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Label-Free Electrical Detection of Cardiac Biomarker with Complementary Metal-Oxide Semiconductor-Compatible Silicon Nanowire Sensor Arrays

Jay Huiyi Chua, Ru-Ern Chee, Ajay Agarwal, She Mein Wong, and Guo-Jun Zhang*

Institute of Microelectronics, Agency for Science, Technology and Research, 11 Science Park Road, Singapore 117685

Arrays of highly ordered silicon nanowire (SiNW) clusters are fabricated using complementary metal-oxide semiconductor (CMOS) field effect transistor-compatible technology, and the ultrasensitive, label-free, electrical detection of cardiac biomarker in real time using the array sensor is presented. The successful detection of human cardiac troponin-T (cTnT) has been demonstrated in an assay buffer solution of concentration down to 1 fg/mL, as well as in an undiluted human serum environment of concentration as low as 30 fg/mL. The high specificity, selectivity, and swift response time of the SiNWs to the presence of ultralow concentrations of a target protein in a biological analyte solution, even in the presence of a high total protein concentration, paves the way for the development of a medical diagnostic system for point-of-care application that is able to provide an early and accurate indication of cardiac cellular necrosis.

Human cardiac troponin-T (cTnT) is a key protein biomarker that is present in elevated concentrations in the bloodstream of a patient suffering from acute myocardial infarction. It is regarded, together with human cardiac troponin-I (cTnI), as the most cardiac-specific of currently catalogued biochemical markers.[1] The damage to the myocardium can be directly related to the indicative concentrations of the cTnT protein and may thus be used as an effective differentiator between a patient suffering from unstable angina or a more serious case of heart attack. Troponin detection has been proposed as a sensitive marker for the indication of myocardial necrosis and thus has been well studied for its prognostic value in patients that suffer from acute coronary syndrome,[2−4] especially given its wide temporal diagnostic window: troponin concentrations may remain at elevated levels in the bloodstream for 4−10 days after the onset of acute myocardial infarction.[5]

The development of efficient diagnostic sensor devices will conceivably rely on the analysis of blood or serum samples obtained from patients subject to symptoms relating to heart failure. Current techniques of protein detection such as the enzyme-linked immunosorbent assay (ELISA) are not able to allow for the label-free and highly targeted detection of sub-10 pg/mL concentrations of cTnT.[6] Furthermore, such techniques often require highly skilled laboratory personnel as well as a significant investment of time and resources into procuring specialized equipment. The early detection and subsequent treatment of a patient suffering from conditions relating to heart disease will likely require the timely identification of abnormal cTnT concentrations.

Molecular electronics opens the door to new types of ultrasmall sensors. Electronic properties of DNA and even a single molecule wired between a source and drain electrode have been studied.[7,8] The importance of biosensors for diagnostic applications has attracted substantial attention to the research and development of silicon nanowire (SiNW) field effect sensors. SiNWs have demonstrated key capabilities in the sensitive identification of biological species, such as viruses,[9] DNA/miRNA,[10−16] proteins,[17,18,21]

* Corresponding author. Phone: +65-6770-5390. Fax: +65-6774-5754. E-mail: zhanggj@ime.a-star.edu.sg.

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alkali-metal ions,[17,19] and small molecules,[20] based on the importance of a portable and integrated system for clinical applications. As sensing elements, nanowires have been touted to offer key advantages in the diagnosis of complex diseases such as cancer, which requires simultaneous detection of biomolecular markers associated with various levels of disease pathogenesis.[21] They combine significant advantages such as a label-free process, rapid response rates, as well as the ability to detect subpicomolar concentrations of a target species. These key advantages are significant for the development of bedside applications such as in the rapid detection of abnormal concentrations of protein biomarkers in medical diagnostics.

