

SiNW-FET in-Air Biosensors for High Sensitive and Specific Detection in Breast Tumor Extract

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Abstract—The sensitive analysis of proteins is central to disease diagnosis. The detection and investigation of angiogenic and inflammatory ligands in the tumor tissue can further improve the level of knowledge of the cancer disease by capturing the heterogeneity and the complexity of the tumor microenvironment. In previous works we demonstrated that high quality Silicon Nanowire Field Effect Transistors (SiNW-FETs) can be used to sense very low concentration (fM) of pathogenic factors in controlled Phosphate Buffered Saline (PBS). In this work, we show SiNW-FETs as biosensors for the detection of cancer markers in tumor extracts, as proof of our technology to successfully work on real patients' sample. In particular, we achieved the detection of exogenously added rabbit antigen in a much more complex environment, i.e. a human breast tumor extract. Our results show specific and high sensitive antigen detection with p-type SiNW-FETs in the femto-molar range. Further and most importantly, the wires sense rabbit antigen molecules in the presence of a 100.000 mass excess of non-specific protein, indicating that the sensor is extremely resistant to noise.

Index Terms—SiNW-FETs, biosensors, breast tumor extract, femto-molar sensitivity, specificity

I. INTRODUCTION

THE detection and the quantification of angiogenic and inflammatory ligands present in a tumor, combined with biomedical data, is of paramount significance to guide patient diagnostic and therapeutic decisions. To date, the major limitation to the characterization of the tumor microenvironment is the inability of the current technologies to reach the sensitivity required for the analysis of very limited amounts of patient specimens. Human tumor specimens are indeed often of small size, unique, and thus precious biological samples. Moreover, they are sites of uncontrolled deregulated cellular proliferation that induces very fast changes in the protein landscape under investigation. Thus, to date, the tumor microenvironment remains ill-defined

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due to its complexity and its instability.

Thanks to their nanoscale, nanowire-based biosensors functionalized with antibodies have the potential to fulfill the technological need for this characterization. Indeed, semiconducting *silicon nanowire* (SiNW) based *field-effect transistors* (FETs) have exhibited high sensitivity to detect specific molecules [1]. The sensitivity of the SiNW-FET nanosensors arises due to the nanowire small size and large surface-to-volume ratio [2]. These features make SiNWs sensors with single molecule resolution and thus optimal for the analysis of low concentrated factors in tumor tissue specimens.

The successful detection of antigens with antibody-functionalized NW-FETs has been recently achieved with femto-molar sensitivity in an assay buffer solution by several groups [3], [4]. On the contrary, protein analysis using nanowires has not been yet adopted to detect cancer in real human tumor samples due to the low signal-to-background. To the best of our knowledge, only few works have been published on SiNW-based sensing in extracts from tumor cells. They all demonstrate DNA detection [2], [5], and not the more challenging antibody/antigen based detection.

In this paper, we present our recent results on the successful, high sensitive detection of antigen in a complex environment, i.e. a breast tumor extract.

First, we demonstrate that the fabricated high quality SiNW-FET based sensors achieve the detection of VEGF-A (Vascular Endothelial Growth Factor) femto-moles when diluted in a controlled physiological environment (Phosphate Buffered Saline, PBS). The measurements are carried out in air after sensor incubation in PBS solution containing the cancer markers, thus enabling higher sensitivity thanks to the increased Debye screening length of the thin liquid film formed at the interface [6], with respect to the one of bulk liquid in standard biosensing systems.

We then show that with the same devices and improved specificity we detected low concentrated antigen molecules in a real tumor landscape. We present the occurring detection of immuno-recognition events in tumor extract solutions having a 100.000 mass excess of non-specific protein. We demonstrate the reproducibility of the measurement estimated by taking into account the noise introduced in the detection measurement by the salt and nonspecific proteins constituting the tumor extract solution. We also show that non-specific antigen molecules do not affect the conductance of the sensor proving the measurement specificity.

