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Carbon nanotube-based sensing devices for human Arginase-1 detection

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ABSTRACT

A new carbon nanotube-based device for detection of Arginase 1 (ARG-1) was produced. Multi-walled carbon nanotubes (MWCNTs) were deposited between electrodes by dielectrophoresis (DEP) in an accurate and reproducible way. This deposition method has the advantages of low cost and room temperature conditions and therefore, can be used on different kinds of substrates (silicon, glass, plastics) allowing for large scale production of chemical or biological sensors. Scanning electrical microscope (SEM) and electrical characterization have been performed on the biosensors before and after protein exposure. The devices were tested in the present work for the detection of ARG-1. They show high sensitivity and reproducibility, and can be easily and suitably modified to detect other proteins.

1. Introduction

The development of methods and devices for identification of specific bio-markers is one of the major challenges in the biosensor field and in clinical diagnosis.

The detection of proteins by bioassays usually includes methods such as ELISA (enzyme-linked immunosorbent assay), RIA (radioimmunoassay), and electrophoretic immunoassay. Most of these techniques, however, require sophisticated instrumentation and time-consuming procedures, and most of the reagents employed in immunoassays such as antibodies, enzymes, and fluorescence labels are very expensive. Therefore, there is an increasing interest for the development of new, simple, sensitive, reliable, and cheaper diagnostic methods.

Immunosensors are a specific type of biosensors and can be defined as compact analytical devices that yield measurable signals in response to specific antibody–antigen interactions. A large number of immunosensors have been developed using different kinds of transducers that exploit changes in mass, heat, electrochemical, or optical properties [1]. In particular, electrical detection technique has several advantages such as simple and convenient measurements, which enables miniaturized and inexpensive biosensors [2,3].

Carbon nanotubes (CNTs) have been widely discussed as materials with enormous potential for a wide range of in vivo and in vitro bioapplications, ranging from drug delivery to highly sensitive biosensors, owing to their superior electronic and mechanical properties along with nanoscale dimensions [4]. Significant progress has been made in exploring CNTs as electrodes and further as biosensors. Many different CNT-based sensors can be developed using the same approaches mentioned above for the detection of various clinically significant biomolecules. Based on the mechanism of transduction, the CNT-based immunosensors can be grouped into (1) field effect transistor (FET) immunosensors and (2) other electrochemical immunosensors such as CNT-resistors or amperometric immunosensors.

In this work, CNT-resistors have been produced, in which the CNT layer (sensitive layer) is deposited by dielectrophoresis (DEP) between two Pt electrodes on a silicon dioxide/silicon substrate. This kind of immunosensors exploits the resistivity change of the CNT layer after antibody–antigen binding for antigen detection.

DEP has been considered as a reliable, cheap and efficient CNT deposition techniques, and it involves the deposition of solution-dispersed CNTs between electrodes. The alignment and density of the deposited

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CNTs can be controlled by the AC parameters and the concentration of CNTs [5,6]. Although chemical vapor deposition (CVD) is a common method for the direct growth of CNTs or a network of CNTs, and CVD-grown CNTs have shown the best performance, DEP is generally much simpler and more cost-effective, and does not require special materials and high temperature for the growth.

The analyte chosen for testing the biosensing properties of the devices is Human Arginase 1 (ARG-I or liver-type arginase), a 35 kDa protein circulating in blood probably as a homotrimer. It is most abundant expressed in mammalian liver, but is also found in non-hepatic tissues, for instance red blood cells, lactating mammalian glands, and the kidney. In addition to its involvement in ammonia detoxification via the urea cycle, arginase plays a role in other processes, for instance macrophage-mediated cytotoxicity due to arginase release and inhibition of lymphocyte proliferation. It shows high activity in growing tissues, wound healing, proliferating lymphocytes and tumors. Furthermore, ARG-1 acts as a modulator of the immune response. Besides this, arginase plays a role in allergen challenged lungs, in autoimmune inflammation in the central nervous system and in acute liver injury. In plasma of healthy individuals ARG-1 is present in levels of 1.8–30 ng/ml [7,8] which increases approximately 10 fold during acute phase responses. ARG-1 blood levels elevate in cancerous patients and correlate with cancer stages and poor prognosis. In particular it is detectable in peripheral blood as serum biomarker (Arg-1) for hematological malignancies, including Hodgkin's Lymphoma and Multiple Myeloma (MM) [9–11] and in urine as renal cell carcinoma biomarker [12]. Recent studies by quantitative urinary proteomics have also identified ARG-1 as a potential candidate molecule involved in the development of obstructive nephropathy in newborns [13]. It was shown that an elevation of arginase in a patient's blood is very harmful to the host immune system, more than having effect in the promotion of the tumor cell growth [14].

