Electronic Anabolic Steroid Recognition with Carbon Nanotube Field-Effect Transistors

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ABSTRACT A proof of concept of the electronic detection of two anabolic steroids, stanozolol (Stz) and methylboldenone (MB), was carried out using two specific antibodies and arrays of carbon nanotube field-effect transistors (CNTFETs). Antibodies specific for Stz and MB were prepared and immobilized on the carbon nanotubes (CNTs) using two different approaches: direct noncovalent bonding of antibodies to the devices and bonding the antibodies covalently to a polymer previously attached to the CNTFETs. The results indicated that CNTFETs bonded to specific antibodies covalently or noncovalently are able to detect the presence of steroids. Statistically significant changes in the threshold voltage and drain current were registered in the transistors, allowing the steroids to be recognized. On the other hand, it was determined that the specific antibodies do not detect other steroids other than Stz and MB, such as nandrolone (ND) because, in this case, statistically significant changes in the transistors were not detected. The polymer prevents the aggregation of antibodies on the electrodes and decreases the transistor hysteresis. Nevertheless, it is not able to avoid the nonspecific adsorption of streptavidin, meaning that nonspecific adsorption on CNTs remains a problem and that this methodology is only useful for purified samples. Regarding the detection mechanism, in addition to charge transfer, Schottky barrier, SB, modification, and scattering potential reported by other authors, an electron/hole trapping mechanism leading to hysteresis modification has been determined. The presence of polymer seems to hinder the modulation of the electrode—CNT contact.

KEYWORDS: biosensing · immunosensing · carbon nanotubes · field-effect transistors · anabolic steroids

The broad applications of nanotechnology make it one of the most rapidly growing areas of research in contemporary science and medicine.[1,2] The unique chemical and optical properties of nanomaterials in terms of particle aggregation, photoemission, electrical and heat conductivity, and catalytic activity have paved the way for development of nanobioelectronic devices.[3,4] Micro- and nanofabrication has allowed the production of ultrasensitive, portable, and inexpensive biosensors. These devices generally rely on chemical or biological receptors that recognize a particular compound of interest and transfer this recognition event effectively.[5] Recently, one-dimensional nanostructures, such as carbon nanotubes and semicondutor nanowires, have been successfully demonstrated to be sensitive biological sensors.[6]

CNTs show unique features that are being used for the development of nanometer scale materials with outstanding potential technological applications. Owing to their structural and electronic uniqueness, carbon nanotubes have been proposed either in advanced electrochemical devices or as molecular-sized electrodes for very fast electrode kinetics research and for sensing and immunosensing.[7]

On the basis of the specific antibody−antigen interaction, immunosensors provide a sensitive and selective tool for estimation of proteins. Immunosensors incorporate an antibody or antigen integrated within or intimately associated with a physiochemical transducer. A large number of immunosensors have been developed using different transducers based on changes in mass, heat, electromechanical, or optical properties.[8] One promising approach is the direct electrical detection of biological macromolecules using semiconducting nanowires or CNTs configured as field-effect transistors, CNTFETs, which change conductance when charged macromolecules are bound to receptors linked to the device surfaces.[9−12]

CNTFET immunosensors have been prepared by joining the antibodies directly to the CNT or through the use of aptamers. Park *et al.*[13] have immobilized monoclonal antibodies against carcinoembryoic antigen. Chen *et al.*[12] have developed CNTFETs for biomarker detection for the early diagnosis

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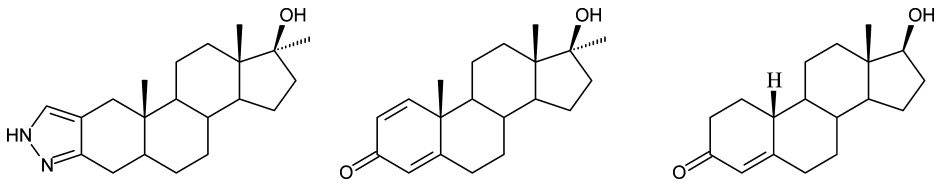

Figure 1. Anabolic steroids: methylboldenone, stanozolol, and nandrolone.

of cancer.[14] Li *et al.*[15] have demonstrated a novel FET immunosensor for the detection of prostate-specific antigen. Utilizing a monoclonal antibody against immunoglobulin (IgE) immobilized in the device, the IgE concentration was estimated using a CNTFET.[16]

This present paper reports the first-time use of CNTFET for anabolic androgenic steroid sensing. Anabolic androgenic steroids (AAS) are banned substances in different fields. The World Anti-Doping Agency (WADA)[17] and the European Commission[18] have prohibited the utilization of AAS compounds for enhancing athletic performance and as a growth promoter in cattle owing to their being considered a public health risk.

