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Memristive biosensors based on full-size antibodies and antibody fragments

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ABSTRACT

For the first time memristive biosensors developed by the incorporation of scAbs (single-chain antibody fragments) enabling label-free detection of free Prostate Specific Antigen (fPSA) for early-stage detection of prostate cancer (PC) are reported. In addition, the first ever-published report of a direct relationship between the size of the bio-probes used to transfer specificity to the sensor and the voltage gap is here included. The proposed bio-detection is achieved by means of a voltage gap acquired in the electrical response of memristive sensors obtained by the bio-modification of silicon nanowire arrays. A comparison between memristive biosensors based on full-size antibodies and antibody fragments is also demonstrated by analysis of the surface morphology via Scanning Electron Microscopy. This new kind of biosensors provide excellent analytical sensitivity with a limit-of-detection down to 0.144 pg/mL on PSA.

1. Introduction

Reliable, specific, selective and ultra-sensitive detection of biomolecules can be achieved by the implementation of miniaturized instrumentation and nano-sensors, which are attracting a growing interest from within the biomedical field, particularly for point-of-care applications. More specifically, miniaturized bioassays based on nanowire structures facilitate biomarker detection at early stages of the disease, leading to reduced misdiagnosis due to their high sensitivity [1]. In addition, such nanostructure-based bioassays are far more cost-effective for clinical sample analysis, with resultant improvements in healthcare diagnostics [2]. Meanwhile, the incorporation of recombinant antibodies provides a highly novel approach for the detection of biomolecules and biomarkers of cancer. While traditional polyclonal and monoclonal antibodies can provide good detection capabilities, the utilization of recombinant antibodies provides significant advantages. These include the capacity to engineer the specificity of the antibody and to develop antibodies with tags and structures that may promote better immobilization, thus generating surfaces with much improved sensing abilities [3,4]. In this research, single-chain antibody fragments are coupled with nanowire devices that exhibit memristive properties toward successfully biosensing purposes for the first time.

The memristor concept was first introduced by L. Chua in 1971 [5], and since then, nanoscale devices exhibiting memristive properties have been proposed for various applications [6,7]. Most importantly, nanoscale memristive devices can be leveraged as extremely sensitive biosensors, known as “memristive biosensors”, and facilitate the detection of extremely low concentrations of target biomarkers [1,7]. The hysteretic properties in the electrical conductivity of those nanodevices depend on the carrier rearrangements at the nanoscale due to externally applied potentials. Upon bio-functionalization of the nanowire surface with specific probe-molecules, a voltage difference in zero current crossing appears in the current/voltage characteristic of the device, giving rise to the so-called voltage gap in the semi-logarithmic curve of the device (Fig.1) [7].

Target molecule uptake, typically due to binding of the specific antigens, changes the value of the voltage gap with respect to its initial

Abbreviations: PC, prostate cancer; (f)PSA, (free) prostate specific antigen; LOD, limit of detection; scAb, single-chain antibody fragment; scFv, single-chain fragment variable; VH/VL, variable heavy/variable light regions; IMAC, immobilized affinity chromatography; NiSi, nickel silicide; SOI, silicon-on-insulator; DRIE, deep reactive ion etching; PBS, phosphate buffered saline; SEM, scanning electron microscopy; CLMS, confocal microscopy