SiNWs can be fabricated either via top-down lithographic techniques or by using catalyzed bottom-up growth methodologies. Ease of production and reconfigurability of produced SiNWs are afforded by the bottom-up growth methodology; however, the approach inherently suffers from limitations such as scalability and the difficulty of mass production, since the integration with external circuitry necessitates precise and individual positioning of the nanowires. On the other hand, the "top-down" procedure allows for the mass production of SiNW devices as it provides for significant compatibility with established CMOS lithographic manufacturing technologies. The top-down procedure has also demonstrated good sensing results in literature, as demonstrated by Stern et al. in the label-free detection of sub-100 fM concentrations of antibodies using CMOS-compatible SiNW devices manufactured via lithographic procedures.[18]

Herein we present a CMOS-compatible SiNW array platform for the label-free, ultrasensitive, real-time detection of cTnT in the assay buffer as well as in undiluted serum samples. In designing the SiNW manufacturing process, we focused on the utilization of conventional optical lithographic techniques and self-limiting oxidation processes that can reliably reproduce the SiNW dimensions used in our sensing experiments. The ability of our SiNW devices to sense specific biomarkers such as cTnT is derived from the appropriate functionalization of the silicon surface which can be coupled with biomolecular moieties for the detection of specific protein biomarkers such as cTnT. The sensitive nature is due to the high surface area to volume ratio that the nanowire dimensions confer. The nanoscale geometry of the nanowire device also negates the necessity of large sample volumes which are often critical in traditional systems that perform spectrum analysis of enzyme-catalyzed products, further reinforcing its future promise as a resource-efficient early detection sensor technology. We demonstrate the protein sensing capability to 1 fg/mL concentrations in the buffer solution, as well as 30 fg/mL concentrations in the serum samples, indicating the practicable development of rapid, robust, high-performance, and low-cost diagnostic systems that afford substantial improvement over existing protein-identification methodologies such as ELISA or Western blot.

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EXPERIMENTAL METHODOLOGY

Materials. Monoclonal mouse anti-human cardiac troponin-T and human troponin-T were purchased from HyTest Ltd. (Turku, Finland). Bovine serum albumin (BSA), human serum (from platelet poor human plasma), and all other chemicals were purchased from Sigma-Aldrich, Inc. The proteins were used without further purification and diluted to the desired concentrations with the assay buffer. Centrifugal filter devices (Centricon Ym-3, 3 000 molecular weight cutoff (MWCO)) were purchased from Millipore (Billerica, MA).

SiNW Device Fabrication. The silicon nanowire devices were fabricated on a n-doped silicon-on-insulator (SOI) substrate with a 145 nm buried oxide layer. The wafers were n-doped with a implant dose of 5×10^{13} cm^{-2} at 30 keV and the dopants activated via rapid thermal annealing. With the use of deep ultraviolet lithography, a 50−80 nm silicon layer was patterned and etched to define the fins from which the nanowire arrays were derived. Individual nanowires were further formed via the thermal oxidation of the fin structure at 900 °C in O_2 for ∼4 h, followed by n+ doping of the nanowire ends for contact metal definition and subsequent passivation with Si_3N_4/SiO_2. Because high temperature (900 °C) destroys the metallization, all the thermal processes were accomplished before metallization in the fabrication of the device. Finally, the nanowires were released by dry etching of the Si_3N_4/SiO_2 passivation layers followed by wet etching of the remaining SiO_2.

Surface Functionalization. In order to chemically modify the surface of the SiNWs to allow binding of biological probe molecules, the SiNW array chips were immersed in a solution of 2% 3-aminopropyltriethoxysilane (APTES) in a mixture of ethanol/H_2O (95%/5%, v/v) for 2 h, as previously reported.[22] APTES molecules bind to the hydroxyl-terminated SiO_2 surfaces of the nanowires, resulting in an amine-functionalized surface. A bifunctional linker, glutaraldehyde, was then covalently attached onto the amine-terminated surface by immersing the array chips in a solution of 2.5% glutaraldehyde in water for 1 h. Antibody attachment on the SiNW surface was carried out by applying antibody solution in phosphate buffered saline (PBS) at room temperature for 2 h, followed by thoroughly washing with the same buffer. In order to prevent nonspecific binding of proteins in the detection step, the unreacted aldehydic groups on NW surface were passivated by applying 100 mM ethanolamine solution in PBS for 1 h, followed by washing with the same buffer. In various experiments, solutions of human anti-cTnT at concentrations of 10 μg/mL in buffer solution were used. Figure 1 provides a schematic depiction of the above functionalization procedure.