Given the high number of substances to be controlled, analytical screening methods are necessary to reduce the time invested in confirmatory methods, which have to be performed using chromatography coupled with mass spectrometry.[19] These methods require time-consuming sample preparation and sophisticated instruments that have to be operated by highly skilled personnel. Immunological methods have demonstrated their usefulness for screening purposes in different areas of clinical toxicology.[20] The feasibility of immunochemical techniques as a screening method to measure AAS, such as stanozolol[21] and tetrahydrogestrinone,[22] has been demonstrated. In the field of immunosensors, devices using an electrochemical transducer[23,24] or optical transducer[25,26] have been published. More recently, a hapten microarray has been developed for the immunochemical screening of AAS.[27] In this paper, we propose the utilization of field-effect transistor arrays based on carbon nanotubes as immunosensors for the detection of stanozolol (Stz) and methylboldenone (MB) (Figure 1). Stz and MB are two AAS that are banned by WADA and the European Commission. The methodology used takes advantage of the specifity of the immune assays but with the added value of the electronic measurements being faster and direct.

A large array of back-gated carbon nanotube devices was fabricated using conventional microfabrication techniques. Antibodies specific for Stz (As147) and MB (As143) were immobilized on the carbon nanotubes (CNTs) either directly or bonded covalently to the polymer poly(methylmethacrylate$_{0.6}$-*co*-poly(ethyleneglycol)methacrylate$_{0.15}$-*co*-*N*-succinimidyl methacrylate$_{0.25}$) (Figure 2). The objective of using the polymer was to avoid an aggregation of antibodies on the electrodes that could modify the metal work function and the Schottky barrier and also to prevent the nonspecific adsorption of proteins and biomolecules on the CNTFET. Strong binding between the nanotubes and proteins has been reported by several authors.[6,28−31] This is an important issue when the detection is carried out in a serum environment.

A schematic representation of the bonding of antibodies and steroids to single-walled nanotubes (SWNTs) is shown in Figure 3. The chips containing the CNTFETs with the attached antibodies were placed in contact with the corresponding steroids for their recognition.

The specificity of the detection was checked on chips bonded with the polymer and the antibodies *versus* the steroid ND (Figure 1) to determine if other steroids are able to bond the antibodies specific for MB and Stz. Additionally, streptavidin was used to check the nonspecific adsorption of proteins to the device after bonding the polymer. Streptavidin is a water-soluble protein that displays hydrophobic domains within its structure,[32] and it has been demonstrated to bind to the nanotubes strongly.[28]

RESULTS AND DISCUSSION

The devices were electrically characterized before and after anchoring any chemicals or biomolecules. In order to see the effect of the antibodies and steroids on the electrical characteristics of the transistors, statistical analyses of a large array of devices were performed. For steroid detection, only devices having good transistor behavior showing an on/off current ratio higher than 100 were considered in the study.

The parameter more frequently used for determination of device characteristics is the dependence of the source−drain current on the gate voltage. In this paper, the threshold voltage and the hysteresis were also characterized. The threshold voltage V_{th} is taken as the *x*-intercept of the line tangent to the steepest part of the I_d-V_g curve. Because the threshold voltage depends on the sweep direction of the back-gate voltage, a separate V_{th} is extracted for the forward and the reverse sweep.

The bare devices showed type "p" behavior indicating hole conduction (Figure 4) and an important shift

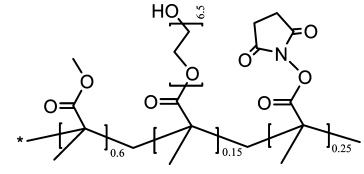

Figure 2. Poly(methylmethacrylate$_{0.6}$-*co*-poly(ethyleneglycol)methacrylate$_{0.15}$-*co*-*N*-succinimidyl methacrylate$_{0.25}$).

