanowire is selectively to target molecules and the nonspecific binding and adsorption of BSA protein molecules may effectively be omitted from consideration.

Figure 3.4 The silicon nanowire modified with leptin antibodies (a) or resistin antibodies (b) did not response to bovine serum albumin (BSA) (100 $\mu\text{g/ml}$ or 1.4 μM in PBS) and bovine serum (10% v/v in DMEM medium). The black line represents the nanowire conductance measured in 1 mM PBS without pre-treatment. The red line represents the nanowire conductance measured in 1 mM PBS after 30-minutes incubation with BSA solution. The blue line represents the nanowire conductance measured in 1 mM PBS after 30-minutes incubation with serum-containing medium.

Adipocytes differentiated from 3T3-L1 fibroblasts are a popular cell model to study the physiology of adipocytes. Using our nanowire chips, the kinetics of leptin and resistin secretion from 3T3-L1 adipocytes was monitored simultaneously on the same chip, under the resting condition or in presence of stimulus (Figure 3.5a and 3.5b). The flask of 3T3-L1 culture was first replenished with fresh culture medium ($t = 0$ minute, free of leptin and resistin). Subsequently, the culture medium (containing both secreted leptin and resistin) was collected every 30 minutes for determination of leptin and resistin concentrations over a period of several hours using a nanowire chip that had been functionalized with both leptin and resistin antibodies in two separate sensing regions. The protocol of sample collection was repeated to the same flask of cells but with addition of a stimulation cocktail containing 25 mM glucose, 0.25 μM insulin and 1 mM pyruvate (GPI stimulation) into the culture medium. In parallel with the measurements on the control samples, this set of samples was assayed in another dually functionalized nanowire chip.

Figure 3.5 Kinetics of leptin (a) and resistin (b) secretion from 3T3-L1 adipocytes are shown. Two dually functionalized nanowire chips were used to analyze the aliquots of culture medium collected from a flask of cell culture over a time period of 4.5 hours with (red triangles) and without (black circles) GPI stimulation, respectively. Each data point was averaged from the measurements on 5 nanowires on the same chip. The error bars represent the standard deviations. Leptin and resistin concentrations were determined based on the changes in nanowire conductance and the calibration curves presented in Fig. 3. Each data set is fitted by a sigmoidal function: $C_{\text{sat}}/(1+\exp((t_{\text{half}} - t)/\tau))$ to obtain the saturation concentration (C_{sat}), half-time (t_{half}), and rate constant (τ). For basal leptin secretion, $C_{\text{sat}} = 2.54$ pM, $t_{\text{half}} = 166.4$ minutes, $\tau = 33.5$ minutes. For GPI stimulated leptin secretion, $C_{\text{sat}} = 7.98$ pM, $t_{\text{half}} = 204.5$ minutes, $\tau = 34.7$ minutes. For basal resistin secretion, $C_{\text{sat}} = 3.09$ pM, $t_{\text{half}} = 169.6$ minutes, $\tau = 20.6$ minutes. For GPI stimulated resistin secretion, $C_{\text{sat}} = 0.59$ pM, $t_{\text{half}} = 149.3$ minutes, $\tau = 17.8$ minutes.

In the typical experiment demonstrated in Figure 3.5a, the leptin concentration steadily increased over time even under the resting condition. It suggests that adipocytes constitutively secrete leptin molecules without the necessity of stimulation. Presumably, this is important in maintaining the basal circulating leptin concentration in blood to suppress appetite (or to avoid constant hunger). The constitutive secretion of leptin reached sigmoidally to an apparent plateau. The sigmoidal fitting of the data points indicates a saturation concentration of ~ 2.54 pM and a rate constant of secretion of ~ 33.5

minutes. It seems that there is certain self-limiting (auto-inhibition) mechanism to control the basal leptin level, likely through the leptin receptors expressed in adipocytes themselves [105]. It has been shown that GPI can potently stimulate leptin secretion from adipocytes [106]. This is confirmed by our observation that GPI caused significant increase in leptin secretion (to 3.36 ± 1.28 folds in 3 hours, 3 cell cultures). In the example shown in Figure 3.5a, leptin secretion was enhanced to 2.73 folds by GPI. Our data agrees well with the kinetics of leptin secretion reported previously using RIA [106] and immunoblotting [107].

The secretion of resistin is even less investigated as compared to that of leptin. Figure 3.5b presents quantitatively, likely for the first time, the kinetics of resistin secretion. As shown, 3T3-L1 adipocytes also constitutively secreted resistin and the concentration of resistin increased sigmoidally to a saturation level of ~ 3.09 pM with a rate constant of ~ 20.6 minutes. Similar to leptin secretion, resistin secretion appeared to be self-limiting although resistin receptors have not been found in adipocytes. Interestingly, we discovered that, in contrast to its stimulatory effect on leptin secretion, GPI significantly inhibited the secretion of resistin (to $43.5 \pm 19.4\%$ in 3 hours, 3 cell cultures). As leptin and resistin have distinct effects on insulin sensitivity, it is perhaps not surprising that insulin regulate their secretion oppositely. Despite that GPI was influential on the extent of leptin secretion and resistin secretion, it did not significantly affect their secretion kinetics (i.e., the rate constant and half-time of secretion were not significantly altered by GPI treatment as shown in Figure 3.5a and 3.5b).

3.4 Conclusions

In summary, we have demonstrated a silicon nanowire approach to detect adipocytokines with femtomolar sensitivity, adding a novel tool to study adipobiology. Electronic detection realized by SiNWs is simple, fast, and label-free. Moreover, given the small size of the sensing element (nanowire), this approach requires only a tiny amount of sample for measurement. The array format of our nanowire chips enables parallel multiplexed sensing for multiple species and experimental protocols. For our specific application, in principle, secretion of multiple adipocytokines in response to various stimulations can be assayed simultaneously on one chip. This would help to understand the interplays between these adipocytokines: how they play regulatory roles in concert for a physiological function, and how a metabolic syndrome is resulted from (or causes) alteration of the adipocytokine profile. As the silicon nanowire array was made using standardized CMOS compatible techniques, large-scale and low cost production, easy integration with electronics can be realized. Using the nanowire approach, the secretion kinetics of leptin and resistin from 3T3-L1 adipocytes was revealed quantitatively. And it was discovered that, in contrast to its stimulatory regulation on leptin secretion, insulin inhibits the secretion of resistin.

3.5 Acknowledgements

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Chapter 4

Nanoelectronic detection of triggered secretion of pro-inflammatory cytokines Using CMOS compatible silicon nanowires

4.1 Introduction

Field-effect transistors (FETs) based on silicon nanowires (SiNWs) [108] have been employed to electrically detect various biomolecules [5, 23] and cellular bioelectricity [70, 109]. Comparing to conventional biochemical, immunofluorescence and biophysical assays, such nanoelectronic biosensing [110] offers simple, label-free and rapid detection with exquisite sensitivity. Using CMOS-compatible top-down fabrication techniques standardized in the semiconductor industry, perfectly aligned silicon nanowire arrays were fabricated. And they were used to parallelly detect the secretion kinetics of two pro-inflammatory cytokines (tumor necrosis factor- α and interleukin-6) from macrophages or the dynamic change of their serum levels in rats, following the stimulation by bacterial endotoxin (lipopolysaccharide), with femtomolar sensitivity and high specificity.