Fluid Exchange. Acrylic wells designed for the attachment of Tygon tubes for fluid exchange were adhered onto the surface of the SiNW array chips prior to antibody attachment. The flow setup is similar to the one reported by the M. A. Reed group.[18] More specifically, a rectangular macroscale solution chamber (inner size, $\sim 0.8 \times 0.4$ cm^2) instead of a microchannel was used, which avoids the diffusion issue but may increase detection sensitivity because a larger amount of solution is applied. The simple design involves a reservoir which contains a volume of

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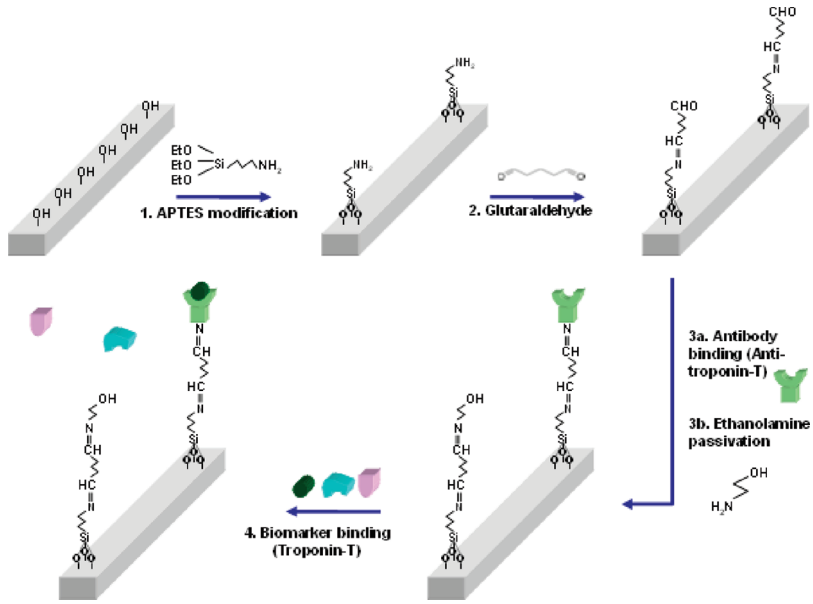

Figure 1. Schematic diagram of the chemical process for surface functionalization of SiNW devices. The hydroxyl-terminated silicon dioxide surface of the nanowire binds to the ethoxy groups of APTES. Glutaraldehyde converts the amine-terminated surface to an aldehyde-terminated one, which is able to bind with the N-terminus of the anti-troponin-T antibody. Antigen−antibody interactions cause troponin-T to bind specifically to the antitroponin-T probes on the surface, causing changes in SiNW conductance.

buffer solution sufficient to maximize binding efficiency while avoiding the diffusion limitations that microchannel systems inherently suffer from. A buffer solution of 0.01× PBS (pH 7.4) was used for cTnT detection experiments. For all the sensing experiments, the volume of liquid in the solution chamber was 20 μL, and another 20 μL of various protein solution was added by a syringe pump under a flow rate of 1 mL/min.

Electrical Characterization. Electrical measurements of SiNW conductances were performed using an Alessi REL-6100 probe station (Cascade Microtech, Beaverton, OR) in conjunction with a HP parameter analyzer (HP-4155A). A two-probe system was set up in which a controlled voltage could be applied to the source electrode of the SiNW device with the drain electrode referenced to ground. To verify the quality of the SiNW devices before real-time detection was performed, the current−voltage ($I-V$) curve of the SiNW with back-gate bias at various stages during the functionalization procedure was obtained by sweeping the applied voltage from −0.5 to +0.5 V. SiNWs that demonstrate ohmic $I-V$ characteristics were selected for biotesting.