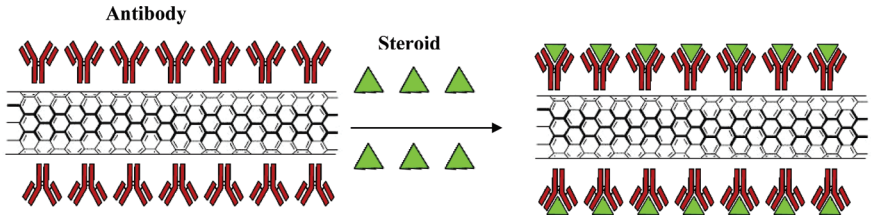

Figure 3. Schematic representation of the bonding of antibodies and steroids to SWNTs.

of the reverse sweep with regard to the forward sweep. This hysteresis is characteristic of the CNTFETs.[33] Figure 5 shows the plot of drain current (I_d) *versus* gate voltage (V_g) of typical devices for the antibody−steroid bonding when the antibodies are joined directly to the CNTFETs. Figure 6 shows the I_d-V_g transfer curves when the antibodies were bonded through the polymer. In the first approach, when the antibodies As147 (specific for Stz) and As143 (specific for MB) are bonded noncovalently to the CNTs, the transistors change from "type p" to ambipolar behavior after attaching the antibodies (Figure 5). This is probably due to the electron-donating ability of amine groups[28,34,35] in the antibodies. When the antibodies are covalently joined to the polymer with which the CNTFETs have been previously functionalized noncovalently, the transistors type p behavior remains.

In the recognition step, when the steroids are bonded to the antibodies, the I_d/V_g plots shift toward more negative values in the forward sweep and toward lower positive voltage in the reverse sweep for both detection processes in the absence of polymer. In the presence of polymer, I_d/V_g plots shift toward more negative voltage in the forward sweep, indicating charge transfer mechanism. These shifts of the transfer curves point out a charge transfer mechanism as reported by Grunner.[28] The binding site of the antibody is formed by a few amino acids from the variable region of the antibody (MW = 150 000 Da), and the recognition of the antibody *versus* analyte (MW = 300 Da) promotes a conformational change in its structure. This conformational change seems to entail some charge transfer that is detected in the transistor.

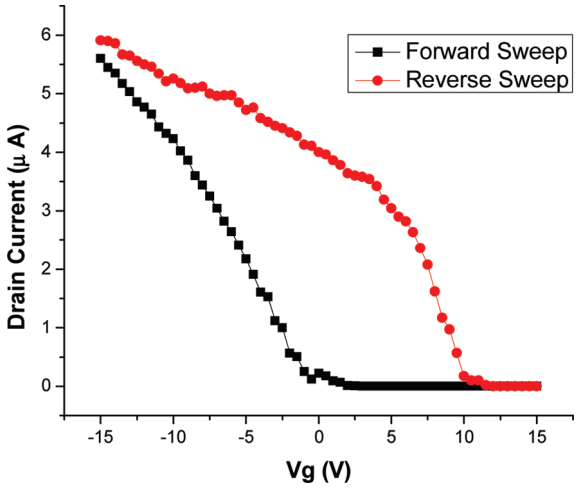

Figure 4. I_d/V_g plot for the forward (red) and reverse (black) sweep of a typical device.

The mean V_{th} values for the forward and reverse sweep for each of the Stz and Mb detection steps are plotted in Figures 7 and 8. In Figures 1 and 2 of Supporting Information the V_{th} distribution histograms of each of the detection steps for the steroid recognition are also shown.

In the process of Stz detection, the incorporation of the antibody As147 to the CNTFET shifts the mean V_{th} of the forward sweep (Figure 7, left) to more negative values whether in the presence or absence of polymer, indicating charge transfer from the antibody to the CNT channel. In the reverse sweep (Figure 7, right), in the absence of polymer, V_{th} shifts to more positive values in the reverse sweep when the antiboby is bonded, evidencing hole traps and increasing considerably the hysteresis (see Figure 9).

In the recognition step when the Stz bonds the specific antibody immobilized in the CNTFET, the mean V_{th} also shifts to more negative voltage for both the forward and reverse sweep, in the presence of polymer. In the absence of polymer, in the forward sweep, the mean V_{th} shifts also to more negative voltage and in the reverse sweep to lower positive values, indicating electronic doping of CNTs and hole reduction. The statistical analysis of the data indicates that the changes in V_{th} in the recognition step are significant at the level of 5% for the forward and reverse sweep without polymer and in the forward sweep with polymer (Table 1).

