Ultrasensitive and Selective Multiplexing Detection
of Cancer Markers Using Nanowire Nanosensors

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We report a generic sensor scheme by covalently immobilizing antibodies onto semiconductor nanowire surface for selective detection of virtually any protein. Here this new device architecture is applied to the ultrasensitive detection of cancer markers. Both free form and complex form of prostate specific antigens, currently considered as molecular tracers for prostate cancer, were detected with high specificity and ultrahigh sensitivity down to femto-gram/ml level. This approach is further extended to carcinoembryonic antigen (a colorectal cancer marker) sensing to demonstrate the generality of this sensor scheme. These sensors provide an ultrasensitive, fast, and label-free method for detecting cancers at its earliest stage, monitoring the progression of malignancies, and evaluating effects of treatment. Lastly, multiplexing experiments of free form and complex form indicate that there is no interference in detecting multiple proteins simultaneously, representing a major step towards nanowire array-based high throughput biological sensing.
Detecting chemical and biological analytes using nanoscale structures or nanoscale principles (such as nanomechanics) has recently attracted vast attention because of the potential for extreme sensitivity and high integration, and the capability of label-free sensing. For instance, carbon nanotubes (NTs) have been demonstrated as sensitive NO$_2$ and NH$_3$ gas sensors due to the conductance change via the electron-withdrawing or electron-donating effect.\(^1\) However, the uncontrollable synthesis of metallic and semiconducting NTs makes the systematic study difficult and the flexible methods for the modification of NT surfaces, which are required to prepare interfaces selective for binding a wide range of analytes are not well established. Microcantilevers have been used to specifically detect DNA and proteins.\(^2\)\(^-\)\(^3\) In this case, the binding of analytes induces surface stress and is translated into a nanomechanical response. But the sensitivity in this approach is limited to ~ng/ml for protein detection and high integration is less likely due to the large size of the cantilever (~100 µm). Polycrystalline Pd metal wire (~100 nm diameter) arrays, when exposed to H$_2$ gas, undergo resistance change caused by the expansion of palladium grains in the wires to function as a H$_2$ sensor.\(^4\) In addition, electro-deposited Cu quantum wires showed that the quantized conductance decreases to a fractional value or increases to a higher quantized value upon the adsorption of different small organic molecules such as amine, attributed to the scattering of electrons by adsorbates or the strong binding-induced structure modification.\(^5\) However, the possible applications of these nanoscale metallic wires are limited to a very small range of chemical species.
In our laboratory we have utilized semiconductor nanowires in making nanoelectronic and nano-optoelectronic devices such as field effect transistors (FETs),

pn junctions, light emitting diodes, photodetectors, logic and computation devices. Furthermore, we have demonstrated that Si nanowires (SiNWs) could be used as highly sensitive sensors and have significantly overcome other sensors’ limitations. In our sensor approach, SiNW FETs can be configured into a nanosensor by modifying the NW surface with molecular receptors. The binding of charged or polar species results in the NW surface potential change and causes depletion or accumulation of carriers in the NWs. Thus the binding can be monitored by a direct change in conductance. As a proof of concept, we have demonstrated sensing pH, metal ions and detecting the specific binding of proteins such as streptavidin down to 10 pM range using biotin-modified nanowire surface. Many advantages are combined into semiconductor nanowire nanosensors: (1) High sensitivity: They are always semiconducting unlike NTs, and their small size requires only small number of binding molecules to produce detectable signal. In addition, the doping concentration can be controlled precisely during synthesis, which enables the sensitivity to be tuned and even reach the point that single molecule detection is possible; (2) High specificity: NW surface is covered with 1-3 nm intrinsic oxide. The existence of vast knowledge of oxide surface chemical modification provides well-established strategies to link the nanowire with specific receptors for sensing selectively a wide variety of chemical and biological species (proteins, nucleic acids, etc.); (3) The small size of NWs and the recent advances in nanostructures assembly suggest that densely integrated arrays of sensors could be prepared; (4) Others: The detection is in real-time. There is no need to label analytes with fluorescence or
radioactive tags, and only a very small amount of analyte is needed for detection, making it compatible with current research in proteomics. Now the critical issues come up: can we develop a generic NW sensor scheme such that we can detect any proteins for proteomics and disease diagnostics? Can we detect multiple proteins at the same time to realize highly efficient multiplexing detection? Herein, we address these critical issues by exploiting NW sensors in cancer marker detection.