Real-Time Detection. Real-time detection was performed using the probe station system described earlier. A short pulse voltage of 0.1 V was applied to the SiNW device, and the current through the SiNW was measured. One data point was taken every 1 s in order to reduce heating effects due to power dissipation through the SiNW device. A buffer solution was injected into the SiNW device region through the inflow tube. Monitoring of the current through the SiNW device while immersed in buffer solution proceeded for between 100−200 s subsequent to the time of addition of buffer in order to establish the baseline current relative to which changes in conductance would be measured. Upon establishment of the baseline current, the biomolecule analyte solution was added into the SiNW device region through the inflow tube, resulting in a change in conductivity due to specific binding of the biomolecule to the functionalized surface. Monitoring of the current through the device proceeded for between 100−200 s subsequent to addition of the analyte solution in order to obtain a stable current representing the conductance of the SiNW after the binding event.

The charge on the protein molecule may potentially be screened by the counterions that exist within the buffer solution. Various studies[23] have clearly demonstrated the importance of the Debye screening effect on field effect sensors; thus, we have deliberately chosen the concentration of buffer solution in which to perform the biomolecule detection experiments so as to minimize this counterion screening effect. The Debye length, λ_D, of 0.01× PBS is calculated to be ~7.3 nm,[24] which is sufficiently long to ensure effective sensing during the capture process.

RESULTS AND DISCUSSION

SiNW Design and Characteristics. In this study, the SiNW biosensors for detection of cardiac biomarkers were realized by immobilizing specific antibodies onto the SiNW surface after device fabrication. The details are illustrated in Figure 1. The native oxide coating on SiNW surfaces is an effective passivation layer which can usually be functionalized with target molecules of interest. A conventional silane chemistry, as described earlier, was employed to generate an amine-modified SiNW surface as the technique is well understood and the reagents easily accessible.[22] The freshly prepared antibody-functionalized SiNW chips proceeded immediately to electrical testing.

The device consists of SiNW clusters including electrically addressable and well ordered arrays, contact lines and pads, and an acrylic reservoir for solution exchange (Figure 2a). The SiNW array chip consists of 36 clusters of electrically addressable and well-ordered SiNWs separated by a 200 μm spacing; each cluster is composed of 5 individually addressable nanowires. A schematic

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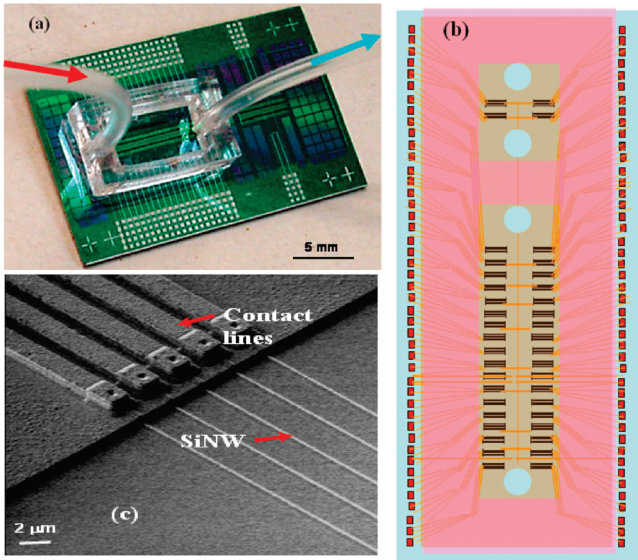

Figure 2. Clockwise from top, (a) Image of SiNW device array chip, integrated with microfluidic system for fluid exchange, used in experiments. Fluids are deposited into the acrylic well through the inflow tube on the left (red arrow) and removed from the outflow tube on the right (blue arrow). (b) Schematic showing the layout of the SiNW device array on the chip. A total of 36 clusters of 5 nanowires each are available for use, potentially allowing for simultaneous, multiplexed detection of assorted proteins. (c) SEM image of a cluster of nanowires. Each nanowire is individually addressable by oxide-passivated metal contact lines running out to the external edge of the chip.

of the array layout is provided in Figure 2b. The dimensions of the design layout were chosen to be compatible with the capabilities of robot spotting technology, allowing for the immobilization of different capture probes at different clusters in order to provide multiplexed detection capabilities with electronic read-out. Scanning electron microscopy (SEM) micrographs of the SiNW devices were undertaken to document the physical characteristics of the device. As seen in Figure 2c, the SiNW arrays were observed to be well-shaped, highly uniform, and well-aligned, averaging 90 μm in length and 2 μm spacing in between two wires.