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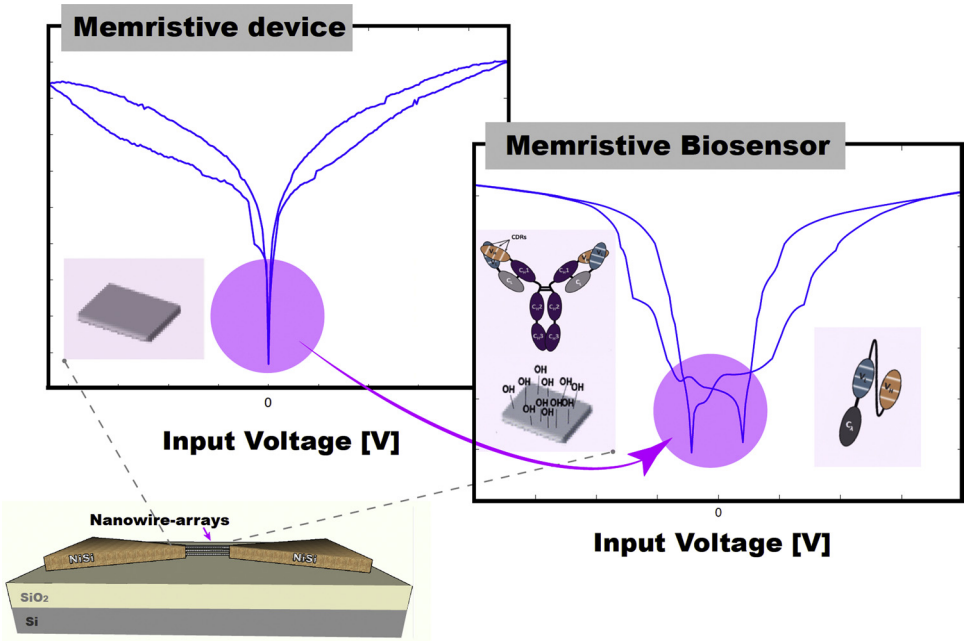

Fig. 1. Electrical characteristics before and after the immobilization of binding molecules. The position of the current minima for the forward and the backward regimes changes after the surface treatment introducing a voltage difference in the semi-logarithmic current to voltage characteristics.

value. More specifically, it is found that this voltage gap modification is highly dependent on the concentration of the bound target molecule, giving rise to a label-free detection method for biological sensing [7]. In the present work, proof-of-concept memristive biosensors are developed based on antibodies and antibody-fragments bio-functionalization to provide new tools for enhancing Prostate Cancer (PC) diagnosis at early stages of the disease. With respect to other electrochemical antibody-based biosensors reported by the literature, the superior performance for the detection on free Prostate Specific Antigen (fPSA) is demonstrated through a remarkably low limit of detection (LOD) for PSA equal to 0.144 pg/mL, obtained by using the memristive devices in conjugation with antibody fragments. Scanning Electron Microscopy further reveals different features in surface morphology due to the presence of the different type of functionalization on the nanostructures' surface. A direct relationship between the voltage gap and the kind of bio-recognition element (antibodies, antibody fragments, or aptamers) is then reported in this paper for the first time in literature.

2. Material and methods

2.1. Antibodies engineering

The single-chain antibody fragment (scAb) was engineered from a single-chain fragment variable (scFv) antibody obtained from a chicken immune library, as previously described [8]. Briefly, the scFv genes are cloned in an 'in-house'-developed version of the pMoPac16 vector [9] for expression of the scFv fused to a chicken constant kappa domain. The scAb antibody fragment consists of the variable heavy (VH) and variable light (VL) regions and an additional constant region attached either to the VL or VH region. While a full-size antibody (divalent structure) has a molecular weight of 150 kDa, scAbs are approximately 42 kDa, with associated smaller dimensions [10]. The scAb constant domain increases the stability of the antibody and facilitates an optimal orientation for antigen binding. The reformatted antibody was expressed in E. coli and purified by immobilized affinity chromatography (IMAC) followed by gel filtration.

2.2. Fabrication of memristive nanowires

Vertically stacked, two-terminal, Schottky-barrier silicon nanowires anchored between nickel silicide (NiSi) pads are acquired through a top-down nanofabrication process using lightly doped, Silicon-on-Insulator (SOI) wafers. First the NiSi pads that play the role of the electrodes for the electrical characterization of the devices are defined by e-beam lithography and generated through Ni evaporation, lift-off and annealing procedures. Then, the nanowires are defined by e-beam lithography using a negative tone resist mask. The nanostructures are finally etched through Deep Reactive Ion Etching (DRIE) of the silicon. A Bosch process, alternating repeatedly between a standard, nearly isotropic plasma etch-mode and a deposition of a passivation layer mode, allows the creation of high aspect ratio vertical structures, etching the silicon layer and creating the suspended nanowires.