Typically the detection of such proteins can be performed by enzyme-linked immunosorbent assay (ELISA) which can be used in the 1 to 300 ng/ml range in serum and culture supernatants [7,8,15].

In this work we describe a method to develop biosensors with high sensitivity and low cost for ARG-1 detection. One of the major challenges in the fabrication of CNT-based immunosensors is to attach antibodies or antigens to the CNTs. To do so, a molecular recognization function has to be added by a suitable functionalization that allows immobilizing the receptor on the sidewalls of the nanotubes, as we have done in the present work [16].

Since this kind of devices is based on the electrical response of functionalized carbon nanotubes to antibody–antigen binding, they can be easily turned to detect other proteins by functionalizing CNTs with appropriate antibodies. Numerous studies revealed that proteins can interact in a non-specific way with the sidewalls of acid-oxidized CNTs [17–19], affecting negatively the specificity of the CNT-based immunosensors, and this has to be kept into account when working with these systems. A suitable rinsing out of the sensor after antibody–antigen binding should remove antigen in excess, not chemically bound, reducing the signal due to non-specific interaction.

2. Materials and methods

2.1. Biomolecules and chemical reagents

Multi walled carbon nanotubes (MWCNTs) with purity >95% and diameter of 20–30 nm, ethyl 4-aminobenzoate, isopentyl nitrite, o-dichlorobenzene, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) were purchased from Sigma Aldrich. The other materials used were: Dulbecco's Phosphate-Buffered Saline (D-PBS) (Euroclone, ECB4004L), Bovine Serum Albumin (BSA) (Sigma, 05470-1G), Anti-ARG-1 antibody (Sigma, SAB 4200510), Arginase 1 (ARG-1, Kit ELISA Bio Vendor) and Tween20 (Sigma, P9416-50 ml).

2.2. CNT functionalization

Diazonium salt treatment was used to create sp^3 hybridization sites along the nanotube, ending in carboxylic acid groups, following a previously reported procedure [20]. A suspension of MWCNT-COOH (50 mg) in dimethylformamide (DMF) (10 ml) was sonicated in a water bath for 10 min. Then 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC·HCl, 12.8 mg, 0.067 mmol) and *N*,*N*-diisopropylethylamine (11 μl, 0.067 mmol) were added and the mixture was left stirring at room temperature for 1 h. Then *N*-hydroxysuccinimide (NHS, 6.7 mg, 0.067 mmol) was added and the mixture was stirred at room temperature for 24 h. The MWCNTs–NHS mixture was then filtered under a vacuum, on a 0.1 μm Millipore membrane and the residue was washed several times with DMF, isopropanol and diethyl ether. The obtained nanosystems were dried under a vacuum at 40 °C. The functionalization process is summarized in Fig. 1.

The functionalization degree of pristine MWCNTs before functionalization, MWCNTs-COOH and MWCNTs–NHS samples was obtained by comparing the thermogravimetric analysis (TGA) data by thermal decomposition of the inserted surface groups, under inert atmosphere. TGA was performed using a temperature ramp of 10 °C/min on a TA Q500 instrument. The pristine MWCNTs do not show any significant weight loss while the weight losses of MWCNT-COOH and MWCNT–NHS samples, calculated at 700 °C, reached the values of 10% and 14%, respectively (Fig. 2).

The difference of weight loss of 4% between MWCNT-COOH and MWCNT–NHS samples, calculated at 700 °C, demonstrated the success of the NHS-ester functionalization reaction. The degree of functionalization is strictly related to the sensor response since it represents the amount of NHS groups which could bind with the anti-ARG1 antibody.