Detection of Human Troponin-T in Buffer Solution. To develop a SiNW array chip able to detect ultralow concentrations of cTnT in biological samples, detection and differentiation between various concentrations of cTnT in buffer solution were carried out. Solutions of cTnT of various concentrations were prepared by dissolving and serially diluting pure cTnT in 0.01× PBS. SiNW devices were functionalized with anti-cTnT antibodies and individually used in experiments to detect cTnT (pI $\sim$ 5.0)[25] in these analyte solutions. Figure 3a demonstrates the real-time response of conductance upon injection of various concentrations of cTnT, where cTnT concentrations are decreased from 1 ng/mL to 1 fg/mL. The data was normalized by computing $|G/G_{t\ =\ 0}|$ and plotted on the same axes for an effective comparison of the relative change in conductance for the SiNW devices used to detect the different concentrations of cTnT. Known concentrations of cTnT were added after a stable reading with 0.01× PBS buffer was achieved. Addition of 1 ng/mL, 1 pg/mL, 100 fg/mL, and 1 fg/mL solutions of cTnT resulted in conductance decreases of ~27%, ~20%, ~11%, and ~5%, respectively, and the SiNW response as a function of the log of cTnT concentration is shown in Figure 3b. The relative standard deviation (RSD) of the data is calculated to be 9.7%, which indicates a good degree of agreement between the nanowire sensors. The ultralow levels of protein that have been demonstrated to be detectable may establish a lower limit of protein concentration required for electrical detection using SiNW technology.

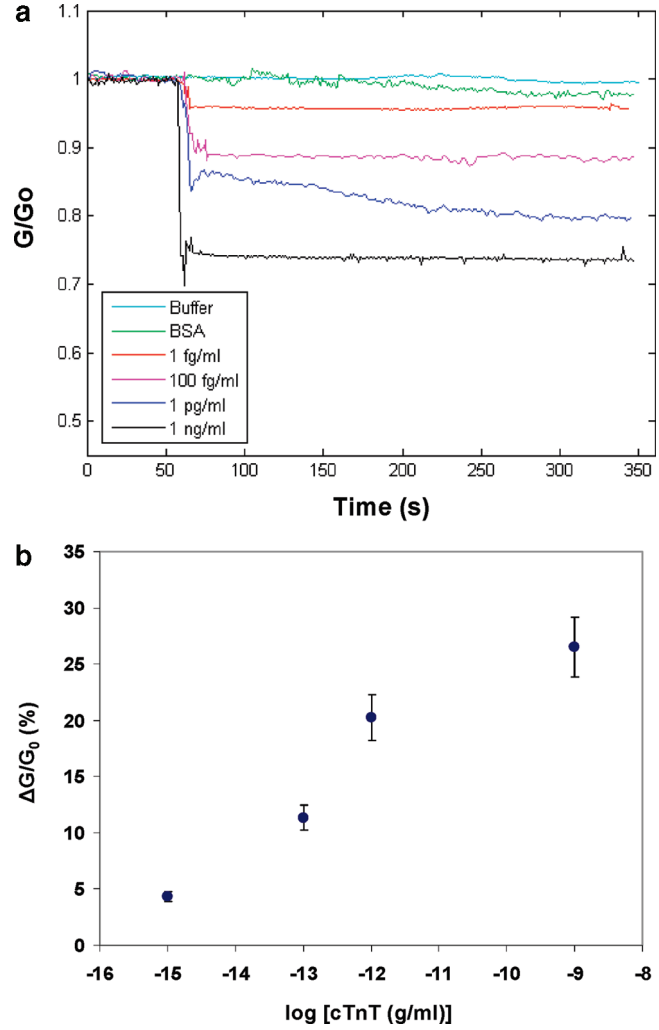

Figure 3. (a) Graph of conductance of SiNW devices functionalized with anti-cTnT showing the detection response with decreasing cTnT concentration. (b) Sensitivity vs logarithm of cTnT concentration.