2.3. Bio-functionalization and antigen uptake

The fabricated memristive silicon nanowires are first incubated in a piranha solution (H_2O_2:H_2SO_4 in ratio 3:1) heated at 70 °C for 20 min in order to clean the sample surfaces from any organic residues as well as to generate more surface hydroxyl-terminating groups to enable chemical attachment of the biomolecules. Subsequently, different substrates of silicon nanowire arrays are functionalized by exposure to antibody solution via overnight incubation at 4 °C in the dark. Diluted solutions of 5 μg/ml in PBS (Phosphate Buffered Saline, pH 7.4, filtered and sonicated) are prepared from the following antibody stock solutions: (a) full-size immunoglobulin (IgG) anti-PSA antibody from Abcam (ab10185) and (b) antibody fragment anti-PSA scAb engineered as described in Section 2.1.

In addition, immobilization of DNA-aptamers is performed as described in supplementary information. Furthermore, a substrate of nanowire arrays is separately incubated overnight at room temperature and in the dark, in 50 μg/mL of FITC-labelled anti-mouse IgG from Sigma (F9006). Subsequently, all chips are washed twice with PBS in order to remove any non-reacted reagents, and, once with deionized water to remove from the surface any possible remaining salts originating from the buffer. Then the chips are gently dried under a nitrogen (N_2) flow.

Antigen uptake is performed by implementing the memristive biosensors obtained with the different antibody formats (full-size antibody structures and the antibody-derived fragments). Solutions of increasing PSA concentrations (PSA, 30 kDa Kallikrein protein, purchased from Merck, Millipore Angebot R-1939458.1; 539,834) are freshly prepared in PBS, spanning the range of concentrations from 330 aM to 33 nM. First, incubation of the chips in the solution of the lowest PSA concentration is carried out for 45 min at 4 °C. Then, the chips are gently washed and dried, as previously described, before the initiation of measurements. Further incubations and electrical monitoring of each chip with increasing concentrations of antigen solution is carried out.

2.4. Equipment and measurement procedure

The electrical characteristics of the memristive biosensors are obtained by measuring the current-to-voltage characteristics of the device structures in air, on dried samples, using a Signatone 1160 probe station, a Keithley 6430 connected to a national Instrument PXIe-1071. The measurements are carried out at room temperature in a controlled humidity environment and with a drain-to-source voltage sweep in the range -2.4 V to +2.4 V.

Measurements on bare (non-functionalized) devices are first performed as reference measurements. This is followed by the electrical measures on the memristive biosensors functionalized with different types of antibodies and, then, after the antigen uptake (PSA) with increasing concentrations. The data obtained from the measurements is then analyzed in order to obtain the statistical average relationship between the mean voltage gap value and the antigen concentration for each one of the probes molecules under investigation.

Morphological analysis on the nanofabricated and bio-functionalized structures is also carried out by Optical fluorescent Microscopy and Scanning Electron Microscopy. The SEM MERLIN analysis tool from Zeiss is used with two accelerating voltages at 1.2 kV or 1.7 kV for devices of mean width 77.5 nm and of mean width of 34 nm, respectively. For the side views, the stage holder is tilted at 40.0° in order to clearly visualize the nanostructures. Confocal microscopy (CLSM) is performed using a ZEISS LSM 710 for obtaining a 3D fluorescent signal distribution of the biomolecules for devices of 150 nm width.

3. Results and discussion

3.1. Structural characterization

SEM analysis of the memristive biosensors reveals the morphological features of the nano-bio-structures (Fig. 2). In addition, 3D fluorescent signal intensity depicts a dominant rise at the nanostructure's region with respect to the neighbouring areas of the electrodes and the substrate providing a confirmation of the presence of the proteins at the nanosensor's area (Fig. 3).

Top view and side views acquired by SEM for nanofabricated and bio-functionalization structures with two different probes (full-size antibodies structures and antibody-derived fragments) are shown in Fig. 2. Due to the DRIE process, the width along the structures is not perfectly homogenous. However, the surface roughness finally enhances the binding of the proteins on the device by increasing the potential binding area. Bare nanostructures have a mean width of 77.49 ± 3.96 nm and length of ≈ 980 nm. Nevertheless, an increase in the device diameter is depicted due to the adsorbed probes on the nanowire surface. Statistical analysis on the increased diameter after functionalization on several devices depicts a mean width increased to 91.45 ± 4.47 nm for the case of full-size antibodies. This increase of about 13.96 nm on top of the initial mean width is coherent with the expected thickness of the bio-layer as due to the size of antibodies, which are close to 14 nm for the full-size antibodies (Size = 150 kDa, height ≈14 nm × width ≈ 8.5 nm). It is worth mentioning that non-homogeneous protein aggregations, mainly consisting of protein clustering with an average size reaching 45 nm, are also observed in some cases.