I_d values show statistically significant changes in the forward and reverse sweep only in the absence of polymer, decreasing approximately an order of magnitude. I_d reduction has been attributed to some contribution of potential scattering mechanism[28] and to modulation of the electrode−SWCNT contact as reported by Gui *et al.*[36] In the presence of polymer, the change of the I_d value is not significant and the type p behavior remains, which seems to indicate that the I_d variations in the absence of polymer could be due to variations of the contact resistance that are hindered in the presence of polymer. Gui *et al.*[36] have demonstrated an important contribution of the junction to the I_d by performing photoresist blocking of the contacts.

For the MB detection, the changes of the mean V_{th} values in the forward sweep (Figure 8, left) are more important for the recognition step than when the antibody bonds the polymer. For the recognition step, the mean V_{th} shifts toward more negative values in the forward and reverse sweep in the presence or absence of polymer, the shifting being higher in the

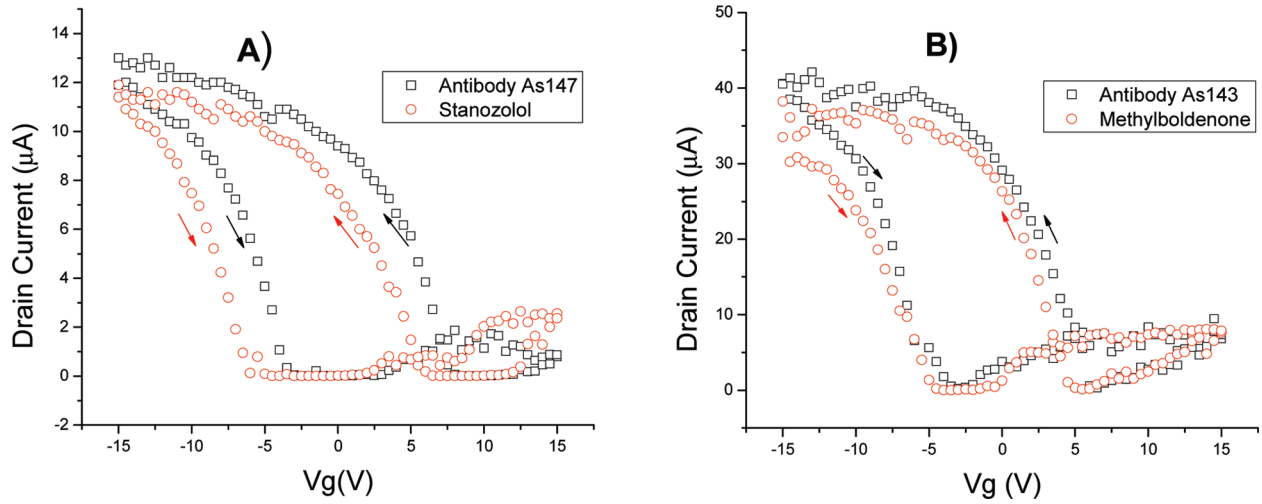

Figure 5. I_d/V_g plots for the forward and reverse sweep of a typical CNTFET. (A) After anchoring As147 antibody (black) and after anchoring stanozolol (red). (B) After anchoring As143 antibody (black) and after anchoring methylboldenone (red).

presence of polymer. For the reverse sweep in the recognition step, Figure 8 right, the mean V_{th} in the presence of polymer shifts toward more negative values and toward lower positive values without polymer. As for the Stz detection, V_{th} values show significant changes at the level of 5% for the forward and reverse sweep without polymer and in the forward sweep with polymer.

I_d values for the MB recognition step show significant changes at the level of 5% for the forward and reverse sweep with polymer (Table 1). The mean I_d values without polymer change slightly (increasing in the forward and decreasing in the reverse sweep), whereas in the presence of polymer, the I_d values decrease significantly. In the absence of polymer, the changes of the I_d values due to the contact resistance and scattering could offset, and only in the presence of polymer the I_d changes are significant due possibly to the hindering of the contact resistance variations by the polymer.

The hysteresis calculated as the difference between the mean V_{th} value in the forward and reverse sweep is much lower in the presence of polymer for both MB and Stz detection (Figure 8). The hysteresis in the recognition step, when the steroid bonds the antibody, increases slightly for Stz and MB recognition, indicating a possible contribution of electron trapping mechanism to the detection.