II. EXPERIMENTAL APPARATUS

A. Clean-room processing

Nanowire FET devices were fabricated from *Silicon-On-Insulator* (SOI) wafers using a conventional top-down fabrication approach [7]. Conventional 4-inch SOI wafers from SOITEC France, with 340 nm thick top silicon layer with a crystalline lattice of (100) and 400 nm thick buried oxide were used. In order to optimize the device sensitivity for biosensing purposes, p-type FET devices were created by Boron implantation with doping concentration in the range of 10^{-16} - 10^{-17} cm^{-3} and of 10^{-19} cm^{-3} , in the body and in the contact regions, respectively. Boron activation inside Si was carried out directly after the implantations step through an annealing process. SiNWs were then defined by *plane-dependent wet etching* (PDE) [8]. The most important steps for PDE definition of SiNWs are depicted in Fig. 1. *Plasma-Enhanced Chemical Vapor Deposition* (PECVD) oxide and nitride were deposited on the wafer to form isolation layers. The contact pads and the region with the SiNW were opened using 0.55% HF. The last step is the alloying process at 400°C in mixture of nitrogen/hydrogen gas mixture (3:0.5) for 20 minutes for a better contact to the highly doped Si layers. The final structure of the fabricated SiNW-FET is schematically reproduced in Fig. 2.

Both individual SiNW-FETs and arrays of SiNW-FETs were fabricated and used for biosensing.

B. Topography characterization

Morphological analysis of the fabricated SiNW-FETs was done by using a Hitachi S7800-H to acquire *Scanning Electron Microscopy* (SEM) images.

Further investigation of the SiNW-FETs surface topography was performed by using a Brukers Dimension FastScan *Atomic Force Microscopy* (AFM) system (Santa Barbara, CA, USA). The imaging was done in tapping mode with the use of a FastScan-A tip characterized by a resonance frequency of 1400kHz. The images were acquired by scanning a few μm squared area, with 512 scan points and a scan rate of 0.5Hz.

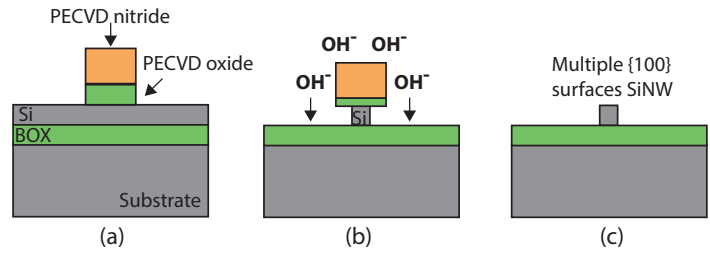


Fig. 1. Process-flow for the fabrication of silicon nanowires. (a) Silicon oxide and nitride hard mask patterning; (b) Silicon etching with KOH; (c) Nitride and Oxide removal ($\text{H}_3\text{PO}_4/\text{BHF}$).

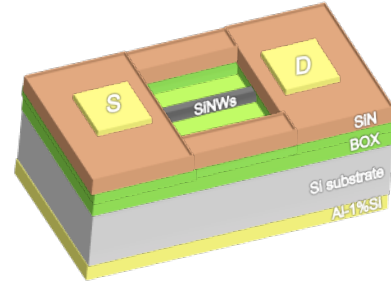


Fig. 2. Schematic representation showing the final structure of the fabricated SiNW-FET. The passivation is opened on the active area of the device for having free access of the antigens to the SiNW modified surface.