An increase of 7.27 nm with respect to the initial mean width is instead acquired on device functionalized with scAbs, which indeed correspond to an average width of 84.76 ± 2.89 nm. These findings are again in agreement with the theoretical expected size of the antibody fragments, that is approximately 8 nm in the case of scAbs (Size = 42 kDa, height: (8 nm × width ≈ 4 nm) [14]. Furthermore, measurements performed for nanodevices with initial mean width of 33 ± 32 nm have shown that the average width upon bio-functionalization with scAb increases to 41.15 ± 3.04 nm, returning a difference with respect to bare of 8.15 nm.

3.2. Sensing performance

The presence of biological substances around the freestanding

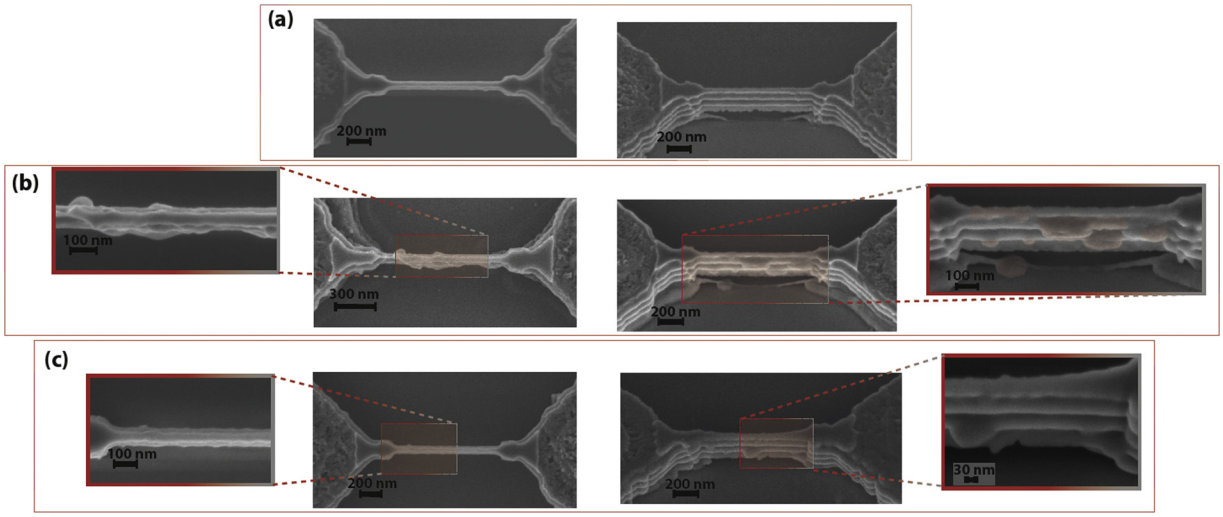

Fig. 2. Top and tilted view SEM images of the nanofabricated structures before bio-functionalization (a), after the bio-functionalization process with direct adsorption of full-size antibodies (b) and after the bio-functionalization process with direct adsorption of antibody fragments (c). The biological reagents are visible, on the surface and sidewalls of the nanostructures.

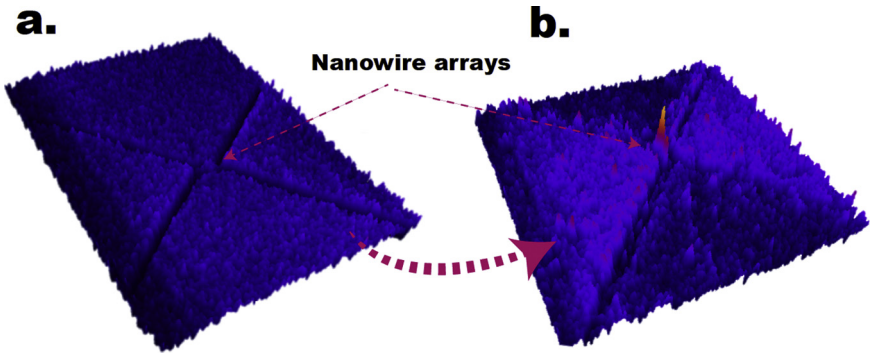

Fig. 3. 3D fluorescence signal distribution acquired using CLSM from the nanofabricated structure before (a) and after (b) the bio-modification with FITC-conjugated antibodies. The bright regions in the right image correspond to the accumulated biomolecule in the sample.