Cytokines are an important class of intercellular messengers produced within tissues following infection, trauma and diseases [111]. In the family of cytokines, tumor necrosis factor- α (TNF- α) secreted mainly by macrophages and interleukin-6 (IL-6) secreted mainly by macrophages and T cells are two pro-inflammatory cytokines

essentially involving in immune responses (e.g., to invasion of pathogens) and other physiological functions [112-115]. They often act collaboratively, particularly in acute immune reactions. Dysregulations of TNF-alpha secretion are implicated in a variety of diseases such as asthma, rheumatoid arthritis, and cancers [116, 117] while abnormalities in secretion of IL-6 are associated with pathological conditions such as diabetes, atherosclerosis, systemic lupus erythematosus, and rheumatoid arthritis [118]. Therefore, detection of these cytokines, preferably at the same time, is not only instrumental to the study of immunity but also important to diagnosis or to monitor drug treatment of a pathological condition.

In a recent study, antigen triggered immune responses of T cells were indirectly monitored using SiNW-FETs based on SiNW response to the extracellular acidification resulting from the discharge of acidic metabolites or the activation of membrane transporters [40]. Here, we directly detect pro-inflammatory cytokines by functionalizing SiNWs with cytokine-specific antibodies. We use it to parallelly study the secretion kinetics of TNF-alpha and IL-6 from macrophages or the dynamic change of their serum levels in rats, following the stimulation by bacterial endotoxin (lipopolysaccharide). As compared to the conventional analytical methods (ELISA, Western blot, radioimmunoassay, and biochemical assays on cytokine-sensitive cells), this nanoelectronic approach is able to resolve the dynamics of the triggered secretion of multiple cytokines on one chip with femtomolar sensitivity, small consumption of samples (20 nl of analytes in 40 μ l buffer solution) and time (minutes per data point).

4.2 Materials and Methods

4.2.1 Molecules and chemicals

Polyclonal antibodies against TNF-alpha were purchased from Santa Cruz Biotechnology (USA) and polyclonal antibodies against IL - 6 were purchased from Abcam, MA, USA. Murine or rat TNF-alpha and IL - 6 were purchased from Biovision CA USA. Lipopolysaccharides (*Pseudomonas aeruginosa* 10), (3-Aminopropyl) triethoxy-silane (APTES), ethanolamine, glutaraldehyde and other chemicals were obtained from Sigma Aldrich (USA). Polyethylene tubing (1.57OD x 1.14 ID) was purchased from Warner Instruments (USA).

4.2.2 SiNW functionalization

The SiNW chips were first soaked in 0.5 % hydro-fluoric acid solution for 7 minutes and rinse with copious amount of water. The nanowires were then left in ambient for 2 days to allow growth of a thin native SiO₂ layer that enables silanization. The SiNW chips were immersed in solution of 2% APTES (in absolute ethanol) for 30 minutes. The APTES forms a self-assembled monolayer on the SiNW surface, providing terminal amino groups for subsequent steps. The chips were then rinsed thoroughly with absolute ethanol and incubated in an oven at 150°C for an hour. The silanized SiNW chips were subsequently treated with 5% glutaraldehyde (in diH₂O) at room temperature for 2 hours. The TNF-alpha antibodies (Santa Cruz Biotechnology, USA) or IL-6 antibodies (Abcam, MA, USA) prepared in solution (20 µg/ml in 10 mM sodium bicarbonate, pH 9.0) were incubated with the SiNWs at 4°C overnight in two separated sensing regions, followed by

thorough rinsing with 0.5 % Tween-20 and subsequent incubation with 100 mM ethanolamine (pH 9.0) for 30 minutes. Finally, the SiNW chips were washed with copious amount of 0.5% Tween-20, and nitrogen was used to blow the chamber (less than 5 seconds). Immediately, the nanowire chip was incubated with proteins solution for subsequent step.

4.2.3 Electrical measurement

With 40 μ l measurement buffer solution on top of the nanowire chip, nanowire current was monitored at bias voltage (V_{ds}) of 200 mV with Ag/AgCl reference electrode immersed in the solution using Multiclamp 700B (Axon Instruments). The current was low-passed filtered at 400 Hz and sampled at 2 kHz.

4.2.4 Cell culture

Murine macrophage cell line (RAW 264.7, American Type Culture Collection, MD, USA) was cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO Labs) supplemented with 10% fetal bovine serum (FBS, HyClone) and grown at 37 $^{\circ}$ C under a 5 % CO₂ atmosphere in T75 flask.

4.3 Results and discussion

The antibodies against TNF-alpha and antibodies against IL-6 were crosslinked onto SiNWs in two separated sensing regions (Figure 4.1a) through a series of functionalization steps (Figure 4.1b). The effectiveness of each functionalization step was indicated by the predicted change of nanowire conductance. Firstly, APTES was conjugated onto SiNW through the covalent reaction between its silicon ethoxide terminal and the -OH group on nanowire surface. It caused increase of nanowire conductance due to electrostatic gating introduced by APTES's positively charged amino group. Then, the secondary linker molecule glutaraldehyde reacted with the amino group of APTES via its aldehyde group on one end. This reaction led to decrease of nanowire conductance due to reduction of the free amino groups on APTES. Finally, antibodies against TNF-alpha or IL-6 were covalently conjugated in the two separated sensing regions on the SiNW chip through the reaction between the amino groups on the protein and the other aldehyde group on glutaraldehyde. This caused increase of nanowire conductance because both TNF-alpha antibody and IL-6 antibody are positively charged in our pH 6.0 buffer solution. As determined by zeta potential measurement the isoelectric points of TNF-alpha antibody and IL-6 antibody are pH 7.81 and pH 8.82, respectively. Finally, the remaining reactive groups on the SiNWs were passivated by ethanolamine to prevent non-specific reactions or bindings.

Figure 4.1 (a) Schematic illustration of SiNW chip dually functionalized with antibodies against TNF-alpha or IL-6. (b) The percentage change in SiNW conductance ($\Delta G/G_0$) before (1. reference) and after each functionalization step (2, 3, and 4).

To enhance the nanowire response, the analyte solutions (cytokine stock in 1x PBS, or cell culture medium, or serum sample) were diluted 1000 times into the measurement buffer solution which is 0.001x PBS titrated to pH 6.0. At the low ionic strength of the final analyte supplemented buffer solution ($\sim 0.002\times$ of physiological ionic strength), the Debye length of charge screening is increased to ~ 16.9 nm as compared to ~ 0.7 nm in physiological solutions. Consequently, the nanowire response to the charge effects of the binding molecules is greatly enhanced. Because TNF-alpha (isoelectric point = pH 5.08) is negatively charged and IL-6 (isoelectric point = pH 6.91) is positively charged in our pH 6.0 buffer, it is expected that binding of TNF-alpha would cause conductance decrease of the n-type nanowires whereas binding of IL-6 would increase the nanowire conductance.

