Label-Free, Electrical Detection of the SARS Virus N-Protein with Nanowire Biosensors Utilizing Antibody Mimics as Capture Probes

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ABSTRACT

Antibody mimic proteins (AMPs) are polypeptides that bind to their target analytes with high affinity and specificity, just like conventional antibodies, but are much smaller in size (2~5 nm, less than 10 kDa).

In this report, we describe the first application of AMP in the field of nanobiosensors. In2O3 nanowire based biosensors have been configured with an AMP (Fibronectin, Fn) to detect nucleocapsid (N) protein, a biomarker for severe acute respiratory syndrome (SARS).

Using these devices, N protein was detected at subnanomolar concentration in the presence of 44 μM bovine serum albumin as a background.

Furthermore, the binding constant of the AMP to Fn was determined from the concentration dependence of the response to our biosensors.

Keywords: biosensor; nanowire; nucleocapsid (N) protein; antibody mimetic protein

INTRODUCTION

Biosensors based on nanowire/carbon nanotube transistors have made the transition from proof of concept[1] to highly selective, ultrasensitive devices capable ofdetecting specific proteins and DNA sequences in recent years.[2~8] These devices utilize a capture agent on the sensor surface to selectively bind the target biomolecules. The captured biomolecules affect the electronic properties of the nanowires/nanotubes, resulting in an electronically readable signal. Capture agents commonly used in nanobiosensorsinclude antibodies, oligonucleotides, and small ligands (*e.g*., biotin).[1~4,8]

Antibody mimic proteins (AMPs) are a class of affinity binding agents developed by *in vitro* selection techniques.[9,10] These AMPs can be evolved/engineered to improve recognition properties such as selectivity and binding affinity, with the potential to surpass antibodies and nucleotide aptamers. In contrast to typical antibodies, AMPs are stable to a wide range of pH and electrolyte concentrations, and are relatively small (usually 2~5 nm, less than 10kDa). Moreover, it is expected that these peptide based affinity agents can be produced in large quantity, at relatively low cost. The combination of low cost, high binding affinity, chemical stability, and small size makes AMPs particularly attractive for use with nanowire/nanotube biosensors.

In this report, we introduce evolved AMPs as a new class of capture agents for nanowire/nanotube biosensors. These for virtually any biomolecule with high sensitivity/ selectivity, as demonstrated here for a protein related to severe acute respiratory syndrome (SARS), using devices based on In2O3 nanowires. Metal oxide nanowires, such as In2O3, ZnO, and SnO2, can be easily derivatized and their surface do not possess an insulating, native oxide layer (*e.g*., SiO2 on Si nanowires) that may decrease the nanowire sensitivity.8 Thus, it is worthwhile to investigate metal oxide nanowires as alternative nanomaterials to silicon nanowires for biosensing applications. We demonstrate that our technology platform based on In2O3 nanowire FETs combined with AMPs, can be used as a diagnostic tool with the potential to serve as a cost-effective, rapid, portable system. A fibronectin-based protein (Fn) was employed as an example of AMP capture agent to selectively recognize and bind the nucleocapsid (N) protein. The N protein is a biomarker associated with the SARS coronavirus.[11] Our platform is capable of specifically detecting the N protein at subnanomolar concentrations, in the presence of 44 μM bovine serum albumin (BSA) as a background. This sensitivity, while comparable to current immunological detection methods, can be obtained in a relatively short time and without the aid of any signal amplifier, such as fluorescence labeled reagents. Ultimately, we show that our platform can also be used to accurately determine the dissociation constant of the N protein and Fn by applying a conventional Langumir model to the concentration-dependent sensing response.

RESULTS AND DISCUSSION

A schematic illustration of our fibronectin-based capture agent anchored to an In2O3 nanowire fieldeffect transistor is shown in Figure 1a. The evolved portion of N-protein is highlighted in red in Figure 1a. The Fn probe was also engineered to have a single cystine residue near the C-terminus of the protein, remote from the binding site. This unique thiol group allows the Fn anchoring to the nanowire to be carried out selectively, since the chosen linker molecule/chemistry gives a nanowire surface that is reactive only toward sulfydryl groups. This conjugation strategy allows every bound Fn to retain full activity, a

clear advantage over antibodies, which are often bound to the nanowire surface *via* amine containing residues, randomly distributed on the antibody surface.[2~7]