The ambipolar behavior after bonding the antibodies in the absence of polymer is thought to be due to enhanced tunneling, probably caused by the accumulation of charges at the drain by aggregation of antibody near the electrodes and the contact resistance change of the metal−SWCNT junction. In the absence of polymer, the device changes seem to be due to variations in the Schottky barrier as well as electron charge transfer and electron/hole trapping that increases the hysteresis when the antibody As147 is bonded to the transistor and decreases when the As143 is bonded to the transistor.

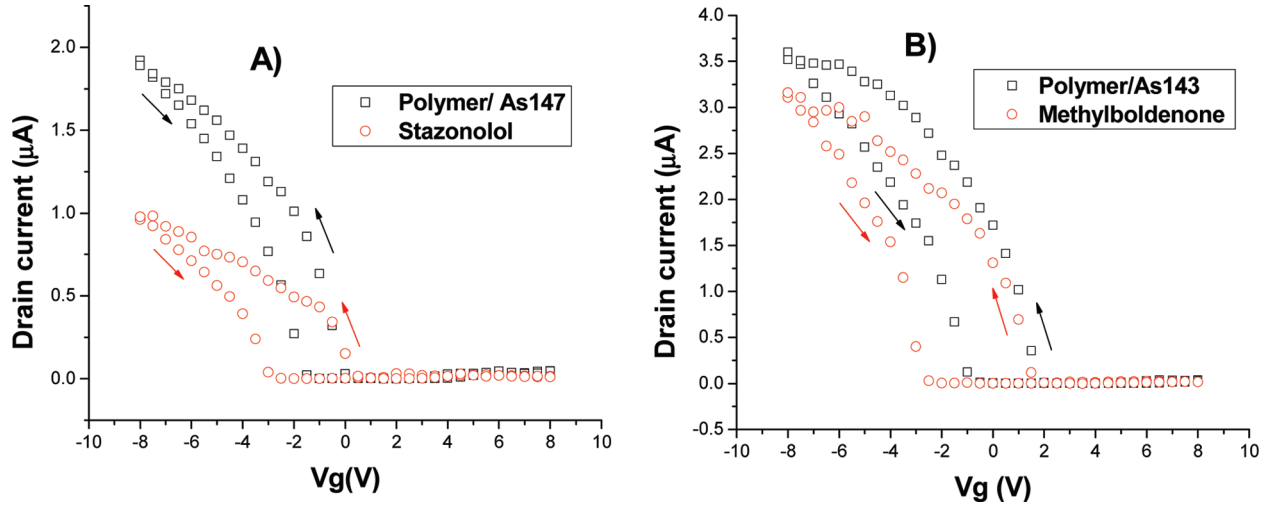

Figure 6. I_d/V_g plots of typical CNTFETs. (A) After anchoring the polymer and As147 antibody (black) and after anchoring stanozolol (red). (B) After anchoring the polymer and As143 (black) and after anchoring methylboldenone (red).

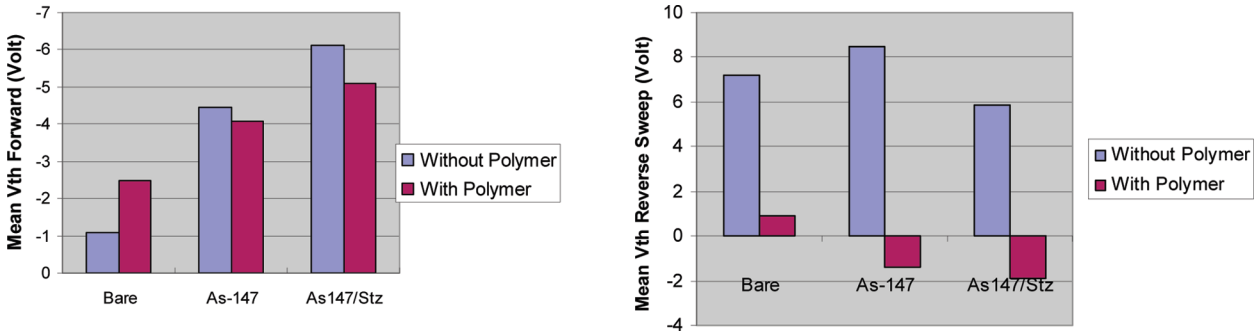

Figure 7. Left: Mean V_{th} values for the forward sweep through the different Stz detection steps: bare transistors, after adding the antibody (As147), and after the Stz recognition (As147/Stz). Right: Mean V_{th} values for the reverse sweep through the different Stz detection steps: bare transistors, after adding the antibody (As147), and after the Stz recognition (As147/Stz).