2.3. Device fabrication and DEP

A silicon dioxide (SiO_2) layer was thermally grown on a silicon substrate and two metallic electrodes (Pt 100 nm/Ti 20 nm) were defined by optical lithography and lift-off process [21]. Dielectrophoresis, which allows the manipulation of polarizable micro and nano-sized neutral particles using non-uniform electric fields, has been used to accurately control the deposition of the MWCNTs–NHS between electrodes [5,22]. By a suitable choice of the DEP parameters (voltage, frequency, time, CNT concentration in solution) it is possible to tune the final CNT layer resistivity. The device geometry, the scheme of the DEP system and a SEM image of a typical CNT layer deposited between the electrodes are reported in Fig. 3. In this case, the CNT concentration in water solution was 1 μg/ml. A voltage of 20 V and a frequency of 100 kHz were set, with a deposition time of 300 s, obtaining a CNT layer resistance of 22 kΩ. Higher resistance values obtained for the other devices under investigation were achieved by reducing the deposition time without changing the other parameters. In particular, 100 kΩ was reached after a deposition time of 120 s and 115 kΩ after 90 s. For deposition processes performed with the same experimental parameters we achieved the same CNT film resistance values within a 2% error. SEM analysis is useful to study the homogeneity and uniformity of the CNT film (Fig. 3b).

2.4. Electrical characterization of devices

Several devices were produced with different CNT layer resistance values. Electrical characterization was performed by a Source Meter Unit (SMU), Keithley 6430, and the current values were acquired for a voltage range between −1 V and 1 V. The stability of the devices, after DEP deposition, was verified in dry state by repeating the electrical characterization more times consecutively and after 12 h. As an example we report in Fig. 4 the I–V characteristics of one device, showing shelf stability and indicating that CNT-NHS functionalization is not

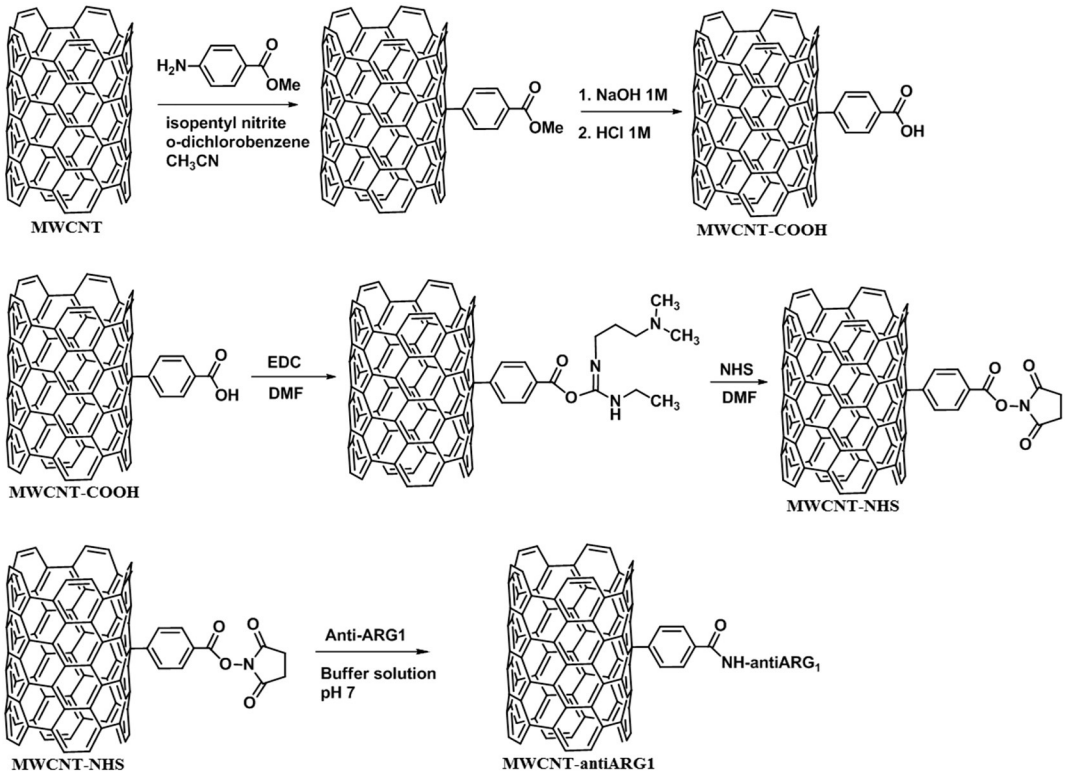

Fig. 1. Scheme of the CNT functionalization process.