nanowire contributes to extra charges surrounding the device creating a virtual all-around gate, and consequently an electric field surrounding the channel. Under these conditions the conductivity of the nanostructures channel is affected, inducing the introduction of a voltage gap in the semi-logarithmic electrical characteristics. The voltage gap is considered as the detection parameter and can be defined as:

$$D = -\Delta Vds = -(Vds_{Backward_{min}} - Vds_{Forward_{min}}) \quad (1)$$

where $Vds_{Backward_{min}}$ and $Vds_{Forward_{min}}$ stand for the drain-to-source voltage values where the current minima occur during the forward and the backward voltage sweeps, respectively, and ΔVds is the difference between these voltage values. The mean value of the voltage gap obtained for six bare (un-modified) nanodevices (Fig. 4 –inset) is initially close to zero, increases after bio-functionalization to a signal-difference percentage of 2.7% and stays almost constant after the incubation in PBS (< 10% signal-difference), demonstrating the stability of the memristive sensors after bio-functionalization as well as their specificity to antigen binding. Namely, the sensor behaves as predicted and the voltage gap upon bio-functionalization is not affected by the blank response but only by the upcoming antigen uptake. The presence of negative charges, introduced by the antigen uptake on the device surface, brings a masking contribution to the effect introduced by the positively charged antibodies [7]. The positive charge dominance after the bio-functionalization process is going to decrease with increasing antigen concentration in the sample and affecting the width of the voltage gap [7].

A typical calibration curve about the average voltage gap as sensing parameter versus dose-response of fourteen memristive nanosensors is presented in Fig. 4 in the case of antibody-fragments used as probes. The reported error-bars stand for the standard error of measurements related to these nanodevices. For the calculation of the LODs, the method of Armbruster is applied [11]. The obtained dose-responses follow a typical linear fit, with a root-mean-square value (R-Square) of 0.95 and 0.98 for the full-size antibodies and for the antibody-fragments, respectively. A LOD of 4.8 fM (corresponding to 0.144 pg/mL PSA) is then calculated for the memristive biosensors based on antibody-fragments. A slightly higher LOD (difference of 12.8%) is instead computed for the case of full-size antibodies.

Overall, it is found that the memristive biosensors finally result in outstanding sensing performance and LOD compared fairly well to other values reported in literature (Table 1) for antibody-based electrochemical biosensors for PSA. Only two studies were found reporting lower sensitivity for PSA detection. Chuah [12] reported a LOD of 0.1 pg/mL of PSA using gold-coated magnetic nanoparticles as 'dispersible electrodes'. However, they fail to provide appropriate statistical data. Another study was able to detect PSA up to 0.01 pg/mL, using a system that relied on an intricated triple signal amplification strategy using AuNPs–PAMAM dendrimer/HRP-linked aptamer as a label [13]. In comparison to this last strategy, our memristive biosensor allows label-free sensing, simplifying the assay design and facilitating miniaturisation for faster point-of-care applications.

In addition, the applicability of the memristive biosensors for the detection of PSA from real samples was also demonstrated using aptamer-based sensors and serum samples (see Supplementary information). On the other hand, the antibody fragments showed high specificity for PSA, when tested against potential interferants spiked in PBS buffer [8]. These preliminary results support the potential utility of our biosensing approach for the detection of PSA from patient samples.