C. Measurement setup

Electrical characterization of the SiNW-FETs was carried out using a Signatone H-100 Probe Station and a Keithley 6430 Sub-Femtoamp SourceMeter. All the measurements were carried out at relative humidity (rH) of 50% and temperature of 21°C. First, source-to-drain current versus source-to-drain voltages $I_{ds}-V_{ds}$ and source-to-drain current versus back-gate voltages $I_{ds}-V_{bg}$ characteristics were acquired in order to characterize the bare devices after the fabrication. Then, the NW surface was functionalized with antibodies and corresponding changes in the biosensor conductance ($\frac{\delta I_{ds}}{\delta V_{ds}}$) were monitored to acquire the baseline current before exposition of the sensor to target molecules. The devices were then incubated in differently concentrated antigen solutions and, after being rinsed and dried, tested electrically in air. The calibration curve of the sensor was calculated by associating each point of the curve to the mean conductance of individual nanowire devices. The conductance was obtained by averaging the slope points of the $I_{ds}-V_{ds}$ characteristic in the most linear V_{ds} range. 5 different points were averaged within the range 0 to 3 mV.

D. Surface modification of SiNW-FETs

The device were functionalized using a modification of the procedure described by Kim et al. [10]. The nanowire surface was first cleaned with piranha 1:1 solution to make the surface highly hydrophilic and sets OH groups into the surface. It was then functionalized by exposing the surface to a GPTS (glycidoxypopyltrimethoxysilane) solution (1% in ethanol containing 10 mM acetic acid) for 60 min at room temperature. The surface was then washed in ethanol/acetic

acid and dried in oven at 110°C. Anti-VEGF or anti-rabbit antibodies were then covalently attached to the Si surface by incubation overnight with PBS containing 0.5 mg/ml antibody. 10 mM ethanolamine solution was used to block the remaining active GPTS-derived groups. An additional blocking step was carried out with PBS containing 3% gelatin from cold water fish skin, this time with the aim of passivating the sensor surface and avoid non-specific binding [11], [12]. The modified surface was washed and stored in PBS at 4°C until use. Chemicals unless stated otherwise were purchased from Sigma-Aldrich (St-Louis, MO).

E. Tumor extract preparation

To capture the complexity of the tumor microenvironment, a tumor extract was prepared by mechanical disruption of a pool of 10 distinct human breast tumor biopsies. Following breast tumor resection, a fresh tumor tissue was sectioned into small pieces with a scalpel, the fragments were snap frozen in liquid N₂ and homogenized with a mortar and a pestle. The resulting tissue powder was resuspended in sterile water and further homogenized with a tip sonicator. The mixture was then clarified by centrifugation and the protein concentration of the resulting supernatant adjusted at 1 mg/ml. The specific antigen was spiked in the tumor extract using 100.000 fold molar excess of nonspecific tumor proteins.

III. RESULTS AND DISCUSSION

A. SiNW-FET Imaging

The KOH developer etches silicon both vertically down into the wafer until the buried oxide is reached, and also laterally underneath the oxide and nitride mask decreasing the size of the wires. By controlling the etching time, the nanowire diameter can be easily controlled, too. The SiNW-FETs used in this paper measure 2 μm in length and 300nm in diameter. Top-down processing of nanoscale structures often leads to the patterning of very small objects but characterized by high surface rugosity. The fabrication here performed defined high quality structures and surfaces. Indeed, the plane-dependent etching enables the formation of well shaped nanostructures, with smooth and straight sidewalls. In Fig. 3 we show the SEM images of the fabricated SiNW-FETs (a), (b), the SiNW-FET arrays (c), and a tilted view of the straight sidewall of a nanowire (d). As demonstrated by the images, the silicon surface of the nanowire is flat, thus favoring the achievement of good and homogeneous functionalization with bio-receptor monolayers.

AFM imaging was also performed on the fabricated samples in order to have a higher resolution characterization of the nanowire surface morphology. In Fig. 4 the active area of the sensor, corresponding to the passivation opening onto the central part of the nanowire, is shown.