As a control experiment to quantify any background effect due to the nonspecific binding of noncognate proteins to the SiNW device surface, a SiNW device array was functionalized with anti-cTnT capture antibodies in the same method as described previously. Upon the addition of 1 μg/mL bovine serum albumin solution (BSA, pI = 4.7)[26] in 0.01× PBS, the conductance of the SiNW device demonstrated negligible change. This suggests that the nonspecific binding and adsorption of BSA protein molecules, even at a 10^9-fold higher concentration than used in previous cTnT detection experiments, may effectively be omitted from consideration.

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Table 1. Debye Length vs Ionic Strength of Buffer

buffer (pH 7.4)	ionic strength (mM)	Debye length (nm)
1× PBS	180	~0.7
0.1× PBS	18	~2.3
0.01× PBS	1.8	~7.3

We confirmed that the limit of detection of 1 fg/mL cTnT was determined based on the signal that exceeds the background by 3 times. To evaluate the noise signal, blank buffer (0.01× PBS) without cTnT was injected into the liquid chamber after the baseline was stabilized in the same buffer. A blank sensor response ($\Delta G/G_0$) of ~1% ($n = 3$) was obtained. The sensor response of ~5% caused by the 1 fg/mL cTnT is well above 3 times of the blank response. We thus defined the detection limit for cTnT as 1 fg/mL. Our results clearly demonstrate that the large conductance changes in the anti-cTnT-functionalized SiNW devices are due to the specific binding of cTnT target molecules onto the capture antibodies; the effect of other mechanisms that may result in a conductance change in the SiNW devices are small in comparison to the effect of cTnT binding to the SiNW device. We would be confident to report this detection limit for cTnT (MW 37 kDa) also because this detection limit is in agreement with that reported for prostate specific antigen (PSA, an analyte of similar MW size 35 kDa) measured using a similar n-type SiNW FET immunosensor.[27]

Sheehan et al. reported simulation methods for demonstrating detection limit for nanoscale biosensors by determining the total flux of biomolecules on a sensor as a function of time and flow rate for a sensor incorporated into a microfluidic system.[28] The microchannel is 800 μm wide and 100 μm long. However, the chamber employed in this work is a large well rather than a microchannel. The results indicate that the binding times for cTnT vary less than a factor of 2 when concentration varies a million fold. It is likely that the phenomena observed in the experiments can be explained by using a large chamber as fluid exchange, which allows for an increasing flow rate and subsequently an increasing total flux of biomolecules. The results in terms of binding time vs concentration of cTnT are consistent with the one published, where the binding times for streptavidin vary less than a factor of 2 when concentrations vary 10 000-fold.[18]

Influence of Debye Screening on Troponin T Sensing. The Debye length is an important parameter affecting the device performance. The Debye length is estimated using the formula: $\lambda_D = 0.32(\mathrm{I})^{-0.5}$, where I is the ionic strength of the buffer solution used for sensing. Table 1 indicates various Debye lengths with varying ionic strengths of three types of PBS buffer solution. The Debye length is the distance over which significant charge separation can take place. In other words, a longer Debye length is expected to be long enough to ensure less charges screened by using a dilute buffer solution with low electrolyte concentrations. However, excessive dilutions resulting in a low salt concentration may not retain the biological activity of proteins. To investigate the influence of different buffer ionic strengths on detection sensitivity, real time measurements on 1 fg/mL cTnT in 1× PBS, 0.1× PBS, and 0.01× PBS were conducted. As shown in Figure 4, addition of 1 fg/mL cTnT in 0.01× PBS resulted in a decreased conductance after establishing a baseline current in the same buffer solution, whereas addition of 1 fg/mL cTnT in 1× PBS did not give rise to any response. A slight conductance drop was visible when 1 fg/mL cTnT in 0.1× PBS was injected. This is because different ionic strengths give various Debye lengths and an increase in the ionic strength screens most of cTnT's intrinsic charges. At such a low concentration of cTnT, 1 fg/mL cTnT in 1× PBS and 0.1× PBS is not detectable. Not only are the results in good agreement with the previously reported results on the impact of Debye screening on streptavidin sensing[23] but also further verifies that 1 fg/mL cTnT is detectable in optimal conditions.