An extremely interesting relation between the hysteresis modification after bio-functionalization expressed by the voltage gap value

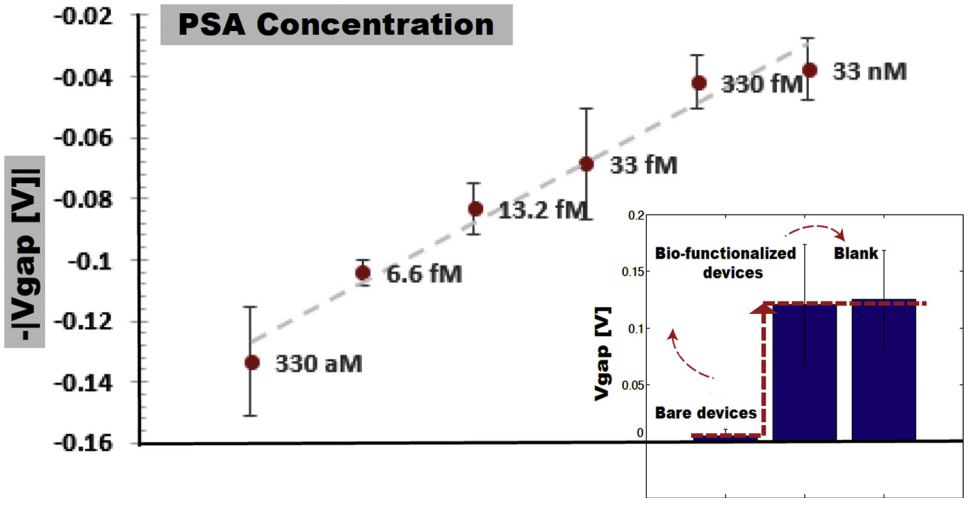

Fig. 4. Sensing performance and effective PSA detection through the electrical hysteresis variations and the average voltage gap response to increasing biomarker concentration (main figure). The voltage gap response before and after binder molecule uptake, depicting no significant difference for the blank measurement (inset figure).

Table 1
Characteristics and limit of detection (LOD) of various PSA immunosensors.

Assay Type	Detection Tool and Types of Electrodes	LOD (pg ml^{-1})	Reference
Sandwich assay with bio-2^{ary} Ab/SA/AP.	CV AuNP-modified SPE (carbon WE)	1000	[14]
Label-free detection using Abs	CV GR-Au modified GCE	590	[15]
Sandwich assay using 1^{ary} scAb/MPs and 2^{ary} Ab/HRP	CA nAu-modified SPE (carbon WE)	500	[8]
Sandwich assay using 1^{ary} Ab/MPs and 2^{ary} Ab/HRP	CA SPE (carbon WE)	100	[16]
Sandwich assay using 2^{ary} scFv/ PtNPs.	EIS AuE with SAM layer	30	[17]
Sandwich assay using 2^{ary} Ab/HRP	DPV ILs/MWCNTs/TH – modified GCE	20	[18]
Sandwich assay using 1^{ary} scAb and lectins	EIS AuE with SAM layer	10	[4]
Sandwich assay using 1^{ary} Ab/AuNPs and 2^{ary} Ab/Si@AuNPs nanocomposite	DPV AuNPs/PEI-PTCA-modified GCE	4	[19]
Sandwich assay using bio-AP/SA/2^{ary} Ab/Au@PBNPs/O-GS	DPV AuNPs/PEI-PTCA-modified GCE	3.4	[20]
Sandwich assay using 2^{ary} Ab/QD-GS	SWV GS-modified electrode	3	[21]
Sandwich assay with 2^{ary} Ab/SiNPs	IES Polymer brush-grafted AuE with SAM layer	2	[22]
Sandwich assay with 2^{ary} Ab/HRP/AuNPs	CA AuE with self-assembled DNA probes	1	[23]
Label-free detection using Abs	EIS Interdigitated Au μ-electrodes with SAM layer	1	[24]
Sandwich assay with ^{2ary} Ab/MSN-Th-AU	DPV NH_2-Fe_3O_4- modified immobilized onto magnetic GCE	0.31	[25]
Label-free detection using scAb	Memristive nanowires	0.144	**Current study**
Sandwich assay with 1^{ary} Ab/Au@MNP and 2^{ary} Ab/HRP	CA Modified Au-MPs as 'dispersible electrodes' with SAM layer	0.1	[12]
Sandwich assay with 1^{ary} Ab and AuNPs–PAMA/aptamer-HRP	DPV GCE	0.01	[13]

