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Nanoelectronic Detection of Triggered Secretion of Pro-inflammatory Cytokines Using CMOS Compatible Silicon Nanowires

Tze-Sian Pui^a, Ajay Agarwal^b, Feng Ye^a, Yinxi Huang^a, and Peng Chen^{a,*}

^aSchool of Chemical and Biomedical Engineering, Bioengineering Division, Nanyang Technological University, 70 Nanyang Drive, Singapore 637457

^bInstitute of Microelectronics, A^{}STAR (Agency for Science, Technology and Research), Singapore 117685*

* Correspondence: chenpeng@ntu.edu.sg

Abstract

Nanotechnology, such as nanoelectronic biosensors, are bringing new opportunities and tools to the studies of cell biology, clinical applications, and drug discovery. In this study, crystalline silicon nanowire based field-effect transistors fabricated using top-down approach were employed to parallelly detect pro-inflammatory cytokines in the complex biological fluids (cell culture medium and blood samples) with high specificity and femtomolar sensitivity. Using this technique, the dynamic secretion of TNF-alpha and IL6 were revealed during the immune response of macrophages and rats to the stimulation of bacteria endotoxin. This technique could provide a unique platform to examine the profile of complex immune responses for fundamental studies and diagnosis.

Keywords: silicon nanowires, nanoelectronic biosensing, biosensors, inflammatory cytokines, bio-nanotechnology, immune response

1. Introduction

Field-effect transistors (FETs) based on silicon nanowires (SiNWs) (Carlen and van den Berg 2007) have been employed to electrically detect various biomolecules (Wang et al. 2005; Zheng et al. 2005) and cellular bioelectricity (Patolsky et al. 2006; Pui et al. 2009a). Comparing to conventional biochemical, immunofluorescence and biophysical assays, such nanoelectronic biosensing (Huang and Chen, 2010) 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 (Hopkins 2003). 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 (Hack et al. 1997; Hirano et al. 1990; Nanes 2003; Pedersen and Akerstrom 2007). They often act collaboratively, particularly in acute immune reactions. Dysregulations of TNF- α secretion are implicated in a variety of diseases such as asthma, rheumatoid arthritis, and cancers (Kriegler et al. 1988; Lin et al. 2008) while abnormalities in secretion of IL-6 are associated with pathological conditions such as diabetes, atherosclerosis, systemic lupus erythematosus, and rheumatoid arthritis

(Akira et al. 1993). 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 (Stern et al. 2008). Here, we directly detect pro-inflammatory cytokines by functionalizing SiNWs with cytokine-specific antibodies. As compared to the conventional analytical methods (enzyme-linked immunosorbent assay - 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 µl buffer solution) and time (minutes per data point).

2. Materials and Methods

2.1 Materials and chemicals

TNF-alpha and IL - 6 were purchased from Biovision. Polyclonal antibodies against TNF-alpha and IL-6 were purchased from Santa Cruz Biotechnology (USA) and Abcam, respectively. Lipopolysaccharides (Pseudomonas aeruginosa 10), (3-Aminopropyl) triethoxy-silane (APTES), ethanolamine, glutaraldehyde and other chemicals were obtained from Sigma-Aldrich. Arrays of crystalline silicon nanowires were fabricated in Institute of Microelectronic (Singapore) using our previously reported CMOS-compatible top-down method (Pui et al. 2009b).

2.2 SiNW functionalization

The SiNW chips were first soaked in 0.5 % hydrofluoric 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 deionized water) at room temperature for 2 hours. The TNF-alpha antibodies or IL-6 antibodies 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 blown dry with nitrogen.

2.3 Electrical measurement

With 40 µl measurement buffer solution (containing different analytes) 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.

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 °C under a 5 % CO₂ atmosphere in T75 flask.

3. Results and discussion

Arrays of crystalline silicon nanowires were fabricated by CMOS-compatible top-down method as previously reported (Pui et al. 2009b) (Figure S1 in Supplementary Information). The nanowire exhibits prominent n-type field-effect, indicating that its conductance is sensitive to the minute electrical perturbation in the solution. The antibodies against TNF-alpha and antibodies against IL-6 were crosslinked onto SiNWs in two separated sensing regions (Figure 1a) through a series of functionalization steps (Figure 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 1)

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 ($\sim 0.002x$ 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, **due to electrical gating effect**.

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) increase (or decrease) of nanowire conductance in the corresponding sensing region (Figure 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). Such sensitivity is several orders better than ELISA measurement (pg/ml). Although it is comparable to the sensitivity of radioimmunoassay, radioimmunoassay suffers from several limitations, such as radiation hazards, stringent requirements on experimental conditions and skills, and short shelf-life of radionuclide-labeled antibodies.

In comparison, cell culture medium similarly diluted in the buffer solution did not cause appreciable nanowire response (Figure 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. Furthermore, TNF-alpha antibody functionalized SiNWs did not respond to IL-6 while IL-6 antibody functionalized SiNWs did not respond to TNF-alpha. And cytokine leptin and resistin could not cause any responses from the two types of functionalized SiNWs (data not shown).

(Figure 2)

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 2. As shown in Figure 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) (Utaisincharoen et al. 2000). 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 (Shalaby et al. 1989). In contrast, without LPS treatment, the concentrations of both cytokines remained low, indicating that the basal release of these cytokines is trivial.

(Figure 3)

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 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 (Shalaby et al. 1989). 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 (Ulich et al. 1991). Our results are comparable with previously reported results based on ELISA (Blanche et al. 1998). 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.

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 (Huang and Chen, 2010; Pui et al., 2010).

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Figure legends:

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

Figure 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.

Figure 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 µg 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 2. Each data point (mean +/- SEM) is the average from 10 SiNWs in 3 chips.

Figure 1

Figure 2

Figure 3