To calibrate the nanowire responses, the buffer solution added with various amount of TNF-alpha or IL-6 were perfused onto the dually functionalized SiNW chip while the source-to-drain current (I_{ds}) through nanowire was continuously monitored at the biasing voltage of $V_{ds} = 200$ mV and solution gate voltage $V_g = 0$ mV. As expected, perfusion of TNF-alpha (or IL-6) onto the SiNW chip resulted in step-like (reaching the plateau in 20s) decrease (or increase) of nanowire conductance in the corresponding sensing region (Figure 4.2). And the amplitude of conductance change was logarithmically proportional to the cytokine concentration. The detection limit for both cytokines is about 100 fg/ml (5.71 fM TNF-alpha or 4.61 fM IL-6). At this low detection limit, a signal-to-noise ratio about 4 can be achieved (~ 90 pA of nanowire response versus ~ 22 pA of noise level). In comparison, cell culture medium similarly diluted in the buffer solution did not cause appreciable nanowire response (Figure 4.2). This result shows that the nanowire detection

of cytokines is highly specific because the culture medium supplemented with fetal bovine serum contains a spectrum of protein species and other substances.

Figure 4.2 Detection of TNF-alpha (a) and IL – 6 (b) with antibody functionalized SiNW chips. Perfusion of TNF-alpha or IL-6 containing buffer solution caused decrease or increase of the nanowire conductance in concentration dependent manner (left panel). Perfusion of buffer solution without cytokine or with serum supplemented cell culture medium which contains a large variety of proteins and other biomolecules did not alter the nanowire conductance. The percentage change of nanowire conductance is linearly proportional to the logarithmic concentration of the added cytokines (right panel). Each data point (mean +/- SEM) is the average from 10 SiNWs in 3 chips.

We further used our SiNW FETs to simultaneously resolve the secretion kinetics of both cytokines from macrophages following stimulation with 100 ng/ml lipopolysaccharide (LPS). LPS is bacterial endotoxin which activates macrophages to release cytokines by complexing with lipopolysaccharide binding protein (LBP) and binding with CD14 receptors on the cell surface. The medium that cultures macrophages was first refreshed right before the application of LPS. Then the culture medium (with secreted molecules from the cells) was collected at different time points over 3 hours following LPS stimulation and diluted by 1000 times to the buffer solution for measurement. The amounts of TNF-alpha and IL – 6 released by macrophages in the culture medium were detected using our dually functionalized nanowire chips and calculated based on the calibration curves presented in Figure 4.2. As shown in Figure 4.3a, the release of TNF-alpha took off in about 15 min and increased exponentially with a time constant of ~35 min. Such kinetics is in agreement with the results reported using cytotoxic assay of TNF-alpha sensitive L929 cells (indirect, yet commonly used, assay of TNF-alpha) [119]. On the other hand, the secretion of IL-6 exhibited a longer delay, slower kinetics, and much higher extrapolated plateau level. These can be partly attributed to the fact that TNF-alpha is able to induce and potentiate IL-6 secretion [120]. In contrast, without LPS treatment, the concentrations of both cytokines remained low, indicating that the basal release of these cytokines is trivial.

Figure 4.3 Time course of cytokine secretion measured from the macrophage culture medium (a) and blood serum from neonatal rats (b) collected at different time points after macrophages were stimulated with 100 ng/ml LPS or after intraperitoneal injected 50 μ g LPS to rat. The concentrations of TNF-alpha (middle) or IL-6 (bottom) in the culture medium or serum were calculated using the calibration curve presented in Figure 4.2. Each data point (mean $\pm$ SEM) is the average from 10 SiNWs in 3 chips.

In addition to analysis at cellular level, we monitored the serum levels of TNF-alpha and IL-6 during LPS triggered immune responses in rats. Neonatal rats (1 day old) were intraperitoneally injected with 50 µg LPS. Blood samples were then taken at different times after LPS injection, and centrifuged at 4000 rpm for 20 minutes at 4 °C. The supernatant without red blood cells was collected for analysis. Again, the serum samples were diluted by a factor of 1000 in 0.001x PBS solution (pH 6.0) for measurement. As demonstrated in Figure 4.3b, the serum level of TNF-alpha increased sharply 50 min after LPS injection, and then quickly decreased after reaching the peak at 180 min. In contrast, the increase of IL-6 level is much potent with a longer delay, partly due to the initiating and facilitating effects of TNF-alpha on IL-6 secretion [120]. The transient characteristics of TNF-alpha and IL-6 response in serum level may be due in part to that IL-6 also possesses anti-inflammatory properties and thus acts as the negative feedback to quench the inflammatory reactions induced by LPS [121]. Our results are comparable with previously reported results based on ELISA [122]. The transient elevation of both cytokines are regulated at complex system level, and are resulted from coordinated secretion from several cell types in addition to macrophages, including T lymphocytes and endothelial cells in blood and kupffer cells in liver. Therefore, the change kinetics of serum level is different from the secretion kinetics from macrophages. On the other hand, both serum and culture medium measurements indicate that IL-6 secretion is more potent and lags behind as compared to TNF-alpha secretion.

As demonstrated, SiNWs are able to resolve secretion kinetics of the cytokines upon triggering of bacterial endotoxin. However, the detection is obtained on collected culture medium at different time points, instead of real-time detection at cell level. For this

particular application, our approach is sufficient as the kinetics of cytokine secretion occur in a time scale of an hour. But in principle, it is possible to realize real-time detection at cell level if cells are directly cultured on the devices and the functionalized antibodies are protected by polymeric cushion.

4.4 Conclusions

In summary, we have demonstrated the use of silicon nanowire based nanoelectronic biosensors for simultaneous and specific detection of TNF-alpha and IL-6 which are important cytokines involving in immune reactions and other physiological functions. Using this nanowire approach, the secretion kinetics of these cytokines upon triggering has been readily resolved at cell level and animal level. In principle, many cytokines can be detected in parallel on a single chip. This would be instrumental to understand the complex immune responses, during which various cytokines are released in concert with defined, yet not fully understood, patterns. We envision that such nanoelectronic biosensing based on nanostructured materials promises novel applications for fundamental biological studies, diagnosis, and drug discovery [110].

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Chapter 5

CMOS-compatible nanowire sensor arrays for detection of cellular bioelectricity

5.1 Introduction

Semiconducting silicon nanowires (SiNWs) configured as field-effect transistors (FETs) have recently emerged as a novel class of nanoelectronic biosensors due to their extraordinary sensitivity and simple detection schemes [108]. In this study, perfectly aligned arrays of long poly-SiNWs fabricated using ‘top-down’ CMOS (complementary metal oxide semiconductor) compatible technology standardized in semiconductor industry were employed for real-time extracellular recording of cellular bioelectricity. Electrocardiogram (ECG) signals from a beating rat heart and spontaneous membrane action potentials from individual rat cardiomyocytes were transduced into changes in nanowire conductance with high signal-to-noise ratio. Nanowire sensors have also detected membrane excitation of rat aortic smooth muscle cells. This technology enables non-invasive, highly sensitive, and long-term electrophysiological measurements at single cell level or from tissues, therefore promising applications in high-throughput drug screening for ion channels.

