High Sensitive Detection in Tumor Extracts with SiNW-FET in-Air Biosensors

F. Puppo*, M.-A. Doucey†, J.-F. Delaloye‡, T.S.Y. Moh§, G. Pandraud§, P.M. Sarro§, G. De Micheli*, S. Carrara*
*Integrated System Laboratory, EPFL, 1015 Lausanne, Switzerland
Email: francesca.puppo@epfl.ch
†Ludwig Center for Cancer Research, UNIL, 1066 Epalinges, Switzerland
Email: Marie-Agnes.Doucey@unil.ch
‡Centre du Sein, CHUV, UNIL, Lausanne, 1066 Epalinges, Switzerland
Email: Jean-Francois.Delaloye@chuv.ch
§Delft Institute of Microsystems and Nanoelectronics (DIMES), TU Delft, 2628 CT Delft, The Netherlands
Email: p.m.sarro@tudelft.nl

***Abstract*— Sensitive analysis of proteins is central to disease diagnosis. Their detection and investigation in the tumor tissue can further improve the level of knowledge of the cancer disease by capturing 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 bioanalytes in tumor extract. 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 range of 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 protein, indicating that the sensor is extremely resistant to noise.**

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 induced very fast changes in the protein landscape under investigation. Thus, to date, the tumor microenvironement remains ill-defined 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 their small size and large surface-to-volume ratio [2].

In previous works, we demonstrated to achieve the detection of fM range concentrations of VEGF-A (Vascular Endothelial Growth Factor), a well known cancer marker, in a controlled physiological environment (Phosphate Buffered Saline, PBS) [3]. The measurements were carried out in air after sensor incubation in PBS solution containing the target molecules, thus enabling higher sensitivity thanks to the increased Debye screening length of the thin liquid film formed at the interface [3], [4], with respect to the one of bulk liquid in standard biosensing systems.

The successful detection of antigen with antibody-functionalized NW-FETs has been recently achieved with the same sensitivity in an assay buffer solution by other groups, too [5], [6]. 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, and they all demonstrated DNA detection [2], [7], and not the more challenging antibody/antigen based sensing with nanowires.

We previously demonstrated biodetection in controlled PBS using an anti-rabbit antibody/antigen pair [8]. In this paper we present our recent results on the successful, high sensitive detection of antigen in a much more complex environment, i.e. a breast tumor extract, with high quality SiNW-FET based sensors. We show 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 prooving the measurement specificity.

II. Experimental apparatus

A. SiNW-FET fabrication

Nanowire FET devices were fabricated from Silicon-On-Insulator (SOI) wafers using a conventional top-down fabrication approach, as described previously [9]. Briefly, conven-

978-1-4799-0162-3/14/$31.00 ©2014 IEEE

tional 4-inch SOI wafers from SOITEC France, with 340 nm thick top silicon layer and 400 nm thick buried oxide, were used. 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. Standard photolithography was used to define micron size slabs. NWs were then formed by gradually reducing the width to 100 nm via low etch rate AZ400k developer consists of of 15% of Potassium Borate in water. The gate terminal was formed at the backside of the device to have the surface available for functionalization and interaction with the mesurand.

B. Modification of the nanowire surface

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 (glycidoxypropyltrimethoxysilane) 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°. Anti-rabbit antibody was then covalently attached to the Si surface by incubation overnight with PBS containing 0.5 mg/ml rabbit antibody. 10 mM ethanolamine solution was used to block the remaining active GPTS-derived groups, followed by an additional blocking step carried out with PBS containing 3% gelatin from cold water fish skin. 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).

C. 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 N_2 and homogenized with a mortar and a pestle. The resulting tissue powder was resuspended in sterile water and futher 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.

D. Sensing protocol

As already described in previous work [3], the sensing measurements were performed in air under controlled humidity and temperature conditions. Fig. 1 illustrates a schematic of the working protocol for detecting biomolecules in tumor extracts. First, a tumor extract was prepared as described in Section II-C directly from human biopsies of breast tumor. The prepared tumor extract is characterized by high concentrations of nonspecific proteins that can affect the sensor performances. We used an anti-rabbit antibody/antigen pair previously used for detection in PBS [8], thus enabling us to compare detection sensitivity levels. Exogenous rabbit antigen was diluted at different concentrations (5 to 200 fM) in the breast tumor extract. The solution was 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 was rinsed to eliminate unreacted antigen molecules, gently dried under N_2 flow and measured in dry conditions.