Currently, the diagnosis of cancer usually follows detection of a mass that is resolvable by anatomic imaging, frequently many years after the earliest stages of cancer development. It is based on gross changes in size and shape of tissues and cells, rather than fundamental changes in the molecular processes that underlie the development of disease. Specificity and effectiveness of diagnosis could be dramatically improved if they were directed towards critical changes in certain molecular processes that result in the cancer phenotype. For instance, prostate cancer is the most common cancer in men and the second leading cause of cancer death in United States. Prostate specific antigen (PSA) level in serum has proved to be an extremely useful marker for early detection of prostate cancer and in monitoring patients for disease progression and the effects of treatments. PSA is a serine protease produced by prostate gland at very high concentration, playing role in semen liquefaction. The amount of PSA released into the bloodstream is small in healthy men but becomes large when prostate is abnormal. PSA in serum has many forms with the free form (fPSA, ~33 kDa) and the PSA-α1-antichymotrypsin (PSA-ACT, ~100 kDa) complex form being dominant. The fPSA and PSA-ACT account for nearly 99% of total PSA level in serum and are the major diagnostic targets for prostate cancer. Although it is age-dependent, the total PSA (tPSA) clinical screening level is usually set...
at 4 ng/ml. In the “diagnostic gray zone” (tPSA between 4 and 10 ng/ml), the patient has 25% probability of having prostate cancer and the systematic biopsies have to be performed. Above 10 ng/ml, more than 50% of the patients have prostate cancer. In addition, the distinction between fPSA and PSA-ACT has been recognized as a clinical relevant feature of the PSA tests. Approximately 20-25% of PSA exists as fPSA form in normal serum, the proportion decreasing to lie between 10% to 0% in prostate cancer. After radical prostatectomy, the serum tPSA concentration should fall to below 10 pg/ml within 4 weeks, and rising above that level is indicative of recurrent disease. The above facts about PSA suggest that it is highly desirable to have sensors to detect serum f-PSA and PSA-ACT level at ng/ml range for clinical screening and very sensitive range (pg/ml) for detecting the prostate cancer at its earliest stage and monitoring the effect of treatment. The current PSA detection techniques are enzyme-linked immunosorbent assay (ELISA) or radioimmunoassay (RIA), both of which require cumbersome fluorescence or radioactive labeling. Herein, we demonstrate the label-free, fast, ultrasensitive multiplexing detection of cancer markers with NW sensors.

The underlying strategy for a generic protein sensor scheme is to immobilize their antibodies onto the nanowire surface and use them for specific antigen (protein) detection. Since virtually antibody to any protein can be raised with current biotechnology, NW can be modified to detect almost any protein. To accomplish this goal, it is necessary to couple antibody onto NW surface in a way that preserves their native conformation. We used a method easily accessible and compatible with our devices structures illustrated in Fig. 1 A through D. The NW FET devices (Fig. 1A) were modified with aldehyde propylmethoxysilane (APMS) to terminate the surface with
aldehyde groups (Fig. 1B). The aldehydes react readily with primary amines on the proteins to form covalent bonds. Because typical proteins such as antibodies display many lysines on their surfaces as well as the generally more reactive α-amine at their N-termini, they can attach to the nanowire surface in a variety of orientations (Fig. 1C), permitting different sides of the protein to interact with antigens in the solution (Fig. 1D). Starting from immobilization of antibody onto nanowire surface, the nanowires and proteins were covered in the microfluidics channel and kept constantly hydrated in aqueous solution to prevent denaturation. All the nanowires used in these studies are single crystal boron-doped (p-type) 10-20nm diameter silicon nanowires (SiNWs). They were prepared by a nanocluster-mediated vapor-liquid-solid growth method described previously. Devices were fashioned by flow alignment of SiNWs on oxidized silicon substrates and then making electrical contacts to the NWs with electron-beam lithography. Linear current versus source-drain voltage behaviors was observed for all the devices exploited in these studies (Fig. 1E), suggesting good Ohmic contact between NWs and leads. The gate voltages produce expected response for p-type nanowires. After the antibody was coupled onto nanowire surface, the nanowire sensitivity in the solution was checked by measuring the conductance versus backgate voltage within small gate voltage range (0.5V) to prevent large electrical disturbance to the system. The high gate response (transconductance ~2700 nS) in Fig. 1F indicates that antibody-modified nanowires are very sensitive to electric field or charge variation close by and are ideal for ultrasensitive antigen detection.