5.2 Materials and Methods

5.2.1 Recording on nanowire chips

Nanowire sensor array chips were wire bonded onto printed circuit board so that nanowires are individually addressable by the amplifier. A solution chamber made of acrylic was fixed onto nanowire chips for cell culturing and recording. Before cell culturing, the chips were sterilized by washing with alcohol / distilled water and subsequent 30-minute UV exposure. The chips were then coated with collagen (0.5 mg/ml) to promote cell adhesion. Collagen coating did not cause significant alterations in nanowire properties.

The current through the nanowire was low-pass filtered at 1 kHz and monitored at 100 μ s sampling rate using an EPC10 patch-clamp amplifier (HEKA Elektronik, Germany). Recordings for cardiomyocytes were made in the culture medium at room temperature. While for smooth muscle cells, recordings were made at room temperature in a bath solution containing (in mM): 140 NaCl, 5.5 KCl, 1 MgCl₂, 2 CaCl₂, 10 glucose, and 10 HEPES (titrated to pH 7.4). For high K⁺ solution used to induce membrane depolarization in smooth muscle cells contains, the KCl concentration is increased to 105 mM whereas NaCl concentration is reduced to 40 mM.

5.2.2 Cell isolation and culture

The hearts of 1 to 3-day-old Sprague-Dawley rats were quickly removed into chilled Hanks' Balanced Salt Solutions (HBSS buffer, Sigma), containing (per liter): 8g NaCl, 0.048g Na_2HPO_4 , 1g D-glucose, 0.4g KCl, 0.06 g KH_2PO_4 , 0.35g NaHCO_3 , 0.14g CaCl_2 , 0.1g $\text{MgCl}_2 \cdot 6 \text{H}_2\text{O}$, 0.1g $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$, with addition of penicillin 50 U/ml and streptomycin 50 ug/ml. The ventricles were cut into 1 to 2-mm cubes and dissociated by the three-step treatment with (1) 0.1% trypsin digestion for 20 minutes at 180 rpm at 37 °C ; and (2) addition of fetal bovine serum in the digestion solution (20%) for 1-2 minutes with gentle pipetting at 24°C; (3) dissociated cells were collected in cold low glucose Dulbecco's Modified Eagles Medium (DMEM) medium supplemented with 3% fetal bovine serum and 1% Insulin-Transferrin-Selenium. The above treatment was usually repeated for several rounds to obtain sufficient number of cells. The collected cell suspension was then centrifuged at 1200 rpm and 4°C for 5 minutes. The resulting cell pellet was subsequently suspended in the supplemented DMEM medium and plated in Petri dish for 1 -1.5 hours at 37°C to allow fibroblast cells to attach onto the Petri dish. The myocyte-containing supernatant was then collected and centrifuged (4°C and 1200rpm for 5 minutes). The myocyte pellets were then re-suspended and plated onto nanowire chips at a density of 2.5×10^5 cells/ml and culture in the supplemented DMEM medium at 37°C and 5% CO_2 . All surgical procedures and protocols were in accordance with the Guidelines for Ethical Care of Experimental Animals and were approved by the NTU Institutional Animal Care and Use Committee.

The rat aortic smooth muscle cells (A7r5) (American Type Culture Collection) were cultured in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal bovine serum and 1% (v/v) penicillin-streptomycin (10 000 units/ml penicillin and 10 mg/ml streptomycin, Sigma-Aldrich) at 37°C in an incubator with 5% CO₂ – 95 % air. The growth medium is renewed every 2 to 3 days. Cells are passaged every 4 – 5 days. In brief, cells grown over 80% of the flask surface area are ready to passage by remove and discard the culture medium. Then, we rinse the cell layer with PBS to remove all traces of serum that contain trypsin inhibitor. We incubate the cells with 0.05% trypsin for 1 minute at 37⁰ C for cell to detach from the wall. After that, we add 2 ml of complete growth medium (DMEM) to inactive the trypsin activity, centrifuge the cells at 1200 rpm for 5 minutes, discard the supernatant, add in growth medium and aspirate cells by gently pipetting. Lastly passage the cell to new flask at split ratio of 1:3 or onto nanowire array at a density of 10⁵ cm⁻².

5.3 Results and discussion

Perhaps the most well known bioelectricity phenomenon is ECG, which is measurable at the body surface as characteristic changes in voltage during the course of a heart beat. In the speculation that the ionic currents accompanying heart activities may produce sufficient voltage variations in the bulk conductive bath solution to modulate nanowire conductance, we first tested whether our nanowire sensors can report cardioelectricity in characteristic changes of their conducting current. Quickly after its removal from a neonatal rat, a beating heart was placed into the recording chamber on the top of the

nanowire chip, as shown in Figure 5.1a. The current was continuously monitored while applying a source-to-drain voltage of 400 mV across the nanowire. In concert with the heart beat rhythm (which was directly observed under the microscope), regular episodes of current spikes were detected (Figure 5.1b).

Figure 5.1 ECG recording from an intact beating heart. a) A beating rat heart was placed in the recording chamber on top of a wirebonded nanowire chip. b) Nanowire current was monitored at a voltage bias of 400 mV. In the left panel, a train of current spikes that was in concert with the heart beating is illustrated. A typical transient current event is shown in the right panel. c) Voltage signals recorded by an impaling nanopipette electrode into the heart. They are similar to the nanowire responses.

In a typical experiment, we also conduct intracellular recording using nanopipette with tip size of ~100 nm. The small tip size reduces damage to the tissue and also limits both the electrical conductivity of the pipette (resulting in a relatively high pipette resistance) and the washout of cytoplasm by pipette fluid. The high resistance is minimized by using a highly conductive solution, 3 M KCL to fill the pipette and inserted with Ag/AgCl

electrode. The pipette electrode was puncturing into a beating heart, and the voltage fluctuations were recorded in the current mode. We were puncturing the beating heart at several location and two distinct types of signals were recorded. One type of them which are biphasic transient signals, we believe, were ECG signals detected in the interstitial space between cells (Figure 5.1c) [123]. The nanowire current spikes and the ECG recordings share similar waveform, suggesting their common origin. The second type of recording obtained from pipette electrode, we believe, was the intracellular recording of spontaneous action potential of a single cardiomyocyte when the recording nanopipette by chance impaled into a cell body. A typical event of such recording is depicted in Figure 5.2c (right), in agreement with those being reported elsewhere [123].