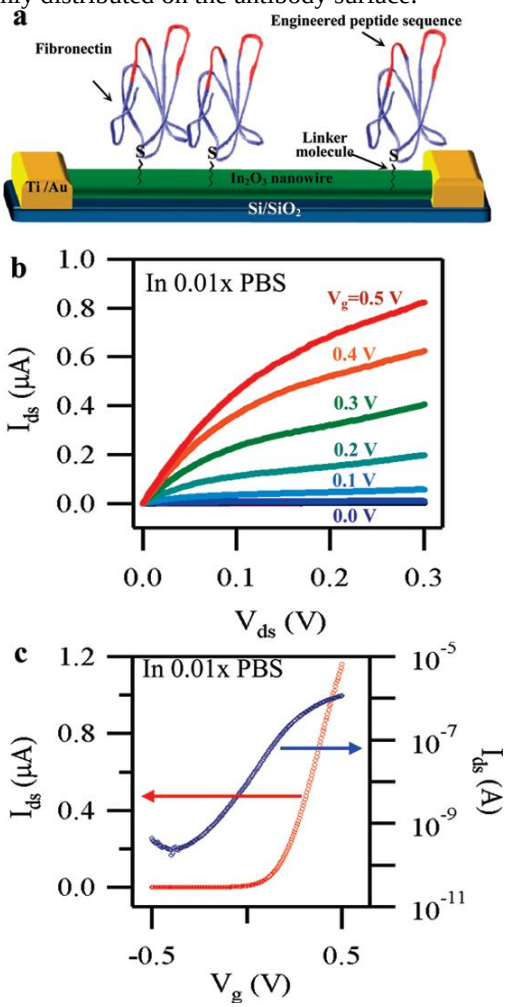

Figure 1. (a) Schematic diagram showing Fn immobilized on the surface of an In2O3 nanowire FET device. The regions of Fn with the engineered peptide sequence are highlighted in red. Fn was attached to the NWs *via* the sulfydryl group of a cysteine near the C-terminus, remote from the binding site. (b) A family of *I*ds-*V*ds curves an (c) a typical *I*ds-*V*g curve (plotted both in linear (red) and logarithmic (blue)) obtained from one of our devices operating with the liquid gate configuration.

In2O3 nanowires were grown *via* a laser ablation CVD method on a Si/SiO2 substrate, following a well-established procedure in our laboratory. The nanowires were suspended in isopropyl alcohol and deposited onto another Si/SiO2 substrate. The position of the source and drain (S_D) electrodes was defined by photolithography, with channel length and width of 2.5 and 780 μm, respectively. Metal deposition on the prepatterned surface followed by liftoff completed the device fabrication. Immediately after cleaning, the nanowire devices were submerged in a 0.1 mM aqueous solution of 6-phosphonohexanoic acid followed by baking in inert atmosphere, resulting in the binding of the phosphonic acid to the surface of the In2O3 NWs. The S_D contacts were then wired to a custom-made printed circuit board, and a mixing cell was assembled on the device chip. This mixing cell was used to deliver and handle all the chemical reagents necessary to complete the surface modification and all the buffer solutions during active measurements.

We have then measured the device characteristics utilizing a liquid gate electrode20 while using the abovedescribedset up. Our In2O3 NW FET devices exhibit excellenttransistor behavior in 0.01x phosphate bufferedsaline (PBS) solution. The linear behavior of the source/drain current *versus* source/drain voltage (*I*ds vs *V*ds) curves at *V*ds up to 0.08 V (Figure 1b) suggests good contact between the nanowires and source/drain electrodes. Strong gate dependence was also observed for a typical device, as shown by the *I*ds *versus* liquid gate voltage (*I*ds_*V*g) curves shown in linear (red curve) and logarithmic scale (blue) in Figure 1c. The on/off ratio and transconductance were ~4.6×10^3 and ~3.6 μS, respectively. These results confirm the stability of our devices under active measurement conditions. Further surface modification of the nanowires conferred our devices with the desired biological recognition properties. The carboxylic acid functional groups on the NW surface were activated with EDC and the activated COOHs were allowed to react with BMPH, resulting in the formation of a NW surface reactive toward the unique thiol present on the Fibronectin probe molecule. The functionalized devices were stored submerged in 1x PBS at 4 °C.