As a first example of cancer marker detection, we have looked at fPSA sensing using monoclonal anti-fPSA antibody (PSA-AB1) modified nanowires. Measurements of
nanowire conductance as a function of time (Fig. 2A) demonstrate that the conductance increases rapidly to a nearly constant value upon flowing in 500 pg/ml fPSA (region 1) and returns to the initial low level after replacing fPSA with buffer solution. The increase in conductance is consistent with the binding of a negatively charged species to the p-type SiNW surface and the fact that fPSA (pI 6.1) is negatively charged at the pH of our measurements. The decrease of conductance with addition of pure buffer suggests that the binding between fPSA and PSA-AB1 is reversible. To ensure that the binding signal is due to specific binding, we carried out a control experiment: flowing 50 ng/ml bovine serum albumin (BSA) (region 2) did not cause any conductance change. This demonstrates that the binding signal is due to specific binding of fPSA onto its PSA-AB1 instead of unspecific adsorption. The reversible binding between them allows us to do the real-time concentration dependence measurements. Figure 2C shows the dependence of the conductance change values on fPSA concentrations taken on another nanowire, from which the dissociation constant is estimated to be around 0.3 nM (~10 ng/ml), consistent with the literature value. To further probe the sensitivity limit, we performed the fPSA detection on some of our extremely sensitive NW devices. The conductance value over time was recorded under very low concentration regime. As shown in Fig. 1D, the conductance change is linearly dependent on the fPSA concentration. Significantly, we can clearly resolve the binding signal of 0.025 pg/ml fPSA (Fig. 2B), which is 100 times lower than what the current ELISA and RIA can detect.

The accurate screening and precise measurement of total PSA level requires us to be able to sense complex PSA form. We thus carried out the PSA-ACT detection using another monoclonal PSA antibody (PSA-AB2), which is raised against fPSA but has
some degree of cross-reactivity to PSA-ACT complex. In the NW conductance versus time measurements shown in Fig. 2E, the conductance rises to a higher level when 5 pg/ml PSA-ACT solution is flowed in, and returns to the original value upon changing back to pure buffer. Similar to fPSA, the binding of PSA-ACT to PSA-AB2 is reversible. The control experiment done using 50 ng/ml denatured PSA-ACT complex did not show any signal change, indicating the binding of the active PSA-ACT onto PSA-AB2-modified NW is specific. The concentration dependence plot (Fig. 2F) of PSA-ACT reversible binding gives the dissociation constant ~25 pM (2.5 ng/ml).

In the reversible binding of free and complex forms of PSA with their antibodies, it is possible that standard PSA samples are used for calibrating the concentration dependent binding curve of NW sensors. The important PSA levels in the real analyte samples can then be precisely determined because the disease relevant concentrations (~4 ng/ml and pg/ml) of both forms of PSA fall into the linear range of the binding curve as shown in Fig. 2C and F. The ability to accurately sense both forms of PSA has significant meaning for precisely and practically diagnosing prostate cancer from several standpoints: first, the tPSA level; second, the ratio of fPSA to tPSA; third, the real time evolution of PSA level and the ratio of fPSA to tPSA.

The generic strategy of exploiting the antibody for detecting proteins can be extended to virtually any protein. To demonstrate this point, we investigated sensing another cancer marker in serum, carcinoembryonic antigen (CEA, a 180 kDa protein), which is used to monitor colorectal cancer progression and response to therapy in patients, although many other malignancies also change CEA levels in serum.24 We created a CEA sensor by immobilizing its monoclonal antibody onto NW surface. Data
recorded from modified SiNWs (Fig. 3A) showed no change when flowing in 50 ng/ml avidin solution (region 1), suggesting no unspecific binding. Significantly, the conductance increased when 50 ng/ml CEA solution was subsequently flowed through the device and stayed at a constant high value (region 2). After pure buffer replaced the CEA solution, the conductance dropped quickly back to the original value, indicating that the binding of CEA to its antibody under the experimental conditions is reversible. The rise of conductance upon CEA binding is consistent with CEA being negatively charge (pI <3.0, Biacore application note) at our experimental pH. The dissociation constant calculated from the concentration dependence study is ~10 pM.