AuE–Gold electrodes; AP–Alkaline phosphatase AuNPs–Gold nanoparticles; PBNPs–Prussian blue and nickel hexacyanoferrates nanoparticles; MNPs–Peptide-modified magnetic nanoparticles; Bio–Biotynilated; CA–Chronoamperometry; CH–chitosan; CV–Cyclic voltammetry; DPV–Differential pulse voltammetry; EIS–Electrochemical impedance spectroscopy, GCE–glassy carbon electrode; GR-Au– conductive graphene-based gold composite; GS– graphen sheets; HAP–hydroxyapatite nanocomposite; IL– Ionic liquid; mAb–Monoclonal antibody; MP–Magnetic particles; MSN-Th-Au–Mesoporous silica nanoparticles-acetal with encapsulated thionine; O-GS–Onion-like mesoporous GS; pAb–Polyclonal antibody; MWCNTs–Multiwalled carbon nanotubes; PAMA– polyamidoamine dendrimer; PEI-PTCA–Polyethyleneimine functionalized 3,4,9,10-perylenetetracarboxylic acid composites; QD–quantum dots; SA–Streptavidin; Si@AuNPs–AuNPs-coated silica nanospheres; scAb–single-chain antibody fragment; scFv–Single chain fragment variable; SPE–Screen-printed electrodes; SWV–Square wave voltammograms; TH–Thionine; WE–working electrode; 1ary/2ary Ab–Primary/Secondary antibody.

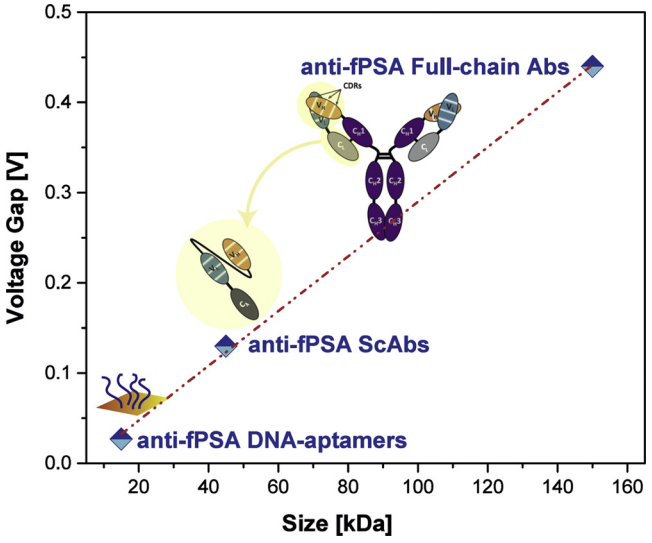

Fig. 5. Indicative voltage gap dependence upon the bio-functionalization reagent: anti-fPSA DNA-aptamers (˜15 KDa), anti-fPSA scAb (˜42 KDa) and full-size anti-fPSA IgG antibody (˜150 KDa) demonstrate different sizes and, therefore, correspond to different voltage gap values resulting in a linear trend for the voltage gap-reagent size relationship.

measured and the size of the bio-recognition element used has been then investigated and here depicted (Fig. 5). The full-size antibodies and the antibody-fragments used in this study as sensing probes present different structures and different sizes. The voltage gap appears proportional to the probe size and definitely coherent with the net positive charge accumulated on the nanodevice surface. Consequently, the value of the virtual bio-gate voltage increases due to the net charge introduced on the nanodevice. This is further proof that the voltage gap depends on net charges on the nanowire surface, as previously demonstrated in literature by Layer-by-Layer experiments with charged polymers on memristive sensors then functionalized with anti-fPSA DNA-aptamers [26].

4. Conclusions

In the present work, memristive biosensors are for the first time developed implementing antibody-fragments as probe molecules. The surface morphology after bio-functionalization with full-size antibodies and single-chain antibody-fragments is analyzed by Fluorescence and SEM investigations. Highly sensitive label-free detection of fPSA, one of the main PC biomarkers, is acquired memristive behavior of the devices as expressed with the appearance of a voltage gap in the semi-logarithmic electrical characteristics. The achieved LOD is down to 0.144 pg/mL PSA. Furthermore, a direct relation between the size of the bio-recognition probes and the registered voltage gap is reported for the

first time in this paper.

Overall, the memristive approach described here linked to the use of antibody-fragments succeeds in an optimum label-free detection of PSA, shows great potential for the further development of novel nano-biosensor platforms and provides a new strategy for understanding of the value and potential of antibody-fragments.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.snb.2019.02.001.

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