In the recognition step, the hysteresis decreases in the absence of polymer but the changes are low particularly for the MB recognition. In the presence of polymer, the detection mechanism seems to be due predominantly to charge transfer through the CNT channels with some contribution of trapping mechanism with slight hysteresis increase. For the MB recognition, the I_d variations seem to also indicate scattering potential in addition to charge transfer mechanism.

The two approaches lead to significant changes in the transistors, but the use of polymer would prevent the accumulation of contaminants or other biomolecules that is known to adsorb nonspecifically on CNTs, leading to false positive responses in the transistors.

In previous experiments, it was determined that CNTs bond nonspecifically with Stz and MB, which could produce false positives if the antibodies do not cover the CNT completely. Nevertheless, when the polymer is bonded with the CNTFET, Stz and MB are not nonspecifically bonded. Nevertheless, it has been determined that the polymer does not avoid the adsorption of other biomolecules such as streptavidin, which means this protocol would be useful for clean samples. In order to suppress the separation and purification steps, further research has to be addressed to avoid nonspecific adsorption of biomolecules on CNTFETs.

Control experiments involving adding NT to devices bonded with the polymer and the corresponding antibodies indicated that there were no statistically significant changes in the transistors in the presence of NT. Figure 3 of Supporting Information shows the V_{th} histograms corresponding to the transistor behavior when NT is added to As143 and As147 antibodies bonded covalently to the CNTFET to the polymer. There

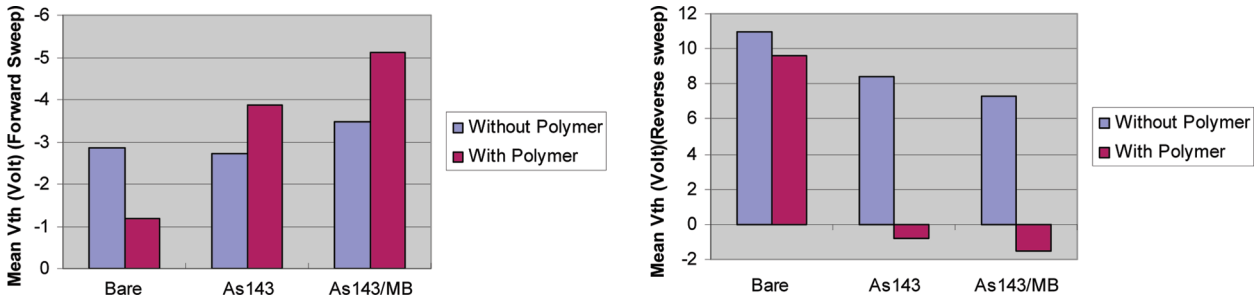

Figure 8. Left: Mean V_{th} values for the forward sweep through the different MB detection steps: bare transistors, after adding the antibody (As143), and after the MB recognition (As143/MB). Right: Mean V_{th} values for the reverse sweep through the different MB detection steps: bare transistors, after adding the antibody (As143), and after the MB recognition (As143/MB).

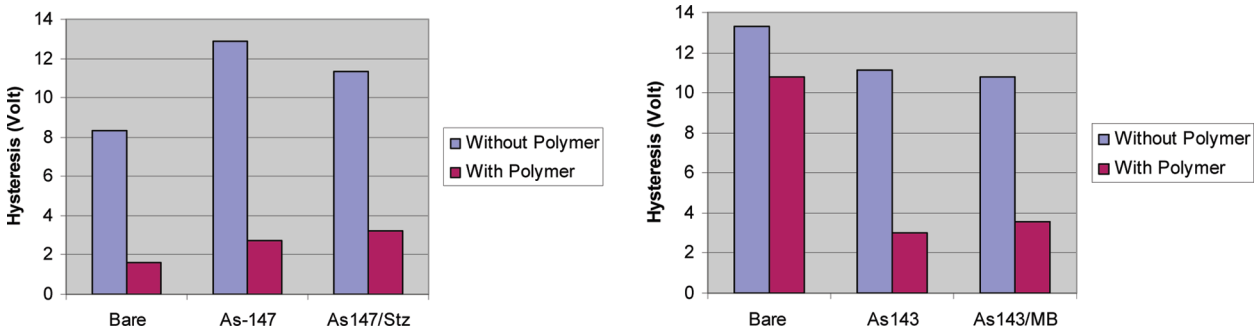

Figure 9. Transistor hysteresis through the different detection steps calculated as the difference between the V_{th} mean value in the forward and reverse sweep. Left: Stz detection. Right: MB detection.