The above results demonstrate that our individual NW sensors modified with antibody are capable of highly sensitive and selective real-time detection of any protein. To take this a step further forward, we need to integrate the nanowires into array-based high throughput sensing, which is important for multiple serum tumor marker screening, currently cost prohibitive. The schematics of this important sensing concept using NW sensors is illustrated in Fig. 4A. The nanowires are assembled into regular arrays and then selectively functionalized with different specific antibodies targeting different proteins. This strategy can enable us to identify each component in complex mixtures (such as human blood and cell lysate) simultaneously. To prove this concept, we investigated the multiplexing detection of fPSA and PSA-ACT. The two NWs on the wafer were independently modified with PSA AB1 and AB2 using two separate microfluidic channels, followed by washing thoroughly with buffer. In the subsequent detection, the flow solution in both microfluidic channels was kept identical at any given time. Measurements of conductance versus time with changes of the solution were recorded as
shown in Fig. 4B. When 50 ng/ml fPSA was flowed in (region 1), the conductances of both NWs went up and were stable at a higher value and decreased back to the original low value after changing back to pure buffer solution. This indicates that both AB1 and AB2 have reversible binding affinity to fPSA consistent with our previous results (Fig. 2). Significantly, adding 50 ng/ml PSA-ACT complex (region 2) and subsequent buffer flow results in the conductance of AB2-modified NW (red curve) increasing to a stable level and subsequently decreasing to the initial low value while that of AB1-modified NW remained stable at the same value. This agrees well with that AB2 has cross reactivity with PSA-ACT but AB1 does not. The signal of PSA-ACT binding onto AB2-nanowire is smaller than that of fPSA at the same concentration, which is due to the partial cross-reactivity of PSA AB2 with PSA-ACT. In region 3, flowing in the mixture of 50 ng/ml fPSA and 50 ng/ml PSA-ACT produces the conductance increase on both NWs similar as in region 1. These data unambiguously demonstrate the multiplexing detection of cancer markers. Furthermore, the multiplexing detection was successfully applied to PSA screening level as shown in region 4, where the signal amplitude of is ~1/6 of that of 50 ng/ml and is consistent with our concentration dependence binding curve (Fig. 2C). The control experiment using 5 µg/ml human serum albumin (HSA) (region 5) did not show any noticeable change and supports that all the binding described above is specific. This also suggests that our NW sensors can potentially be applied to the real blood sample because HSA is a major protein in human blood.

This generic NW biosensor for detection of any protein combine the best and most important features (a) ultrahigh sensitivity (b) high selectivity (c) Complex multiplexing detection, open up extremely exciting opportunities for many directions.
Understanding the functions of proteins and the complex biological pathways in the cell is now not satisfying on a case-by-case basis. It is highly desired to assign the functions on a broader level, which requires miniaturized sensing systems. Ultrasmall NW sensor arrays packaging with biocompatible materials can be implanted into human body, continuously monitoring many molecular makers and transit the “healthy indexes” of the body, which will completely revolutionize the disease diagnostics and medical care.
References and Notes