To detect bioelectrical activities from individual cardiomyocytes, they were isolated from the hearts of neonatal rats and subsequently cultured on the nanowire chips for 1-2 days before the experiments. As presented in Figure 5.2a, a cardiomyocyte grows and adheres on nanowire-II. When this nanowire was biased at 400 mV, rhythmic transient changes in its conducting current were recorded (Figure 5.2b, lower trace). Interestingly, an up-stroke current spike was always followed by a down-stroke current spike. An event of paired current spikes is illustrated in Figure 5.2c (left). Some of the events just like this one showed a slight increase in the current baseline between the paired current spikes. The duration of the events is around 200-300 milliseconds, similar to that of the action potentials measured by intracellular nanopipette recordings (Figure 5.2c, right). Therefore, it is highly plausible that the paired opposite current spikes signal the onset and shut-off of an action potential, respectively. Simultaneous recording on nanowire-I which was not in contact with any cell did not show any transient current events.

Figure 5.2 Nanowire recordings on individual cardiomyocytes. a) The cardiomyocytes were isolated from the hearts of neonatal rats and cultured on the nanowire chip. Two nanowires (I and II) are indicated by dark lines. b) The upper trace depicts the recorded nanowire current from nanowire-I. The lower trace is the recording from nanowire-II which was covered by a contracting myocyte. It showed a train of transient current events. c) A typical episode of the nanowire response from nanowire-II, involving one upstroke current followed by a downward one, is shown on the left. For comparison, an intracellularly recorded action potential using a nanopipette is depicted on the right.

Like cardiomyocytes, other muscle cells are also electrogenic. We interfaced the nanowire sensors with rat aortic smooth muscle cells (A7r5) and sensed their membrane electrical activities. As shown in Figure 5.3a, a confluent sheet of smooth muscle cells covers the entire nanowire array. Each wire was in contact with multiple cells. When a solution of high potassium concentration, which is known to cause membrane excitation (increase in intracellular potential), was introduced into the recording chamber, a train of current spikes was induced after some delay (Figure 5.3b). Such current spikes were not observed as spontaneous events in either culture medium or bath solution. They were also

not observed when applying high potassium solution onto nanowires without the presence of cells. The evoked nanowire responses are biphasic and have durations of ~ 1 ms. The action potentials of A7r5 cells typically last for more than several hundred milliseconds [124, 125]. It is tempting to conceive that the nanowires only responded to the initial rapid take-off of an action potential.

Figure 5.3 Nanowire recordings from aortic smooth muscle cells. a) Rat aortic smooth muscle cells (A7r5) grew confluent on the nanowire chip. Nanowire sensing area is indicated by the dashed square. b) Transient nanowire current signals were induced by membrane depolarizing high K^+ solution. A typical current spike is highlighted in a smaller time scale.

The bioelectrical signals detected by our nanowires differ from the recorded action potentials by intracellular or patch-clamp recordings. The discrepancy is because the later is the direct measurement of intracellular voltage determined by the transmembrane currents through membrane ion channels from the whole cell whereas the former indirectly transduces the local extracellular voltage at the narrow cleft between the adhering membrane patch and the underneath nanowire into nanowire current signals.

And more intriguingly, the membrane properties of a cell are commonly heterogeneous. In other words, the electrical properties of the adhering membrane may differ from the whole cell behaviors because of heterogeneous expression of ion channels. Because of the same reasons, it is not unexpected that different cells types with characteristic membrane properties and adhesion properties produce different FET responses.

Fromherz and colleagues have developed an illustrative model for the interface between an excitable cell and a conventional FET [64, 126, 127], suggesting that the voltage alterations, which influence the conductance of the FET conducting channel buried under a thin dielectric layer, arise from ionic currents and capacitive currents flowing through the adhering membrane patch out of the interface cleft. They have demonstrated that distinct waveforms of FET responses are resulted when capacitive current or Ohmic ion current or voltage gated ion current dominates. According to Fromherz model, the signal from smooth muscle cells can be explained by initial inward sodium current and subsequent outward potassium current [128]. While the sharp positive spike from the cardiac myocytes may be due to a dominating capacitive outward current, and the following small plateau current may be resulted from long-lasting outward potassium current. But the downward spike seems to be mysterious. Obviously, further scrutiny is needed to understand the transduction mechanisms underlying nanowire sensing.

5.4 Conclusions

Nanotechnologies are bringing tremendous opportunities to biosensing and biology [87, 129]. Herein, a nanowire transistor platform for high-throughput electrophysiological recording on live cells is presented. Nanowires were reproducibly fabricated using conventional lithographical technology standardized in large-scale semiconductor manufactory with readily control on both wire diameter and length. Comparing to the state-of-the-art patch-clamp technique which is a laborious and invasive measurement requiring high skills and specialized equipments, nanowire approach provides several orders higher throughput, ease of use, low-cost and the capability of noninvasive and long-term continuous real-time monitoring. On the other hand, comparing to the extracellular recording using metallic microelectrodes, our approach provides exquisite sensitivity and unparalleled spatial resolution given the small size of the nanowire. Array format of SiNW-FET sensors allows spatially and temporally resolved, highly parallel or multiplexed measurements. We are currently developing our nanowire chips into fully functional lab-on-a-chip system with integrated microfluidic system for automatic and small volume fluidic handling and integrated electronics. We envision that the nanowire solution could transform the study of ion channels which underlies the membrane electricity from a specialist niche into a widely accessible “off the shelf” technique, greatly accelerating scientific studies and drug discovery.

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Chapter 6

Non-invasive measurement of cellular bioelectricity based on carbon nanotube devices for high throughput drug screening

6.1 Introduction

Excitable cells (e.g., myocytes, neurons, endocrine cells, and sensory cells) are able to generate bioelectrical signals resulting from the orchestrated activities of ion channels embedded within the cell membrane. Such bioelectrical signals are essential to many fundamental biological processes, such as, communication between neurons, contraction of heart or other muscles, vision, hearing, and so on [130]. Hence, drugs targeting to various ion channels have been developed for critical diseases such as cardiac arrhythmia, hypertension, Parkinson's disease, diabetes, depression, and neuropathic pain [131]. Despite longstanding recognition that these are major therapeutic targets and billions of dollars spent on research, ion channels are still largely under-exploited for drug development. The primary bottleneck in such efforts is the lack of high-throughput electrophysiological screening technologies. Here, we demonstrated that nanoelectronic biosensors based on readily fabricated networks of single-walled carbon nanotubes (SWNT-net) can be used to non-invasively detect cellular bioelectrical signals with high-throughput, high sensitivity, ease of use, low-cost without necessity of micro-/nano-fabrication, and the capability of long-term continuous real-time monitoring. This method promises applications in both fundamental research and high-throughput drug screening targeting on ion channels.

6.2 Materials and Methods

6.2.1 Fabrication of SWNT-net device

Carboxylated SWNTs (Carbon Solutions) were first dispersion in DI water by 30-min sonication (0.01 mg/mL), followed by centrifugation (6000 rpm) to remove large bundles and impurities. The aqueous dispersion of SWNTs was then drop casted onto a glass coverslip and dried in oven (60 °C) for 30 minutes. Silver paste (RS Component) was painted on carbon nanotubes to provide two electrodes (source and drain). Then, silicone rubber was used to make a chamber and insulate the electrodes. Before culturing cells on it, SWNT-net device was sterilized by immersion in 70% alcohol for 2 minutes following by DI water rinsing, and subsequently coated with fibronectin and gelatin.