B. Electrical characterization of SiNW-FETs

The fabrication process described in Section II-A lead to the definition of high quality p-type SiNW-FETs. Fig. 5 shows I_{ds} - V_{bg} characteristic curves of a SiNW-FET defined by etching

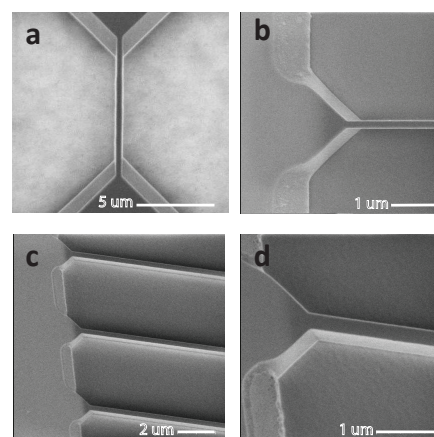


Fig. 3. SEM images of the fabricated SiNW-FETs.

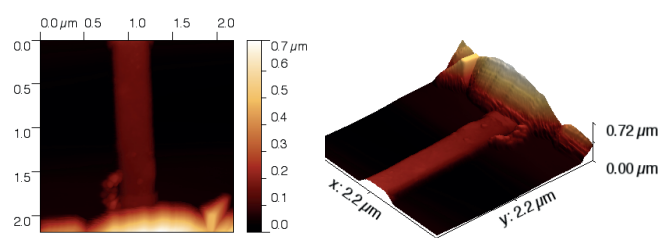


Fig. 4. AFM image of the active area of a fabricated SiNW-FET.

when negative backgate voltage is applied. Accumulation of holes (majority charge carriers) happens when the applied negative backgate voltage is higher than the threshold voltage thus forming a complete channel for the current to pass through and depletion will occur when a positive backgate voltage is applied, as observed in the figure. For positive and increasing V_{ds} , higher current values are observed.

C. Sensing of cancer biomarkers in ideal conditions

The SiNW-FETs have been first tested for sensing biological analytes diluted in a very controlled environment that is the Phosphate Buffer Saline solution. These preliminary experiments were essential to understand the sensitivity of our biosensors and confirm the detection improvements achieved by performing the measurements in air after the incubation of the sensor in the target solution, the washing and drying steps [6].

Vascular endothelial growth factor (VEGF) is a major regulator of angiogenesis, highly active in tumor and in vascular diseases [13]. VEGF was used as target biomarker in this study. The FET devices were covalently functionalized with monoclonal antibody (anti-VEGF) and subsequently incubated with increasing concentrations of VEGF antigen proteins diluted in PBS. After incubation, the sample was rinsed to eliminate unreacted antigen molecules, dried under nitrogen flow and measured in dry conditions. Electrical characteristics were acquired from single wires at different antigen concentrations. The plot reports the data related to an individual device. The error bars stand for the inter-device error calculated as

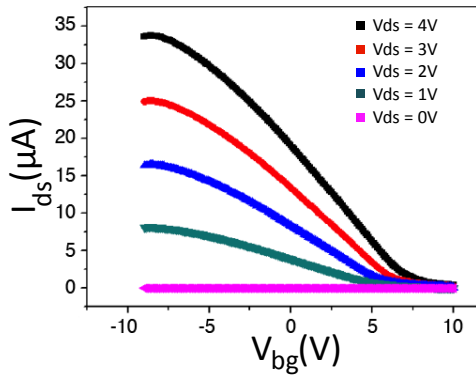


Fig. 5. I_{ds} - V_{bg} characteristic curves of the SiNW-FET.

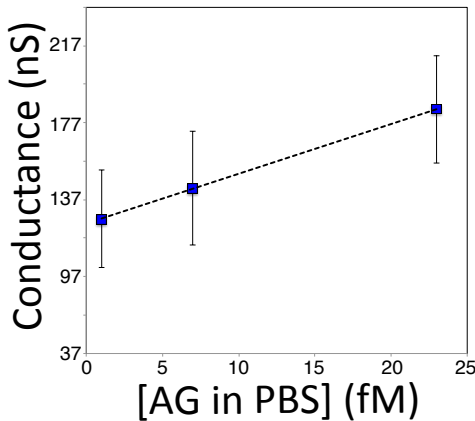


Fig. 6. Sensing of VEGF in PBS.