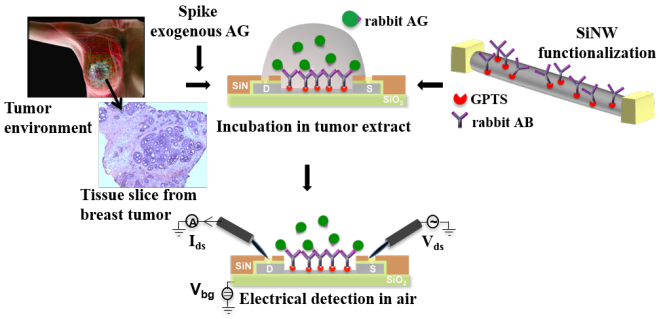

Fig. 1. Schematic of the NW-FET based biosensing method in tumor extract.

I_{ds}-V_{ds} characteristics were acquired in a Signatone H-100 Probe Station and by using a Keithley 6430 Sub-Femtoamp SourceMeter. The back-gate was kept grounded. Changes in the biosensor conductance were monitored before exposition of the sensor to target molecules in order to get the measurement baseline, and after incubation of the sensor in differently concentrated antigen solutions at relative humidity (rH) of 50% and temperature of 21°C.

We first proposed improved sensitivity in dry conditions [3], [4] ensured by an increased Debye length deriving from the absence of counterions from the bulk electrolytic solution [11].

III. Results

To test the capabilities 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 [8], 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 nonspecific proteins, the use of an antigen that is unknown to the tumor landscape can help in ensuring for the specificity of the detection measurement. Using RPP arrays (Reverse Phase Protein Array), we also determined that the antigen is stable for about 2h in the tumor extract at room temperature, thus enabling for sensing experimentation in a much more complex environment than the most frequently used PBS. In order to capture the complexity of the tumor microenvironment, a tumor extract was prepared by mechan-

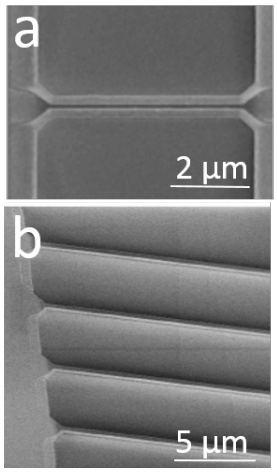

Fig. 2. SEM images of the fabricated SiNW-FETs.

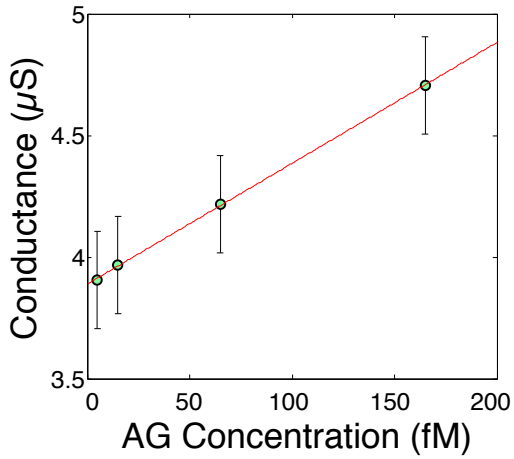

Fig. 3. Increasing conductance as a function of rabbit antigen concentration in the presence of a breast tumor extract. The error bars stand for the error estimated from different sensors after repeated expositions to PBS and tumor extract.

ical disruption of a pool of 10 distinct human breast tumor biopsies as described in Section II-C.

P-type SiNW-FETs with 300 nm wide channel were fabricated and then modified with anti-rabbit antibody (Section II-B). In Fig. 2 we report the SEM images of the fabricated SiNW-FETs (a) and SiNW-FET arrays (b). The images show nanowires with smooth and straight sidewalls. We believe that the obtained flat surfaces can favor the achievement of high quality surface functionalization with bioreceptor monolayer.