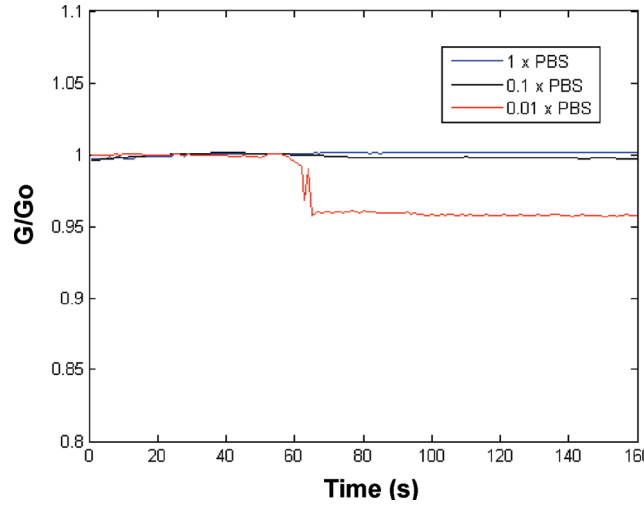

Figure 4. Anti-cTnT-functionalized SiNW device response to 1 fg/mL cTnT of varying buffer ionic strengths.

It is believed that SiNW FET sensors can achieve a very high sensitivity due to the small size and large surface-to-volume ratio. The presence of a few charged molecules on their surface can modulate the carrier distribution over their entire cross-sectional conduction pathway. The detection sensitivity can be further increased by reduced doping, smaller diameter, optimal surface chemistry, and longer Debye length, etc. Cui et al. have pointed out that the detection sensitivity of SiNW biosensor can be changed by the doping concentration and should enable single-molecule detection at sufficiently low concentration.[17] More recently, the same group further demonstrated that single viruses could be detected using antibody-functionalized SiNW FETs.[9] In addition, simulation results also demonstrated the possibility of detecting single biomolecule with SiNW sensors.[29]

Detection of Human Cardiac Troponin-T in Serum. It is of special importance to the development of biosensing devices for medical point-of-care applications that the device is able to accurately detect low concentrations of biomarkers in human blood serum. Experiments designed to test the SiNW device detection capabilities of cTnT in a human blood serum environment were therefore carried out. A sample of raw human serum was obtained from a healthy individual with no prior history of heart disease for use in experiments to demonstrate cTnT detection in serum. The raw serum was desalted using a microcentrifuge filter as reported in the literature[21] and diluted back

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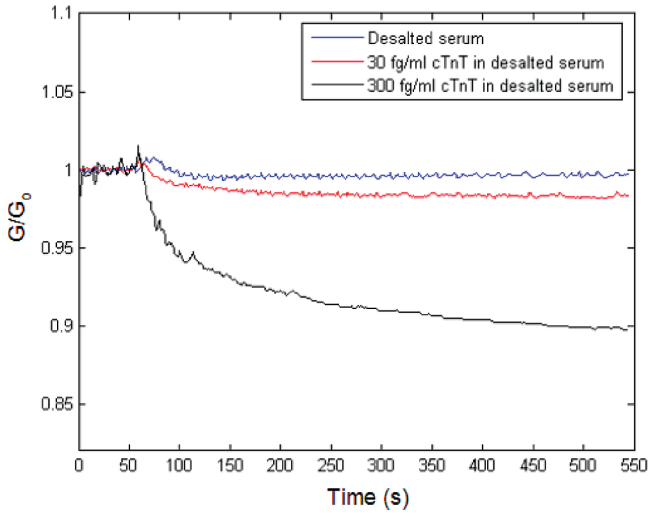