standard deviation of 3 different measurements on the same sensor. The analysis of conductance showed the increase of conductivity as function of VEGF concentration (Fig. 6), thus confirming the detection of occurring immuno-recognition events between antibody and antigen molecules. An according shift of the threshold voltage (V_{th}) was also observed in the I_{ds} - V_{bg} curves [14]. The results confirm data in literature about the possibility of sensing pathogenic factors with SiNW-FET sensors. Here we demonstrate that we succeeded in measuring VEGF in a very low concentration range (fM), taking advantage of the dry environment. The Debye length is increased thanks to absence of counterions from the bulk electrolytic solution.

D. Sensing design in tumor extracts: the challenges

After testing the capabilities of the SiNW-FET sensors in a highly controlled landscape such as a buffer solution, we moved to a much more complex environment that could reproduce the real condition of a human patient sample. Fig. 7 illustrates a schematic of the working protocol for detecting biomolecules in a tumor extract. In order to capture the complexity of the tumor microenvironment, a tumor extract is prepared by mechanical disruption of a pool of 10 distinct human breast tumor biopsies as described in Section II-E. The prepared tumor extract is characterized by high concentrations of nonspecific proteins that can strongly affect the

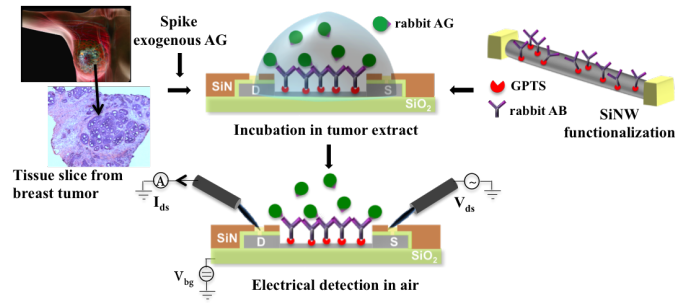


Fig. 7. Schematic of the NW-FET based biosensing method in tumor extract.

sensor performances. To test the potential of the SiNW-FETs for high-sensitive detection of marker proteins in tumor tissue, we first focused on the detection of exogenously added rabbit antigen in a breast tumor extract. We used an anti-rabbit antibody/antigen pair that we previously used for detection in PBS [15], thus enabling us to compare detection sensitivity levels. Moreover, this antigen is spiked at a defined concentration in the tumor extract. In fact, since the behavior of the wires in the presence of tissue extract is unpredictable, due to the high concentration of water and non-specific proteins, the use of an antigen that is unknown to the tumor landscape can help in ensuring for the specificity of the detection measurement. Exogenous rabbit antigen is diluted at different concentrations in the breast tumor extract. The solution is then used to incubate the fabricated sensor for 1h at room temperature and to enable specific immuno-recognition events between the surface immobilized receptors and the rabbit antigen. After incubation, the sample is rinsed to eliminate unreacted antigen molecules, gently dried under N_2 flow and measured in dry conditions. The sensing measurements are performed in air under controlled humidity and temperature conditions [16]. An important issue related to the sensing in a highly active environment such as a tumor specimen, is the presence of enzymes characterized by a very pronounced digesting activity. These factors not only digest the proteins and thus the target molecules that are diluted in the tumor extract but can also eat the antibodies immobilized onto the NW surface thus deteriorating the sensor capabilities. Using RPP arrays (Reverse Phase Protein Array), we determined that the used antigen is stable for about 2h in the tumor extract at room temperature. Since we incubate the sensor for only 1h and then we perform all the measurements in air after getting rid of the tumor tissue residues by multiple washing steps, the SiNW-FET sensors result stable thus enabling for sensing experimentation in a much more complex environment than the most frequently used PBS.