The nanowire based sensors were exposed to tumor extract having rabbit antigen diluted in different concentrations, rinsed and dried after incubation, and electrically characterized, as described in Section II-D. The data show that the incubation with specific rabbit antigen molecules resulted in concentration-dependent conductance changes in SiNW-FET sensors. In particular, best results were achieved in the concentration range 5 to 200 fM, as shown in Fig. 3. Here, the nanowire conductance, measured as slope of the I_{ds}-V_{ds} characteristic at 0.3 V, changes as linear function of the target concentrations in the breast tumor extract.

In order to determine the selectivity of the method, the SiNW-FET sensor response 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 were acquired. Same control tests were carried out by exposing the nanowires to tumor extract solutions that did not contain target molecules. Accurate and repeated washing steps were necessary in order to remove most of the nonspecific proteins from the sensor surface and to reduce their background signal. The best washing protocol for unreacted molecules was found to be a 3 cycle rinsing step of 5 min per wash, with 1×PBS first, and with PBS with lower ionic strength (0.1×PBS) then, in order to remove unreacted proteins and tumor components and to avoid salt deposition onto the modified silicon surface. Small but non-negligible variations in the sensor conductivity were measured as effect of salts and biological components present in the tumor extract and still left onto the sensing device. The error introduced in the measurement was estimated by performing statistics on several sensors for the conductance changes deriving from the exposition to PBS and tumor extract solutions. This measurement error is reported on the data of Fig. 3. Although non negligible, 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 (5 fM, 15 fM, 65 fM, 165 fM). Comparisons with previous works [8] demonstrate the higher detection limit reached 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.

As further investigation of the devices selectivity, we tested the SiNW-FET sensor in competitive binding experiments with a nonspecific protein. An antibody prepared in rabbit was used as nonspecific 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. 4, 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 nonspecific molecule does not induce a conductance change. This result is an evidence of the specificity of the sensor.

We further tested the reproducibility of the fabricated nanosensors with additional measurements. By applying a larger number of concentration points, we confirmed the occurring detection of rabbit antigen in tumor extract within the femto molar range on a single device. Fig. 5 shows the plots of current versus voltage with rabbit antigen at a series of

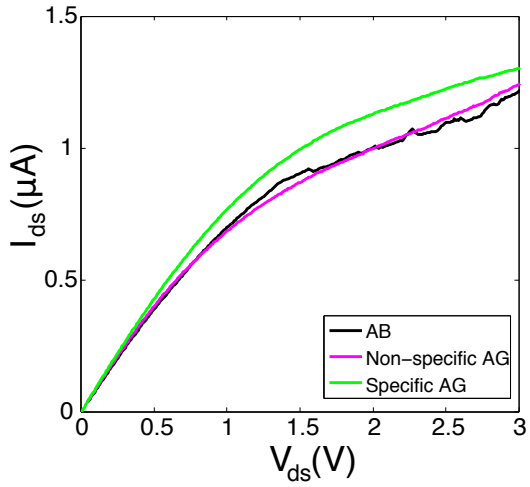

Fig. 4. A non-specific antigen (AG), diluted in tumor extract at same concentration as the specific one (70 fM), determines a smaller conductance signal. In the considered device, the signal is also very closed to the curve acquired on the same wire in the absence of specific AG (AB). This result demonstrates the specificity of the sensor.

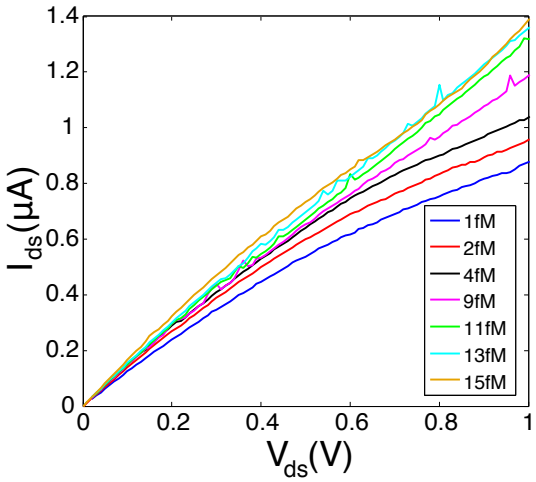

Fig. 5. I_{ds}-V_{ds} characteristics for nanowires exposed to increasing concentrations of solution confirm the detection of rabbit antigen in tumor extract in the femto molar range.

concentrations (1 fM, 2 fM, 4 fM, 9 fM, 11 fM, 13 fM, 15 fM) for an anti-rabbit modified SiNW device. The current clearly increases as function of antigen concentration, confirming the occurring trapping of target molecules.