TABLE 1. Statistical Analysis of the Threshold Voltage (V_{th}) and Drain Current (I_d) for the Bonding Antibody Steroids with and without Polymer for Stanozolol and Methylboldenone in the Forward (Fw) and Reverse (Rev) Sweep

parameter	steroid	polymer	Fw sweep	Rev sweep
V_{th}	stanozonol	no	significantly different	significantly different
I_d	stanozonol	no	significantly different	significantly different
			mean I_d value	mean I_d value
			As147: -1.00824×10^{-5}	As147: -1.05363×10^{-5}
			Stz: -7.8920×10^{-6}	Stz: -8.17139×10^{-6}
V_{th}	stanozonol	yes	significantly different	not significantly different
I_d	stanozonol	yes	not significantly different	not significantly different
			mean I_d value	mean I_d value
			As147EA: -4.7335×10^{-6}	As147: EA -4.7335×10^{-6}
			Stz: -2.9915×10^{-6}	Stz: -2.9915×10^{-6}
V_{th}	methylboldenone	no	significantly different	significantly different
I_d	methylboldenone	no	not significantly different	not significantly different
			mean I_d value	mean I_d value
			As143: -1.50041×10^{-5}	As143: -1.70046×10^{-5}
			MB: -1.74631×10^{-5}	MB: -1.53269×10^{-5}
V_{th}	methylboldenone	yes	significantly different	not significantly different
I_d	methylboldenone	yes	significantly different	significantly different
			mean I_d value	mean I_d value
			As143: EA -1.63808×10^{-6}	As143: EA -1.64651×10^{-6}
			MB: -9.10735×10^{-7}	MB: -9.32149×10^{-7}

are no changes in the mean V_{th} values for the recognition step, and the V_{th} histogram of the antibody–steroid step showed slight changes in the distribution, but according to the statistical analysis, these are not statistically significant, meaning that the antibodies As143 and As147 are unable to join NT.

CONCLUSIONS

The feasibility of electronic detection of two anabolic steroids, stanozolol and methylboldenone, has been demonstrated using CNTFET transistors. The bonding of Stz and MB to the specific antibody produces strong changes in the electrical properties of the CNTFET, showing statistically significant changes in the V_{th} in the forward and reverse sweeps in the recognition step when the antibody is attached directly to the CNT and changes in the forward sweep of the recognition step when the antibody is bonded with the polymer, indicating charge transfer mechanism. When polymer is used to bond the antibodies, the changes in I_d are statistically significant for the forward and reverse steps in the absence of polymer in Stz recognition and in the presence of polymer for the MB recognition.

The presence of polymer reduces considerably the hysteresis and the contribution of SB as indicated by the reduction of the changes in the mean I_d values in Stz recognition. In the MB recognition in the absence of polymer, the changes in I_d are very low probably because the changes in SB are balanced by those of the scattering potential. The presence of polymer seems to prevent the changes in SB and makes visible that the changes in I_d are probably due to potential scattering for the MB recognition.

With regard to the mechanism of detection in the absence of polymer, several overlapped mechanisms could be present. In addition to charge transfer, scattering potential and SB modification reported by other authors,[6,28] electron and hole trap mechanisms with hysteresis decrease in the absence of polymer, and hysteresis increase in the presence of polymer have been determined as previously reported[37] for the electronic detection of DNA hybridization. In the presence of polymer, the changes in SB are possibly of low relevance, making the scattering mechanism and charge transfer the predominant detection mechanisms. Nevertheless, this assumption will have to be demonstrated in future work.

As far as the specificity of the detection is concerned, it has been determined that the CNTFETs do not show statistically significant changes when the antibodies are in contact with other steroids such as nantranone; nevertheless, cross-reactivity with other steroids will have to be studied more exhaustively. The bonding of the polymer with the CNTFETs prevents the nonspecific adsorption of the steroids and antibodies on the CNTFETs, but it has been determined that streptavidin causes statistically significant changes, indicating nonspecific adsorption to the polymer or some kind of conformational changes that produce electron transfer in the transistor. Nonspecific binding is a big challenge particularly in the immunoassay field.