changing in the time interval investigated in dry state. Furthermore, we have tested the resistance values of the devices just after DEP deposition and after some days and no variation was observed. This means that the device can be prepared with CNT-NHS film and stored at least for some days before it is used as biosensor, without any visible change in the electrical properties. This is more convenient in terms of time when the sensor has to be used, by avoiding to waste time for the functionalization of CNTs between DEP deposition and the biological steps.

Furthermore, for the voltage range here explored, it is evident that the behavior is almost linear, since MWCNTs can be considered metallic.

3. Results and discussion

Anti-ARG1 antibody (Ab) with a 1 μg/ml concentration was dispersed in a dilution buffer solution (PBS + TWEEN20 0.005% + BSA 0.4%) and a 10 μl droplet was placed on the MWCNT–NHS sensitive layer and left to incubate for 2 h in humid environment. The functionalized device was rinsed out in washing buffer (PBS + TWEEN20 0.005%) to remove antibodies not chemically bound, and incubated for further 2 h with a 5 μl droplet of dilution buffer containing ARG-1 with known concentrations in the range of 22–360 ng/ml, followed by a

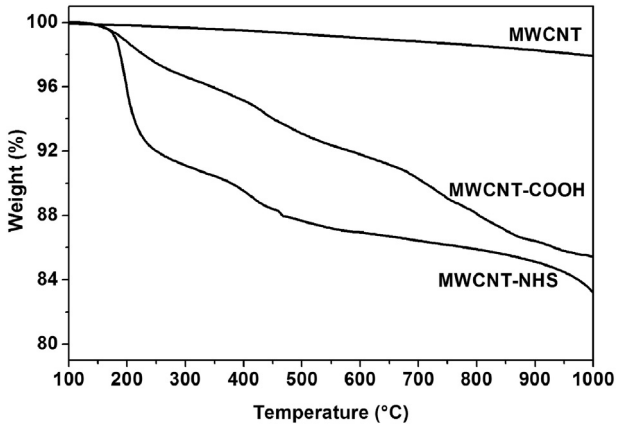

Fig. 2. TGA for pristine MWCNTs before functionalization, MWCNTs-COOH and MWCNTs-NHS samples.

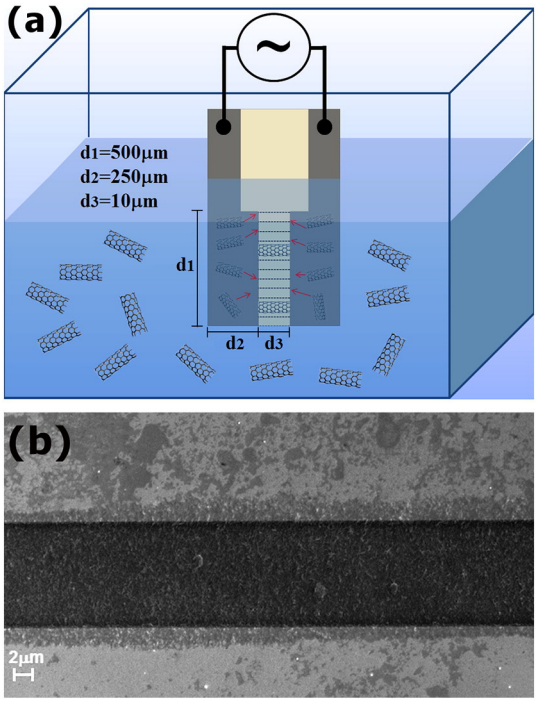

Fig. 3. (a) Scheme of the DEP system and geometrical features of the device; (b) SEM image of the CNT film region between the electrodes in the sensing device.