E. Sensing in tumor extracts: the importance of the washing protocol

P-type SiNW-FETs were fabricated and then modified with anti-rabbit antibody (Section II-D). The nanowire based sensors were exposed to tumor extract having rabbit antigen diluted in different concentrations, rinsed, dried and electrically characterized (Fig. 7).

A very delicate part in the preparation of the sample before loading it into the probe station chamber for measurements was the removal of the non-specific and unreacted proteins from the NW surface. Accurate and repeated washing steps were found necessary in order to remove most of the non-specific proteins from the sensor surface and to reduce their background signal.

In order to determine the selectivity of the method, the response of a set of SiNW-FET sensors taken from the same fabrication batch as of the ones used for the calibration with biomolecular markers was tested under specific conditions before the real detection experiment. The sensors were subjected to repeated incubations in PBS solutions, and subsequent washing and drying cycles. The corresponding conductance variations Δg were calculated as difference between the nanowire conductance acquired before (baseline) and after the sensor exposition to the solutions. Same control tests were carried out by exposing the nanowires to tumor extract solutions that did not contain target molecules.

In Fig. 8 we report the Δg data acquire on different devices incubated in tumor extract and then washed using two different methods. The column height represents the Δg acquired on the chosen device; the error bars correspond to the standard deviation of multiple expositions of the sensor to cleaning solutions of PBS. The same behavior was observed both in single devices (a) and in arrays of SiNW-FETs (b).

Washing the samples with only low concentrated buffer solution (0.01M PBS) did not show good results in term of non-specific material removal. This is demonstrated by the high conductance variation Δg introduced by the incubation with tumor extract and the following washing steps (blue columns in both panels of Fig. 8). Indeed, when compared to the baseline, the conductance resulted increased as effect of the tumor proteins left on the silicon surface as consequence of an unsuccessful cleaning. In order to get rid of the unreacted proteins and tumor components and to avoid salt deposition onto the modified silicon surface, the best washing protocol was found to be a 3-cycle rinsing step of 5 min per wash, with PBS 0.01M first, and with PBS with lower ionic strength (0.1M PBS) then. As clearly shown in Fig. 8, this second washing process enabled a good cleaning of the device surface. The Δg calculated in this case is indeed very small if compared to the previous one (green columns in both panels), and the variability of the data (error bars in graph 8), often appeared reduced, too.

F. Sensing in tumor extracts: results

The data show that the incubation with specific rabbit antigen molecules results in concentration-dependent conductance changes in SiNW-FET sensors. In particular, best results were achieved in the concentration range 5 to 200 fM both for individual devices and arrays of nano-sensors, as shown in Fig. 9. The nanowire conductance changes as linear function of the target concentrations in the breast tumor extract. The error bars reported in the calibration plots define the reproducibility of the results. The repeatability of the measurement is here defined for individual nanowire sensors and not for sets of

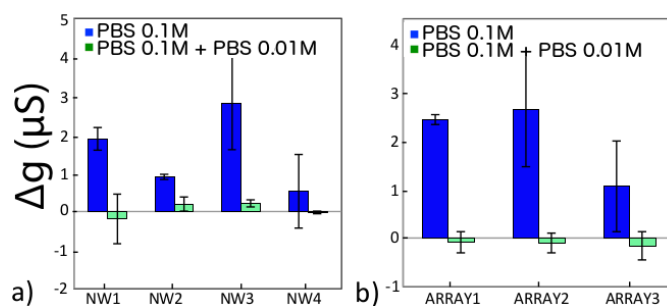


Fig. 8. Statistics on washing steps for non-specific tumor protein removal from the surface of SiNW-FETs (a) and arrays of SiNW-FET (b). The column height represents the conductance variation Δg measured after incubation and washing of the sensors with respect to the baseline acquired before any exposition. The error bars stand for the standard deviations of repeated measurements after multiple washing steps on the sensors.