20. Bare SiNWs were cleaned by oxygen plasma (0.3 torr, 25W power for 60s) to remove contaminants and then immersed into 2% ethanol solution of aldehyde propylimethoxysilanes (United Chemical Technologies, PA) for 2 to 3 hours, followed by thorough ethanol rinse and baking at 120 °C for 10min in the N₂ atmosphere to terminate the NW surface with aldehyde groups. Microfluidic channel (200 µm height and width) made with PDMS (polydimethoxysiloxane) mold, which was pre-coated with polyethylene glycol (MW 5000, Shearwater) to reduce unspecific adsorption of protein, was aligned precisely onto aldehyde-terminated NWs. Covalently linking the antibodies onto the aldehyde-NWs was done in microfluidic channel by flowing 100 µg/ml monoclonal antibody in 50 mM pH 9.6 sodium bicarbonate buffer with 6 M NaCNBH₃ for 2 hrs (fPSA and CEA antibody from Neomarkers, CA; PSA-ACT antibody from Abcam, UK), followed by thorough water rinsing. Lastly, cancer marker (fPSA, PSA-ACT and CEA from Calbiochem, CA) solution was flow in for doing sensing measurements. The conductance of SiNWs was recorded using lock-in techniques. A 31 Hz sine wave with a 30 mV amplitude at zero dc-bias was used in most measurements. In multiplexing measurements, two different frequencies (31 Hz, 110 Hz) were used to avoid cross-talk.


Figure Legends

**Fig. 1** Diagrams and characteristics of NW sensors. (A) through (D), the procedures to convert a NW FET into a generic protein sensor. (A) SEM image of a NW FET consisting of single SiNW covered by two (source and drain) contact electrodes. (B) Aldehyde functionalization of nanowire surface by aldehyde propyltrimethoxysilane (APMS) treatment. (C) Covalent linkage of antibody (AB) onto nanowire surface via aldehyde-amine reaction. (D) Quantitative sensing of antigen (AG) by flowing antigen-containing solution over the sensor surface. (E) I-V characteristic curve of a typical nanowire FET suitable as a sensor (in air). The linear behavior signifies that the contact is ohmic: $V_g = -10$V (yellow), -5V (black), 0V (red), 5V (green), 10V (blue). (F) Change in nanowire conductance in buffer solution with respect to applied gate voltage.

**Fig. 2** NW PSA sensor. (A) Plot of conductance versus time for fPSA antibody-modified SiNW. Region 1 corresponds to 500 pg/ml fPSA contacting with NW, region 2 corresponds to 50 ng/ml BSA. (B) Conductance versus time curve showing the signal of the lowest concentration detectable where region 1 is 0.025 pg/ml fPSA, which corresponds to the lowest concentration data point in (D). (C) Concentration-dependence curve for fPSA antibody-modified SiNW. Each data point has a standard deviation (SD) of 6 nS. (D) Probing the detection limit of NW fPSA sensor below 10pg/ml (SD = 10 nS). The line is the linear fit with $R^2$ value 0.97. (E) Conductance versus time for PSA-ACT antibody-modified SiNW. Region 1 corresponds to 5 pg/ml PSA-ACT, region 2 corresponds to 50 ng/ml denatured PSA-ACT. (F) Concentration-dependence curve for
PSA-ACT antibody-modified SiNW (SD = 10nS). For (A), (B), and (E), unlabeled regions are buffer and arrows indicate the points when solutions were changed.

**Fig. 3** NW CEA sensor. (A) Plot of conductance versus time for a CEA antibody-modified SiNW. Region 1 corresponds to 50 ng/ml avidin solution, and region 2 corresponds to 50 ng/ml CEA solution. Unlabeled regions correspond to buffer. Arrows mark the points when solutions were changed. (B) Concentration-dependence curve for a CEA antibody-modified SiNW (SD = 15 nS). To demonstrate the reproducibility of our experiments, (A) and (B) come from different devices.

**Fig. 4** NW multiplexing sensor. (A) Schematic illustrating the concept of multiplexing. Multiple SiNWs can be differentially modified and the analyte solution can be introduced to all the NWs simultaneously with a common microfluidic channel. The transparent part stands for a microfluidic channel and the blue arrow points toward the flow direction. (B) Plot of conductance versus time for simultaneous real-time detection of fPSA and PSA-ACT. The fPSA antibody-modified SiNW (black) used an antibody specific for fPSA only whereas the PSA-ACT antibody-modified SiNW (red) used an antibody raised against fPSA, but has cross-reactivity with PSA-ACT. Region 1, 2, 3, 4 and 5 correspond to 50 ng/ml fPSA, 50 ng/ml PSA-ACT, 50 ng/ml fPSA + 50 ng/ml PSA-ACT, 4 ng/ml fPSA, and 5 µg/ml HAS, respectively. Unlabeled regions correspond to buffer. Arrows indicate the points when solutions were changed.
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