6.2.2 Culturing HL-1 cells on SWNT-net device

HL-1 cells, a cardiac muscle cell line (HL-1), was a kind gift from Prof. William C. Claycomb (Louisiana State University). They were planted on pre-sterilized and pre-coated SWNT-net device with a density of 2.0×10^5 cells/mL, and cultured till confluence (after 4 or 5 days) in Claycomb medium supplemented with 100 μ M norepinephrine, 2 mM L-glutamine, 10% fetal bovine serum, and 100 U/mL penicillin-streptomycin.

6.2.3 Electrical recording

The cell culture medium was replaced by the bath solution containing (in mM, titrated to pH 7.4): 10 HEPES, 10 glucose, 140 NaCl, 1 MgCl₂, 5.5 KCl, and 2 CaCl₂. A 10 mV source-to-drain voltage was applied across SWNT-net while the SWNT-net current was continuously monitored using a EPC10 dual patch-clamp amplifier (HEKA Elektronik, Germany), with low-pass filtering at 1 kHz and a sampling rate of 10 kHz. In most experiments, action potentials from a cell on top of SWNT-net were also simultaneously recorded using EPC 10.

6.3 Results and discussion

Networks of single-walled carbon nanotube networks (SWNT-net) were simply fabricated by drop-casting aqueous dispersion of carboxylated SWNTs onto a glass coverslip. Due to the interstitial capillary force during water evaporation, SWNTs swim to the edge of the solution drop and form a ring of SWNT-net (~4 mm in diameter) with decreasing density from the edge till disappearance at ~140 μ m away from the edge (Fig. 6.1). Two electrodes (source and drain) were then prepared using silver conductive paint. And silicon rubber was used to insulate the electrodes and provide a chamber for cell culturing and recording. As demonstrated in Fig. 6.1a, the SWNT-net device operated as a liquid-gated FET manifests prominent *p*-type field-effect characteristics. Specifically, while biasing the SWNT-net (drain-source voltage, V_{ds}) at 10 mV, the SWNT-net conductance (or current) can be sensitively modulated by the gate voltage (V_g) applied to

the saline bath solution on the SWNT-net, suggesting that SWNT-net is able to detect minute electrical perturbations in solution occurring in the vicinity of nanotubes.

Figure 6.1 SWNT-net device. (a) Illustration of SWNT-net device configured as liquid-gated field-effect transistor, and relation of source-to-drain conductance versus gate voltage applied to the bath solution from a typical device. $V_{ds} = 10$ mV. (The leak current measured is less than 1 nA) (b) SEM image of part of the SWNT-net ring as indicated by a square in **a** (not to scale). **c.**, **d.** and **e.** Zoom-in SEM images of different regions on SWNT-net as indicated by squares in **b**.

After sterilizing SWNT-net and subsequent coating with adhesion protein (fibronectin and gelatin), cardiac myocytes (HL-1 cell line) were planted onto the SWNT-net device and grew for 4 days to reach confluence before the experiments (Figure 6.2a). Cardiomyocytes periodically produce bioelectrical signals called action potentials (APs) which are transient increase in intracellular electrical potential due to orchestrated transmembrane ionic currents flowing through voltage-gated ion channels on cell membrane. The periodic APs underlie the spontaneous contraction of cardiomyocytes and thus heart beating. It is conceivable that when the transmembrane ionic current flows from the intracellular space into the extracellular narrow gap at the interface between the cardiomyocytes and the underneath SWNT-net or vice versa, the electrical potential at the interface (V_i) alters. And the changes of electrical potential at the vicinity of SWNTs, in turn, influence the SWNT-net conductance, leading to measurable current signals from the voltage biased SWNT-net. This view is in line with the Fromherz model that explains well the coupling between excitable cells and conventional planar FETs [132]. Therefore, it is anticipated that when electrogenic cardiomyocytes are cultured on top of SWNT-net, the transmembrane ionic currents accompanying the periodic action potentials would cause periodic SWNT-net responses.

Figure 6.2 (a) Phase-contrast image of cardiomyocytes grown on the SWNT-net to confluence after 4-days culturing. (b) Illustration of simultaneous extracellular recording using a SWNT-net device and intracellular recording using an impaling nanopipette of bioelectrical signals (action potentials) from cardiomyocytes grown on top of the SWNT-net. (c) Spontaneous and periodic action potentials recorded intracellularly by the nanopipette (top) and simultaneous current responses of SWNT-net (bottom).

We monitored the SWNT-net current and, at the same time, recorded action potentials from a cardiomyocyte on top of the SWNT-net using a quartz nanopipette with a tip size of ~ 100 nm impaled inside the cell (as illustrated in Figure 6.2b). As expected, periodic SWNT-net current signals were observed (Figure 6.2c), and they synchronized with the contraction of cardiomyocytes. All cardiomyocytes in the confluent sheet contract as a

whole due to the synchronization of their action potentials through gap junctions between cells. As shown in Figure 6.2c, The SWNT-net signals are in concert with the spontaneous action potentials recorded intracellularly from a myocyte on the SWNT-net. This unambiguously indicates that each episode of the SWNT-net response corresponds to an action potential generated in cardiomyocytes by sequential activation of sodium, calcium and potassium currents. The action potential of HL-1 cells proceeds with a rapid depolarization phase (i.e., the intracellular potential increases from the resting level ~ -60 mV) due to activation of the inward Na^+ current through the fast voltage-gated Na^+ channels, followed by a slow repolarization phase (i.e., the intracellular potential returns back to the resting level) resulting from the net action of the inward Ca^{2+} current through L-type Ca^{2+} channels and delayed outward K^+ current through delayed-rectifier K^+ channels.

As summarized in Figure 6.3, four distinct types of SWNT-net current spikes have been observed in 43 independent experiments.

Figure 6.3. Four types of signal recorded by SWNT-net.

Type I signal as shown in Figure 6.2c and Figure 6.3a (32.6% of total recordings) exhibits triphasic waveform. When 500 nM tetrodotoxin (TTX), which blocks sodium channels was applied to the cardiomyocytes, the upstroke velocity and the frequency of action potentials were decreased expectedly (Figure 6.4a) [133]. We found that addition of more than 10 μ M TTX had completely eliminate the action potential. In accompany with the slowed rising phase of AP, the amplitude of the SWNT-net response was significantly reduced. It indicates that the amplitude of type I signal mainly reflects the early activation of sodium currents which initiates the action potential and determines its rise slope. As compared to the change in AP, the change in SWNT-net current appears to be a more sensitive indicator. Figure 6.4b presents an experiment in which verapamil, a L-type calcium channel blocker and antiarrhythmic drug, was applied (10 μ M). The amplitude of the SWNT-net response was not sensitive to the application of verapamil, whereas the duration of the signal was shortened. This is consistent with the observation that the duration of AP, which depends on the sustained Ca^{2+} current, was shortened (Figure 6.4b) [134]. Also being an expected effect of verapamil [135], the frequency of AP was decreased as reported by both nanopipette and SWNT-net recording.