similar devices averaged together. In fact, the intra-device standard deviation calculated considering different individual devices was calculated to be 4.7nA; the one related to a set of different arrays of SiNW-FETs resulted 8.5nA. This intra-device error was observed to be evidently bigger than the inter-device variation. The measured discrepancy is due to the variability introduced by the nanotechnology process as well as the bio-modification method, and leads to the necessity of calibrating each single sensors. Because of the difficulties in the normalization of the results, the authors preferred to consider the reproducibility on individual devices. The error bars thus stand for the inter-device error that was estimated by performing statistics on several sensors (single and arrays, respectively) for the conductance changes deriving from the exposition to PBS after incubation in tumor extract solutions. As already seen in Section III-E, the washing steps cannot fully clean the silicon surface; a small but non-negligible variations in the sensor conductivity is present as due to salts and biological components present in the tumor extract that can agglomerate onto the sensing device.

Although non-null, this error does not hide the effect of specific detection events at the nanowire surface and the figure clearly shows an increasing trend of the conductance as function of increasing antigen concentrations. Comparisons with previous works of biosensing of anti-rabbit antibodies [15] and VEGF factors (Section 6) in ideal conditions (PBS solution) demonstrate the higher detection limit of the SiNW-FET sensors in tumor extract. This result is absolutely plausible if considering that the wires sense the antigen in the presence of a 100 000 mass excess of non-specific protein. Occurring pM sensing events in such a complex environment are indeed the proof of how our sensors are sensitive and extremely resistant to noise.

We further tested the capabilities and reproducibility of the fabricated nanosensors with additional measurements. By applying a larger number of concentration points, we demonstrated the occurring detection of rabbit antigen in tumor extract within the femto molar range on a single device. Fig. 10a) shows the plots of current versus voltage with rabbit antigen at a series of concentrations for an anti-rabbit modified SiNW device. The current clearly increases as function of

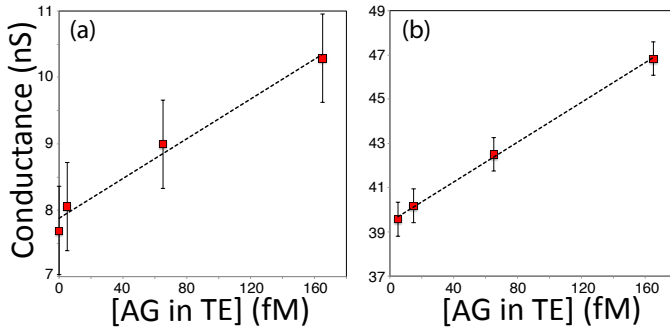


Fig. 9. Sensing of increasing concentrations of rabbit antigen diluted in breast tumor extract. (a) Response of an individual SiNW-FET sensor to the antigen concentration [AG] (0fM, 5fM, 65fM, 165fM). (b) Array of SiNW-FETs. Response to [AG] 5fM, 15fM, 65fM, 165fM. The error bars stand for the inter-device error calculated as standard deviations of repeated measurements after multiple washing steps on the sensor.