Figure 5. Conductance of SiNW devices functionalized with anti-cTnT and used in experiments to detect cTnT in human serum. Serum solution without cTnT registered negligible change.

to the original protein concentration with 0.01× PBS. Various quantities of cTnT were spiked into aliquots of the desalted serum to obtain analyte solutions of required concentrations.

A SiNW device was functionalized with anti-cTnT antibodies as described above and used to detect cTnT in varying concentrations of serum solution. A graph of SiNW conductance against time is given in Figure 5. An initial baseline for the conductance of the SiNW device was established by injecting desalted serum solution not containing cTnT into the acrylic reservoir. Upon subsequent addition of the cTnT-containing serum solution, the conductance of the device immediately decreases and eventually stabilizes at a lower value. We obtained a significant change for the 300 fg/mL cTnT concentration levels and an expectedly smaller change for the 30 fg/mL cTnT concentration solution.

Because the background signal due to the nonspecific adsorption and binding of proteins increases with concentration, the detection of a specific target biomarker protein is limited by both its concentration as well as the concentration of the noncognate proteins in the environment. Blood serum is abundant in proteins such as albumin, which commonly interfere with the resolution of target proteins by traditional biomolecule detection methodologies; thus, it is pertinent to investigate the effect of noncognate protein adsorption on the detection of target biomolecules. To verify that the conductance change observed in the SiNW devices is indeed due to cTnT binding, we applied a blank serum solution in lieu of the cTnT-spiked solutions used for detection experiments and obtained a virtually negligible change in conductance before and after injection. It should be noted the conductance change incurred by the injection of 30 fg/mL cTnT is distinguishable from the control signals. We have thus demonstrated the label-free and real-time detection of cTnT in an undiluted serum environment down to 30 fg/mL, which is 3 orders of magnitude lower than ELISA-related detection methods.[6]

It was observed that the SiNW device used to detect cTnT in serum had a significantly smaller response than that used to detect troponin-T in buffer. This is in accordance with expectations, as the total protein concentration in serum is approximately between 40 and 65 mg/mL, giving rise to a concentration differential of about 12 orders of magnitude. The presence of high concentrations of noncognate proteins interferes with the binding and detection of cTnT to the SiNW device, causing a decreased response and a longer time taken for establishment of the new conductance baseline commensurate with this interference. However, the conductance change is still adequately large and the response sufficiently swift that SiNW device technology can potentially be utilized in the development of a biosensor for point-of-care testing applications.

CONCLUSIONS

We have demonstrated a CMOS-compatible approach to fabricating a SiNW device consisting of 36 clusters of 5 individually addressable nanowires each and an integrated SiNW array biosensor that is capable of sensing 1 fg/mL human cTnT in an assay buffer solution, as well as 30 fg/mL cTnT in an undiluted serum environment. The conductance changes of the individual SiNWs are obtained through direct electrical measurement, thus providing for a method of label-free detection of biomolecular species that will eliminate the need for expensive, highly specialized equipment in diagnostic screening tests. Each individually addressable nanowire in the array is manufactured via a reproducible, top-down lithographic process and thus promises the capability to perform multiplexed detection of a large variety of molecular indicators with biomedical significance. Coupled with the ability of the SiNW array chip to detect ultralow concentrations of biomarkers in human serum solutions, where the total protein concentration exceeds the concentration of the target biomolecule by approximately 12 orders of magnitude, the high sensitivity and rapid response of SiNW technology potentially allows for the development of a platform enabling multiple confirmatory diagnoses to be obtained in a single medical examination. An investigation into the multiplexed-detection capabilities of cardiac biomarkers using the SiNW array device is currently underway.

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