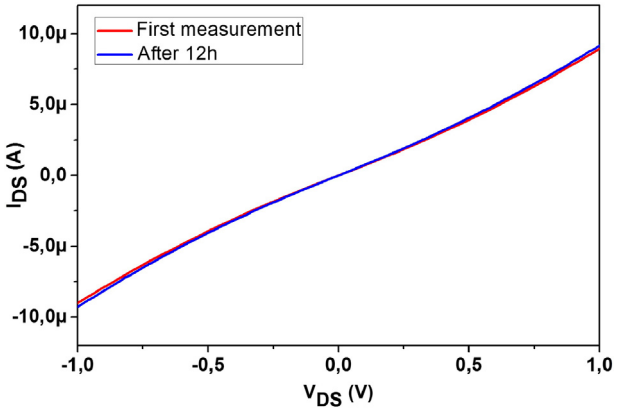

Fig. 4. I–V characteristics of a typical CNT-based device, just after CNT-NHS dielectrophoretic deposition and after 12 h, showing the stability of the device.

careful rinsing out with DI water. The process is schematically illustrated in Fig. 5. The choice of this concentration range has been done keeping into account the ARG-1 values in serum observed in normal (<30 ng/ml) or in pathologic conditions in the case of MM disease (>100 ng/ml). Finally, electrical characterization was carried out and compared with the initial I–V characteristics of the device (before both CNT–antibody and antibody–antigen interactions).

The current–voltage (I–V) characteristic of the CNT-devices was measured before and after Ab incubation and Ab — ARG-1 binding reaction. Each device was used only for a single measurement for a fixed ARG-1 concentration.

In Fig. 6 we report the I–V characteristics, in dry state, obtained for three groups of devices, each group showing the same initial CNT layer resistance (R_0 = 115 kΩ, 100 kΩ, 22 kΩ), after exposure to Anti-ARG1 with 1 μg/ml concentration, followed by antibody–antigen binding reaction. In particular, in Fig. 6a and b the investigated antigen concentration values are 180 ng/ml and 360 ng/ml, whereas in Fig. 6c lower ARG-1 concentrations were used, that is 22 ng/ml and 45 ng/ml. For comparison, the curves obtained in the absence of ARG-1 (0 ng/ml) are reported for each kind of device.

Generally, we notice that the addition of the protein determines a decrease of the current measured in the CNT layer and the higher is the protein concentration the larger is the current decrease (resistance increase). The current (resistance) change respect to the initial value, for a given voltage V_{DS}, can, therefore, be related to the protein concentration present in the solution drop poured on the sensitive layer of the device. Two sets of each kind (R0) of devices were tested and we observed a reproducibility within a variation below 10%.

In Fig. 7 we report a summary of the bio-sensing results that can be extracted by the I–V curves shown in Fig. 6. In particular, for each device we have calculated the normalized change of the resistance $\Delta R/R_0 = (R - R_0) / R_0$, were R_0 and R are the CNT film resistance values at V = 1 V, respectively, before and after the addition of ARG-1 antigen and its binding with the antibody. The results reported in Fig. 7 clearly show that the sensitivity range depends on the initial resistance of the device: in order to detect very low ARG-1 concentrations it seems more convenient to use devices with low initial resistance of the CNT layer, whereas for large protein concentration devices with higher