Figure 6.4 Action potentials (top traces) of HL-1 cell and SWNT-net responses (middle and bottom traces) before (red) and after (blue) application of TTX (a) or verapamil (b).

27.9% of the SWNT-net recordings are type II signal, which is a transient current decrease in phase with the action potential and often ends with a small upward component corresponding to the hyperpolarization phase of action potential (Figure 6.3b). As shown in Figure 6.5a, application of TTX largely eliminated the sharp downward phase of the SWNT-net signal, implying that this initial rapid phase is caused by the sodium current flowing from the SWNT-cell interface gap into the cell. In contrast, Ca^{2+} channel blocker verapamil did not significantly affect the downstroke of the SWNT-net signal (Figure 6.5b). Carbamoylcholine (Cch) is the classic muscarinic acetylcholine receptor (mAChR) agonist which slows the heart beat rate and the duration of AP by enhancing the potassium conductance (Figure 6.5c) [136]. These effects were clearly evidenced by the decreased duration and frequency of the SWNT-net signals after administrating $1\mu\text{M}$ Cch to the cardiomyocytes.

Figure 6.5 Action potentials (top traces) of HL-1 cell and SWNT-net responses (middle and bottom traces) before (red) and after (blue) application of TTX (a) or verapamil (b) or Cch (c).

Type III signal (20.9% of all recordings) is the third derivative of the action potential, manifesting a triphasic downward waveform as Type I. But its duration is much shorter than Type I and it coincides with the sharp rise of AP. Application of TTX completely eliminates Type III signal whereas application of verapamil did not exert obvious effects, suggesting that it is an indicator of early sodium current (Figure 6.6). Propranolol which inhibits beta-adrenoreceptors is an antiarrhythmic compound. It is known that propranolol slows the heart beating rate and reduces the rate of depolarization of cardiac AP [137].

These propranolol effects were observed in both AP and SWNT response. Note the clear decrease in the amplitude of SWNT signals.

Figure 6.6 Action potentials (top traces) of HL-1 cell and SWNT-net responses (middle and bottom traces) before (red) and after (blue) application of TTX (a) or verapamil (b) or propranolol (c).

Type IV signal (18.6% of all recordings) is simply the negative version of Type III (Figure 6.3d). Application of TTX shortens the amplitude of SWNT-net signals while application of verapamil does not cause obvious change to the SWNT-net response (Figure 6.7). Both of the drugs decrease the frequency of firing action potential as expected.

Figure 6.7 Action potentials (top traces) of HL-1 cell and SWNT-net responses (middle and bottom traces) before (red) and after (blue) application of TTX (a) or verapamil (b).

Our SWNT-net devices were also used to detect ECG signals (collective bioelectrical signals from all cardiomyocytes) from beating rat hearts (Figure 6.8). In agreement with the observations using metallic microelectrodes [138], the features of ECG signals recorded by SWNT-net depend on the orientation of the beating heart. When the tip of the ventricles touches the SWNT-net, biphasic current responses were recorded. As the ventricle was lowered closer to the SWNT-net, a smaller biphasic signal corresponding to the repolarization of the ventricle followed the major biphasic current spike. When the

atrium touched the SWNT-net, a preceding smaller biphasic signal due to depolarization of atrium was detected. The duration between the two current spikes represents the propagation velocity of depolarization between the atria and the ventricles. The ECG recorded by a nanopipette impaled into the cell interstice of the ventricle (Figure 6.8a, bottom) synchronized with the major biphasic SWNT-net signal. As shown in Figure 6.8b, application of verapamil (10 μ M) decreased the frequency of AP (thus heart beating rate), and the propagation of bioelectricity between atria and ventricles as evidenced by the elongated gap between the atrium and ventricle spikes in the SWNT-net signal over time. On the other hand, application of TTX (1 μ M) reduced the ECG frequency, slowed the electrical propagation and altered the waveform of the signal originated from the ventricles (Figure 6.8c). Taken together, we show that our SWNT-net devices can detect bioelectricity not only at cell level but also at tissue level.

Figure 6.8 ECG signals detected by SWNT-net FET. (a) SWNT-net response (left) to ECG signal produced by a beating rat heart at different orientations (i, ii, and iii). In the cartoons shown at right, A refers to atrium and V refers to ventricle. In the experiments denoted as iii, a nanopipette impaled into a ventricle was used to detect ECG of the ventricle simultaneously. The ECG signal recorded by the nanopipette coincides with the major spike in the SWNT-net signal. (b) and (c) The frequency and the features of the SWNT-net signal are sensitive to verapamil (b) and TTX (c).

Intracellular recording using a fine penetrating electrode is the classic electrophysiological technique to detect cellular bioelectricity resulting from ion channel activities, while patch-clamp technique derived from the intracellular recording is the state-of-the-art technique that is able to resolve activities of single ion channel. But both methods, particularly patch clamp, are invasive and of extremely low-throughput due to high skills and specialized equipments required, tedious procedures involved, and low success rate. Alternatively, bioelectricity can be recorded extracellularly using metal microelectrodes [58] or conventional planar field-effect transistors (FETs) [65, 139] or more recently silicon nanowire based FETs [70, 109, 140] fabricated in clean-room by micro- or nano- fabrication technologies. In contrast to intracellular recordings, extracellular recordings enable long-term non-invasive monitoring. Here, we demonstrate a simple and novel alternative for extracellular recording using SWNT-net FET devices which can be readily fabricated on bench tops. As demonstrated, SWNT-net FETs are able to report rich information on ion channel activities. In addition, SWNT-net may be made on a soft substrate which can be attached conformally to cardiac tissues or neuronal tissues to detect cellular bioelectricity in situ. Because the sensitivity of SWNT-net FET is greatly compromised by the contamination of metallic SWNTs, it could be significantly improved with advance of new techniques to effectively eliminate (or suppress) metallic SWNTs [141] or to synthesize SWNTs with chiral specificity. The on-going intensive research on developing high-performance SWNT FETs promises better SWNT devices for detection of bioelectricity. In principle, SWNT-net FETs can be miniaturized, for example, with assistance of micropatterning techniques, in order to interface with single cells and detect bioelectricity at single cell level. And FETs based on single or a few

SWNTs may be utilized to achieve high spatial resolution in detection, i.e., local detection of ion channel activities at subcellular domain.

Although the exact mechanisms underlying the SWNT-net responses to cellular bioelectricity warrants further investigations, the signal transduction model proposed by Fromherz [132] and others [65, 139] on coupling between the planar FET and the excitable cells grown on top provide insights to our observations. According to Fromherz model, the electrical potential at the narrow interface between the cardiomyocytes and the SWNT-net alters (ΔV_i) as the ionic currents flows from the solution at the interface gap into the cell membrane through ion channels or out of the cell into the narrow interface space. To describe mathematically, $\Delta V_i = R_i \times \Delta I_{\text{trans}}$, where R_i is the solution resistance of the interface and I_{trans} is the net transmembrane current flowing at the interface. ΔI_{trans} depends on the density and composition of the ion channels on the interface membrane and varies from cell passage to cell passage. It explains why different types of signals are observed from SWNT-net recordings and other extracellular recording methods. But the Fromherz model may not be able to fully explain the SWNT-net responses because, in contrast to the planar FET whose conducting channel is buried underneath the dielectric insulation layer, SWNTs as the conducting channels are in direct contact with the cells. Thus, the alteration of the electrical double layer (only ~1 nm thin) on SWNT surface caused by the transmembrane ionic current may also play a significant role in modulating SWNT-net current.