The normalized electrical response of an Fn modified nanowire device is shown in Figure 2a~c, where we have plotted *I*ds divided by the *I*ds at *t*= 0 s, referred to as *I*/*I*0. The device was operated at *V*ds of 200 mV and *V*g of 100 mV. Under such experimental conditions, a baseline signal was quickly established in pure 1.5mM PBS buffer, as indicated in Figure 2a. We note that the leakage current between source and drain through the buffer is negligible compared to the conduction through nanowires. A shift in the baseline level is often observed when transitioning from pure buffer to protein-rich buffer, attributed to nonspecific binding interactions of proteins with the nanowire device. These nonspecific binding phenomena, if not adequately mitigated, may lead to false positive results. Passivating regions of the device that are subject to nonspecific binding with a "blocking agent", as traditionally used in bioanalytical assays such as ELISA[12,13] is technique useful to minimize false positives during active measurements. We have employed BSA as blocking agent for our nanowire devices including the source_drain electrodes. Aliquots of a 10 mg/mL solution of BSA were used to increase the protein concentration of the buffer in contact with the nanowires. After each BSA addition, the baseline re-equilibrated to a lower value of S_D current, as shown in Figure 2a,b. Saturation of nonspecific binding sites was achieved at 40 μM concentration of BSA, as indicated in Figure 2b. We note that BSA passivation is widely

employed in many standard detection techniques such as ELISA; however, it may be possible that the BSA passivation step can be eliminated by increasing the surface coverage of Fn. The baseline stability in a protein-rich medium was then tested by increasing the BSA concentration by 10% (4 µM) (shown with a black arrow in Figure 2c). The device showed no significant response, which confirmed that sites for nonspecific binding were blocked. The conductance of the device rapidly decreased (4%) upon exposing the nanowire sensor to a solution containing 0.6 nM of N protein in 44 µM BSA. We further tested the response of our devices to higher N-protein concentrations. N-protein solutions were prepared by successive additions of small aliquots of a 100±30 nM stock solution of N-protein in 0.01x PBS containing 44 µM BSA. When the N protein concentration was progressively increased to 2, 5, and 10 nM, we observed a consistent decrease in device conductance of 12%, 22%, and 31%, respectively, relative to the baseline. We note that the sensing mechanism for nanobiosensors is a topic of interest and it is currently under intensive investigations in our research group.

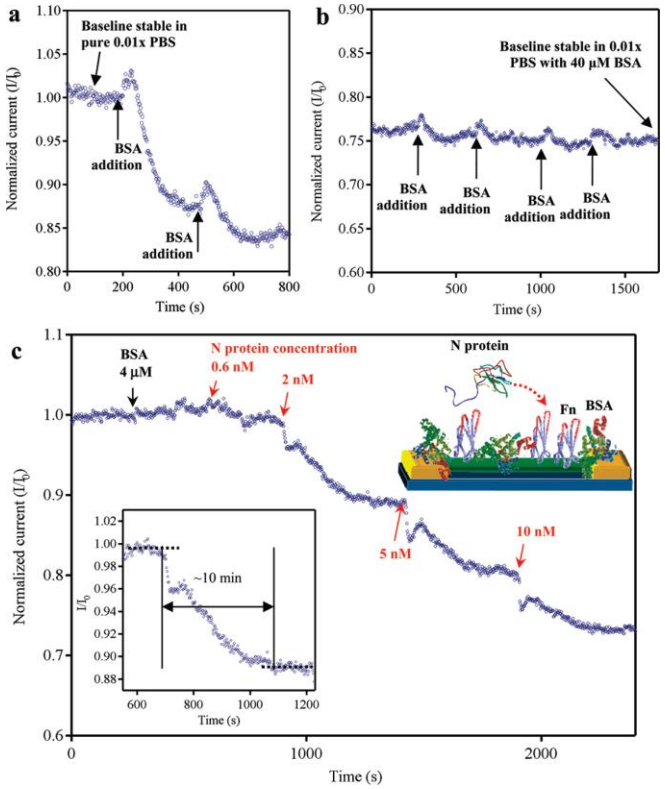

Figure 2. Normalized electrical output (*I*/*I*0) *versus* time of a single operating device. (a-b) Response curves to passivation upon addition of successive aliquots of BSA. (c) Response for a nanowire device functionalized with Fn. The inset on the right side is the configuration of our device during active sensing measurements. The inset on the left side is to show the plateau and the definition of response time.