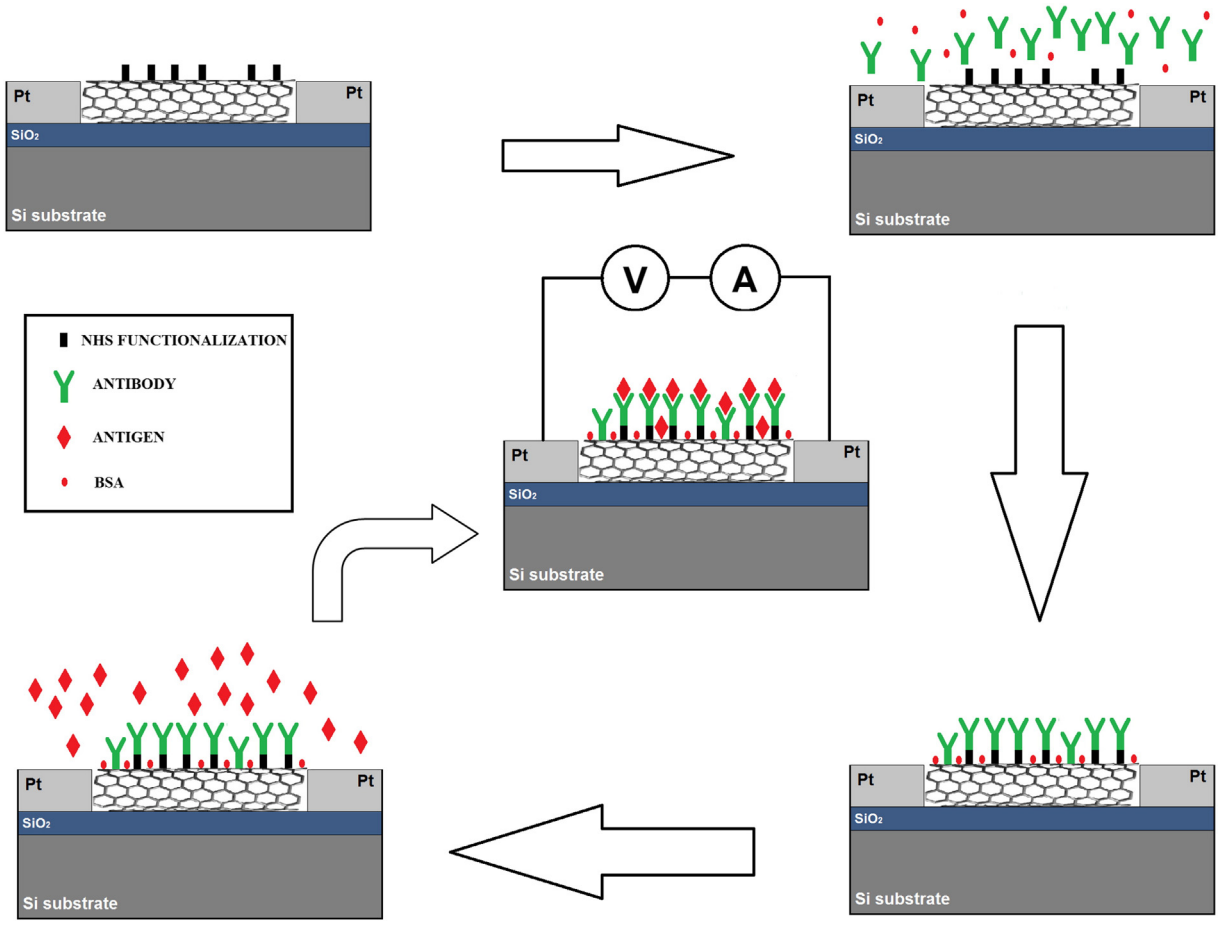

Fig. 5. Scheme of the CNT–antibody–antigen binding reactions and final electrical characterization.