6.4 Conclusions

In Summary, we demonstrate a novel method for extracellular recording of cellular bioelectricity using SWNT-net FETs which can be simply made on bench tops. We show that ionic currents accompanying an action potential can produce characteristic SWNT-net responses. Such nanotube approach enables high-throughput drug screening targeting on ion channels, not only on cardiomyocytes but also on other electrogenic cells such as neurons and neuroendocrine cells. We envision that the interface between nanoelectronics and biology promises various novel applications in both fundamental biology and drug discovery [110].

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Chapter 7

General Conclusions and Perspectives

7.1 General Conclusions

In summary, nanoelectronic biosensors constructed using SiNWs and SWNTs offer promising platforms in resolving dynamic cellular activities, in particular, regulated secretion (exocytosis) of signaling molecules and bioelectricity due to ion channel activities. As demonstrated, these sensors are able to detect the low amount of biomolecules (fM) and minute cellular bioelectricity (mV). As the development of nanoelectronic devices are rapidly advancing due to the world-wide tremendous effort to transcend the current bulk-silicon based microelectronics, we anticipated that nanoelectronic biosensors would be able to achieve even higher sensitivity and capabilities in the near future. Also as demonstrated, nanoelectronic approaches, which provide rapid, non-invasive, label-free, and simple measurement, could be instrumental to biologists serving as novel platforms for high-throughput functional studies. In addition, nanoelectronic approaches could be employed for high-throughput drug screening targeting on various cell functions.

7.2 Perspectives

Although we only demonstrated several examples of the use of nanoelectronic biosensors, nanoelectronic approach can serve as a universal platform to examine a spectrum of cell functions, with properly devised transduction mechanism and well-defined biological context.

Biosignal transduction, often mediated by membrane receptors, may lead to various cellular activities, such as alteration in membrane receptor conformation, activation of ion channels, secretion of biomolecules, changes in metabolic activities, cell morphology, discharge of metabolites or intracellular fluids. These activities could modulate the conductance of the interfacing nanoelectronic sensing element. For example, SWNT- FET was used to detect the extracellular acidification of macrophages (due to release of acidic metabolites) upon their binding to IgG antibodies [2]. We will continue our endeavor to apply our techniques in investigations of other cell functions.

To study the functions of membrane proteins without interference of complex molecular machineries in the native cell membrane, nanoelectronic sensors could be coupled with artificial lipid bilayer (biomimetic membrane) embedded with protein of interest. As an example, an artificial lipid bilayer incorporated with ligand gated and voltage gated peptide ion channels was assembled on the SiNW FET to monitor proton transport through these channels [142].

Due to the small size of the nanostructured materials, small sensing elements could be constructed for spatially resolved detection. In our future efforts, we will take advantage

of this to detect the local biological events mediated by membrane proteins at subcellular level or at microscale membrane functional domains. Although all our devices are made on solid substrates, it is possible to fabricate the devices on flexible substrates. Such nanoelectronic sensors can be readily attached conformally to cardiac tissue or other organs for *in vivo* studies. In a recent study, SiNW-FETs were fabricated on soft polymer substrate to detect bioelectrical signals on a beating heart surface [71]. Carbon nanotubes should also be suitable to make similar flexible sensors.

Although our devices utilize SiNWs and SWNTs as the sensing elements, other semiconducting nanomaterials may also be explored. For example, we will compare the performance of carbon nanotubes with graphene, which is a flat two-dimensional carbon sheet of one atom thick discovered recently in 2004 [143]. Indeed, graphene to detect cellular electrical activity has recently been demonstrated by Lieber's group [144]. It has been shown that graphene offers unique capability of recording signals in both *n*-type or *p*-type mode via tuning the liquid gate voltage across the Dirac point. However, high-yield fabrication of high-performance graphene devices is not yet available and the 2D structure of graphene does not allow nanoscale localized detection. In addition to graphene, other nanostructured nanomaterial could be used, such as In_2O_3 [145], ZnO [146] and ZnS [147].

Despite the successes demonstrated by other labs and in this thesis, the signal transduction mechanisms between the cellular activities and the nanomaterial responses are not fully understood. We will seek to understand it with assistance of modeling and computer simulations. For example, the activities of ion channels can be described by

Hodgkin-Huxley model and the narrow gap between the nanostructured materials and cell membrane can be modeled as a thin resistive solution interface with particular diffusion characteristics. Understanding of the transduction mechanisms would be critical to optimize the sensing capabilities of our devices.

Electronic sensing based on nanomaterials provides many advantages as described in the thesis, such as, high temporal and high spatial resolution, simple and label-free detection, etc. It provides a novel alternative to the conventional methods which would still be indispensable for biosensing. A limitation of nanoelectronic sensing is that the detection depends on measurable modulation of the conductance of the nanomaterial through the local and intimate interactions between the target and the nanomaterial or depends on the availability of recognition elements to specifically recognize the target. Although as described in the thesis, nanoelectronic biosensing provides a versatile platform for many applications, in many cases, it cannot replace other conventional methods which offer their own advantages. In addition, in current stage, nanoelectronic biosensors, although showing great potentials, are still limited to a few laboratories. Tremendous efforts are expected to make commercialized affordable fully-functional devices that are readily available to laboratories and pharmaceutical companies.

Taken together, much effort is still required to advance this emerging, yet promising, field of nanoelectronic biosensing. We envision that this rapidly evolving technology will bring tremendous impacts to various aspects of biomedicine.

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List of Publications

- 1) **Pui TS**, Agarwal A, Ye F, Huang Y, and Chen P, “*Nanoelectronic detection of triggered secretion of pro-inflammatory cytokines using CMOS-compatible silicon nanowires*”, **Biosensors and Bioelectronics**, DOI: 10.1016/j.bios.2010.09059, in press.
- 2) **Pui TS**, Sudibya H, Luan X, Zhang Q, Ye F, Huang Y, and Chen P, “*Non – invasive detection of cellular bioelectricity based on carbon nanotube devices for high – throughput drug screening*”, **Advanced Materials**, 2010, 22(29):3199-3203.
- 3) **Pui TS**, Agarwal A, Ye F, Tou ZQ, Huang Y, and Chen P, “*Ultra-sensitive detection of adipocytokines secreted from adipocytes with CMOS-compatible silicon nanowire arrays*”, **Nanoscale**, 2009, 1(1):1137-114.
- 4) **Pui TS**, Agarwal A, Ye F, Balasubramanian N, Chen P, “*CMOS-compatible nanowire sensor arrays for detection of cellular bioelectricity*”, **Small**, 2009, 5(2):208-212.