We have defined the response time to be the time necessary to achieve equilibrium after a change in the concentration of the N protein. Under our active measurement conditions, the response time turned out to be in the order of ~10 min as shown in Figure 2c inset for one measurement. This response time can be considered relatively short when compared to the time required to produce a signal using other diagnostic technologies such as ELISA (~hours)[14] which requires multistep analysis. Thus, while detecting the N protein in the nanomolar range can also be achieved using current immunological clinical tests, our nanowire sensors offer additional advantages such as label free detection and a comparatively short response time.

Three devices were tested in parallel, and all the devices showed a quantitatively similar concentration dependence for their response. Plots of sensor response *versus* N-protein concentration for these three devices are shown in Figure 3 (dots), confirming the reproducibility of the results. These plots were fitted using a conventional Langmuir isotherm model[15,16] (solid line), and these fits were used to estimate the dissociation constant of Fn to the N protein. In applying this model, we assumed that the response of the sensor is proportional to the number of captured molecules on the sensor surface, such that *I*/*I*0 are in proportion to Fn surface coverage. Application of this analytical model yields a dissociation constant of 4.9±0.4 nM, which is close to the value of the dissociation constant (*K*D~ 3.3 nM) obtained from measurements of surface plasmon resonance (SPR). The close match of the dissociation constant illustrates the validity of our assumption and the Langmuir isotherm model. The small difference may come from the fact that the measurements were done in buffers with different ionic strengths (0.01x PBS for nanobiosensor and 1x PBS for SPR).

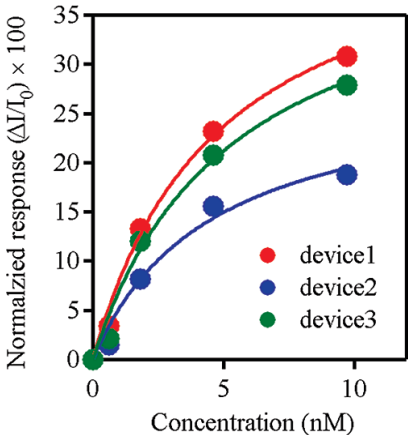

Figure 3. Normalized response from three devices *versus* concentration of N protein (dots). These plots can be fitted using a Langmuir isotherm model (solid line).

We conducted further experiments to confirm the role of Fn as a selective capture probe. A nanowire surface, previously activated for bioconjugation, was treated with 2-mercaptoethanol, prior to Fn. The Fn capture probe is not

expected to bind to the nanowire surface coated with 2-mercaptoethanol moieties, and thus this device should not specifically recognize the N protein. A baseline was established for this device after saturation of any site for nonspecific binding with a 40µM solution of BSA as for the device with Fn. This device was then sequentially exposed to a 2, 5, and 10 nM solution of N protein, while still in the presence of 40 µM BSA. (Figure 4) We did not observe any significant responses, in sharp contrast to the response observed when we used a device functionalized with Fn, confirming that our Fn-based capture probe can selectively capture the N protein.

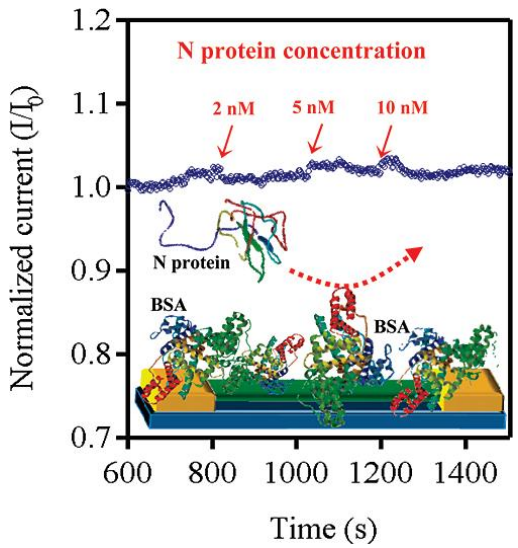

Figure 4. A control device without the Fn capture probe does not respond to the presence of N protein.

CONCLUSION

In conclusion, we have demonstrated that AMPs can be employed as capture agents in nanobiosensors. Sensors based on In2O3 nanowires were modified with a fibronectin-based binding agent that can selectively detect the SARS biomarker N protein. The N protein was detected at a sensitivity comparable to current immunological detection methods (subnanomolar concentration), but obtained within shorter time and without the aid of labeled reagents. We believe that nanowire biosensor devices functionalized with engineered proteins can have important potential applications ranging from disease diagnosis to homeland security. This report also demonstrates the potential for nanobiosensors to be used as an accurate, convenient, and rapid tool to measure the dissociation constants for biological complex systems.

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