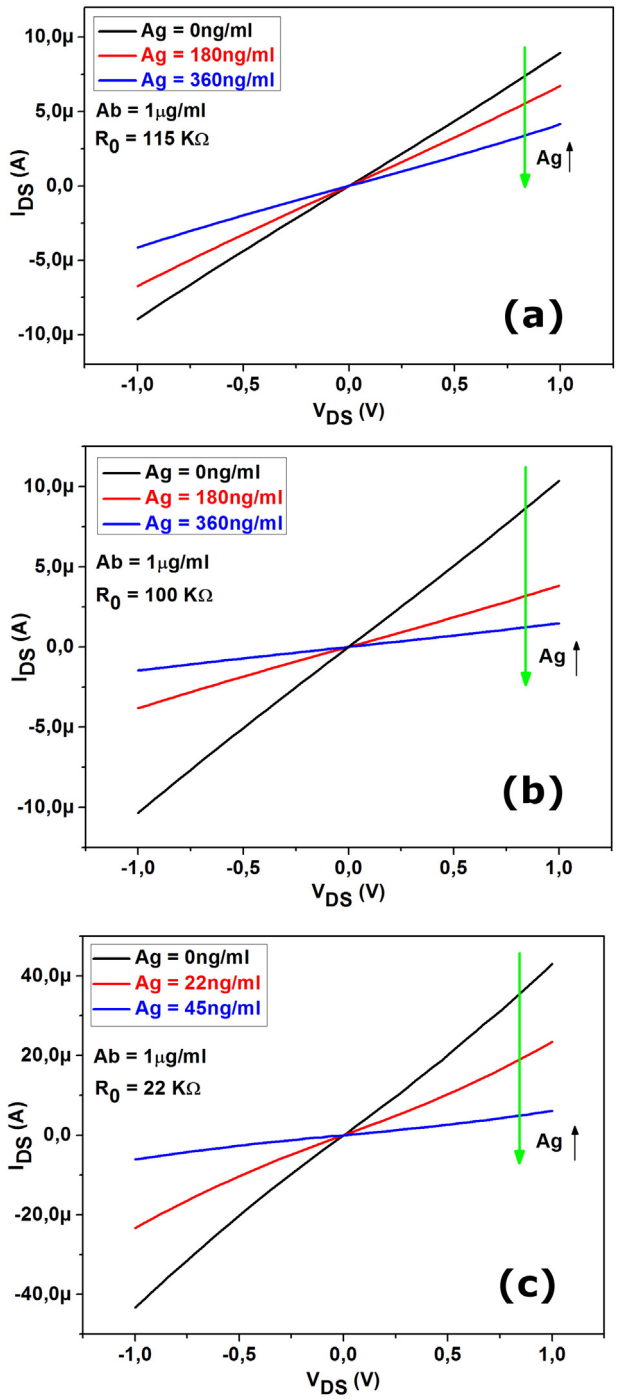

Fig. 6. I–V characteristics of CNT-based sensors with different initial resistance values: (a) 115 kΩ, (b) 100 kΩ, and (c) 22 kΩ. Each kind of sensor was tested in a range of ARG-1 concentrations.

initial resistance should be preferred. Indeed, a lower initial device resistance means (if the same kind of CNTs are used) that a larger CNT density is present between the electrodes and on the whole a larger number of functional groups available for antibody–antigen binding. This allows reaching a higher sensitivity for low antigen concentration values.

In general, a calibration curve can be obtained for sensors with different initial R_0 and in the case of unknown concentrations, as in real samples, it could be useful to use an array of sensors with different initial I–V characteristics and compare their responses. This method allows to perform a cross check of the results given by the different sensors.

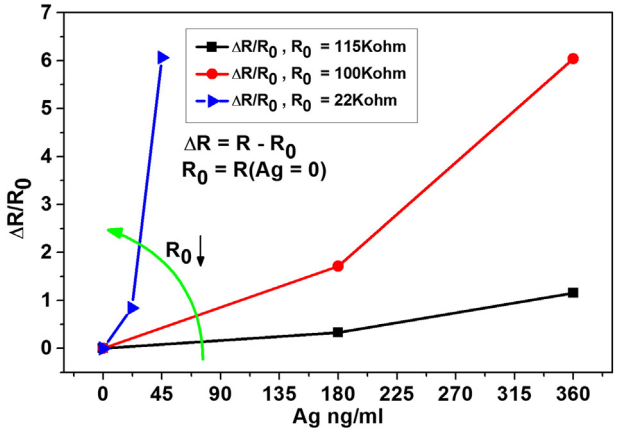

Fig. 7. Changes of the CNT film resistance observed after antibody-antigen binding reaction, for different ARG-1 concentration values, for each group of devices. The plotted results are calculated using the current values at V = 1 V reported in Fig. 6.

4. Conclusions

In this work, CNT-resistors were produced and used as biosensors for the detection of ARG-1, a biomarker related to various pathologies, including hematological malignancies like myeloma and lymphoma.

The production method of the devices is cheap, involves only low temperature processes, like dielectrophoretic deposition of CNTs, and, therefore, is compatible with plastic substrates and with the production of disposable biosensors.

In particular, for the specific application in hematological malignancies, the sensitivity of our devices is appropriate to detect all the protein concentration range from normal values present in serum of healthy individuals (<30 ng/ml), up to higher concentration typical of pathologic conditions (>100 ng/ml). It is comparable to the concentration range detected by commercial ELISA methodology, with the advantage of being less expensive in terms of cost and time-waste.

The sensitivity of each sensor can be tuned by using suitable CNT deposition parameters and, therefore, the use of an array of sensors with different initial electrical characteristics can be exploited for reliable protein detection throughout a very large concentration range.

Conflicts of interest

The authors don't have any conflict of interest.

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