The data reported represent a proof of concept of the feasibility of electronic steroid immune detection. Nevertheless, further research needs to address the development of practical biosensors that prevent nonspecific adsorption of biomolecules such as streptavidin on CNTs and enable direct detection, thus avoiding the necessary separation steps when serum samples are used.

EXPERIMENTAL METHODS

CNTFET Fabrication. A large array of back-gated carbon nanotube devices was fabricated using conventional microfabrication techniques. Starting from a highly doped silicon substrate, a 160 nm thick silicon dioxide layer was grown under dry conditions. The contact to the substrate was defined, followed by the growth of carbon nanotubes on lithographically defined catalyst islands using processes previously reported.[38] Source and drain

contacts were patterned using optical lithography for all nanotube devices simultaneously in a bilayer lift-off stack. Metal contacts were then deposited using e-beam evaporation, followed by lift-off in acetone. The gate length, set by the gap between the contacts, is 2 μm. There are a total of 896 devices per 1 cm^2 chip. The devices were characterized using an Electroglas 2001X automatic probing station in ambient conditions. Figure 4 shows the drain current (I_d) *versus* gate voltage (V_g) of a typical CNTFET.

Preparation of Antibodies. Stanozolol (Stz) and methylboldenone (MB) were purchased from Sequoia Research Products Ltd. (Oxford, UK). Nandrolone was obtained from Aldrich Chemical Co. (Milwaukee, WI). The analytes used as standards were prepared in stocks in DMSO at 10 mM. The antibodies used for Stz and MB demonstrated their functionality as described before in an ELISA development[21] and in a biosensor development based on resonant structures,[25] respectively. The immunoassay developed for Stz (As147/8BSA) has an IC_{50} of 0.16 μg L^{-1} and a LOD of 0.022 μg L^{-1} with a dynamic range from 0.045 to 0.60 μg L^{-1} and a slope of 1.2. The immunoassay developed for MB (As143/15BSA) has an IC_{50} of 1.51 μg L^{-1}. For this study, the antibodies were purified by $(NH_4)_2SO_4$ precipitation.

Protocol for Steroid Recognition by Noncovalent Joining of the Antibodies to the Transistors. After preparation of the specific antibodies, solutions of 10 μg/mL of antibody, either As147, specific for Stz, or As143, specific for MB, were reconstituted in 1× PBS (10 mM). The chip containing the CNTFET devices was immersed in the antibody solution for 90 min. The chip was then washed with 5 mL of 1× PBS (10 mM) five times and dried in a nitrogen flow for a few minutes. After electrical characterization, the chip containing the antibody was placed in contact with a solution of the steroid in dimethylsulfoxide (10 mM) that was diluted to 300 nM in 1× PBS (10 mM). After 90 min in contact, the chip was rinsed with 5 mL of PBS five times. Before electrical characterization in each of the steps, the chips were dried in vacuum for 4 h at room temperature.

The effect on CNTFETs of the 1× PBS buffer with the same concentration of DMSO as present in the steroid solutions was determined by depositing the corresponding solution on the CNTFET noncovalently functionalized with the antibody. This was for the purpose of ruling out whether the solution containing the steroid was responsible for the changes observed in the sensor when adding the steroid.

Protocol for Steroids Recognition by Joining the Antibodies to the Transistors through the Polymer. In a second approach, the polymer poly(methylmethacrylate$_{0.6}$-*co*-poly(ethyleneglycol)methacrylate$_{0.15}$-*co*-*N*-succinimidyl methacrylate$_{0.25}$) (Figure 2) was synthesized according the procedure reported by Martinez *et al.*[36] and noncovalently bonded to the nanotubes. The antibodies were later covalently bonded to the polymer by forming amide bonds between the succinimide groups of the polymer and the amine groups of the antibodies. After joining the specific antibodies, the transistors were treated with ethanolamine (EA) to block any remaining succinimide groups in the polymer and to avoid nonspecific bonding of any accompanying protein or contaminant. After electrical characterization, the devices were placed in contact with the corresponding steroids as indicated previously.

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Supporting Information Available: Histograms corresponding to the threshold voltage distribution of the devices in the absence and presence of polymer for the different steps of stanozolol and methylboldenone detection. Histograms of the threshold voltage distribution of the devices corresponding to the control experiments with nandrolone. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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