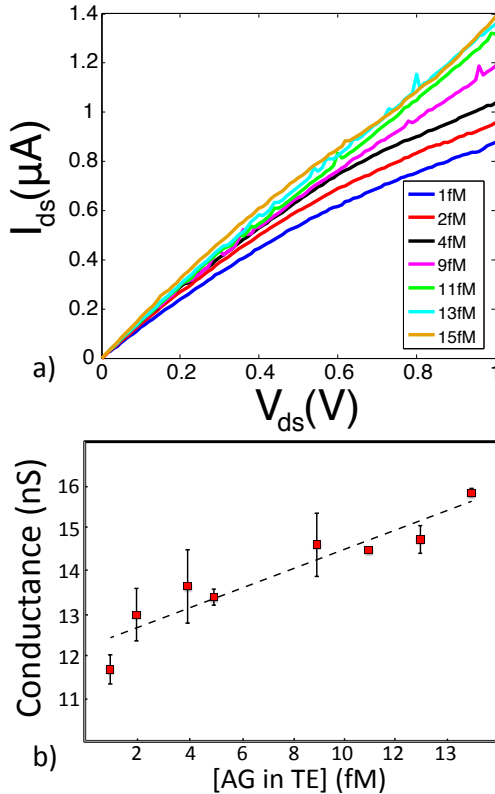


Fig. 10. a) I_{ds} - V_{ds} characteristics for nanowires exposed to increasing femto molar concentrations of rabbit antigen diluted in tumor extract. b) Calibration for the sensor shown in a). The error bars correspond to the standard deviation for 3 different measurements (confidence interval of 68.2%).

antigen concentration, confirming the occurring trapping of target molecules. Fig. 10b) better show this rising behavior of the nanowire conductance as due to the antigen-antibody recognition in tumor extract. Each point represents the conductance value calculated in correspondence of the chosen antigen concentration; the error bars stand for the standard deviation of 3 repeated measurements for each point.

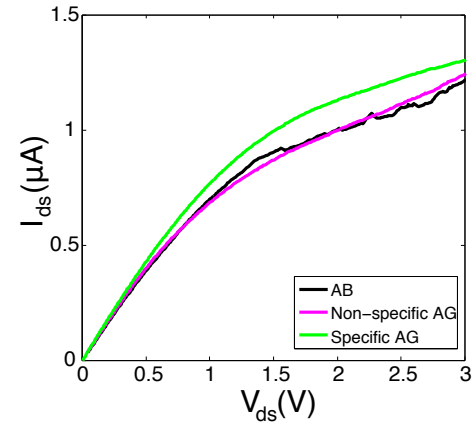


Fig. 11. A non-specific antigen (AG), diluted in tumor extract at same concentration as the specific one (70 fM), determines a smaller conductance signal. In the considered device, the signal is also very close to the curve acquired on the same wire in the absence of specific AG (AB). This result demonstrates the specificity of the sensor.

G. Specificity

As further investigation of the device selectivity, we tested the SiNW-FET sensor in competitive binding experiments with a nonspecific protein. An antibody prepared in rabbit was used as non-specific antigen. It was diluted in an equally concentrated tumor extract, in the same concentration range as the specific anti-rabbit antigen. As clearly shown in Fig. 11, the electrical signal acquired under exposure of the sensor to 70 fM specific analyte resulted increases with respect to the conductance measured for a corresponding concentration of nonspecific protein. Moreover, the curve results superimposed to the one acquired on the same wire in the absence of specific AG (AB), thus demonstrating that exposition to non-specific molecule does not induce a conductance change. This result is an evidence of the specificity of the sensor.

IV. CONCLUSION

In this paper, we have presented recent advances in the development of SiNW-FET biosensors for specific, label-free and highly sensitive immunodetection. In particular, we have demonstrated the feasibility of using SiNW-FET biosensors in real applications with tumor extracts for the early detection of cancer markers thanks to the fM sensitivity of the fabricated high quality nano-devices. After confirming that bio-modified SiNW-FETs can be used to sense very low concentration (fM) of pathogenic factors in controlled Phosphate Buffered Saline (PBS), we have presented the achieved detection of exogenously added rabbit antigen in a much more complex environment, i.e. a human breast tumor extract. The tumor extract was prepared by mechanical disruption of human breast tumor biopsies. Data show specific and highly sensitive antigen detection in the range 5-200 fM. Further and most importantly, the wires sense rabbit antigen molecules in the presence of a 100.000 mass excess of non-specific tumor protein, indicating that the sensor is extremely resistant to noise. The proposed results open the way to future development of nanosensor

based devices for the detection and quantification of angiogenic and inflammatory ligands present in the tumor tissue. Together with biomedical data, this detection system will enable a deeper study of the tumor protein microenvironment thus helping the understanding of the mechanisms leading to the cancer cell activation and proliferation.

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