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. We have previously shown that high quality SiNW based devices can be used to sense very low concentration (fM) of pathogenic factors in controlled Phosphate Buffered Saline (PBS). As direct continuation of the research, we have now demonstrated that we achieved the 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 a pool of 10 distinct human breast tumor biopsies to capture the complexity of the tumor microenvironment. Data show specific and high sensitive antigen detection in the range 5-200 fM on a single nanowire. 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 label-free, high sensitive and specific measurement of proteins directly from the tumor tissue. Together with biomedical data, this detection system will enable a deeper study of the tumor protein microenvironement thus helping the understanding of the mechanism leading to the cancer cell activation and proliferation.

Acknowledgment

This work has been supported by the multidisciplinary FNS grant (CR32I3 135073) and by an ERC grant (ERC-2009-Adg-246810).

References

[1] Y. Cui, Q. Wei, H. Park, and C. M. Lieber, "Nanowire nanosensors for highly sensitive and selective detection of biological and chemical species," *Science*, vol. 209, no. 5533, pp. 1289–1292, 2001.

[2] G. Zheng, F. Patolsky, Y. Cui, W. Wang, and C. Lieber, "Multiplexed Electrical Detection of Cancer Markers with Nanowire Sensor Arrays," *Nat. Biotechnol.*, vol. 23, pp. 1294–1301, 2005.

[3] F. Puppo, M.-A. Doucey, T. Moh, G. Pandraud, P. Sarro, G. De Micheli, and S. Carrara, "Femto-molar sensitive field effect transistor biosensors based on silicon nanowires and antibodies," in *2013 IEEE SENSORS proceedings*, Nov 2013, pp. 1–4.

[4] F. Puppo, M. D. Ventra, G. D. Micheli, and S. Carrara, "Memristive sensor for pH measure in dry conditions," *Surf. Sci.*, vol. 624, pp. 76–79, 2014.

[5] G.-J. Zhang and Y. Ning, "Silicon nanowire biosensor and its applications in disease diagnostics: A review," *Anal. Chim. Acta*, vol. 749, no. 0, pp. 1 – 15, 2012.

[6] M. M. A. Hakim, M. Lombardini, K. Sun, F. Giustiniano, P. L. Roach, D. E. Davies, P. H. Howarth, M. R. R. de Planque, H. Morgan, and P. Ashburn, "Thin film polycrystalline silicon nanowire biosensors," *Nano Lett.*, vol. 12, no. 4, pp. 1868–1872, 2012.

[7] G.-J. Zhang, M. J. Huang, J. J. Ang, E. T. Liu, and K. V. Desai, "Self-assembled monolayer-assisted silicon nanowire biosensor for detection of proteindna interactions in nuclear extracts from breast cancer cell," *Biosens. Bioelectron.*, vol. 26, no. 7, pp. 3233 – 3239, 2011.

[8] S. Carrara, D. Sacchetto, M.-A. Doucey, C. Baj-Rossi, G. D. Micheli, and Y. Leblebici, "Memristive-biosensors: A new detection method by using nanofabricated memristors," *Sens. Actuators, B*, vol. 171172, no. 0, pp. 449 – 457, 2012.

[9] T. S. Y. Moh, Y. Maruyama, C. Shen, G. Pandraud, L. D. Smet, H. Tong, C. Van Rijn, E. Sudholter, and P. Sarro, "IC compatible top down process for silicon nanowire FET arrays with three {100} surfaces for (BIO) chemical sensing," in *Solid-State Sensors, Actuators and Microsystems Conference (TRANSDUCERS), 2011 16th International*, 2011, pp. 1590–1593.

[10] H. J. D. Kim, H.G. Lee and S. Kang, "Single-protein molecular interactions on polymer-modified glass substrates for nanoarray chip application using dual-color TIRFM," *Bull. Korean Chem. Soc.*, vol. 28, no. 5, pp. 783–790, 2007.

[11] E. Stern, R. Wagner, F. J. Sigworth, R. Breaker, T. M. Fahmy, , and M. A. Reed, "Importance of the Debye Screening Length on Nanowire Field Effect Transistor Sensors," *Nano Lett.*, vol. 7, no. 11, pp. 3405–3409, 2007.