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Label-free, chemiresistor immunosensor for stress biomarker cortisol in saliva

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ABSTRACT

Salivary cortisol is commonly used as a bioindicator of the psychobiologic response to environmental and psychological stressors. Current analytical approaches rely on immunoassays performed at distant, centralized laboratories and involve an elaborate specimen collection-processing-transportation-storage-analysis-reporting cycle. To facilitate point-of-use measurement of salivary cortisol levels, we describe the development and proof-of-concept testing of an ultrasensitive, label-free immunosensor based on a single-walled, carbon nanotube-based chemiresistive transducer. Carbon nanotubes were functionalized with a cortisol analog [cortisol-3-CMO-NHS ester] and a monoclonal anti-cortisol antibody was ligated to this receptor. Addition of phosphate buffer as well as artificial saliva spiked with varying cortisol concentrations displaced the anti-cortisol antibody producing corresponding decreases in the resistance/conductance of the nanotube-biomolecule hybrid. The immunosensor demonstrated an ultralow detection limit of 1 pg/ml and excellent binding selectivity for cortisol even in the presence of structurally similar steroids such as 21-hydroprogesterone. The nanotube immunosensor offers attractive prospects for the development of highly sensitive biosensor for rapid, label-free measurement of salivary cortisol in a variety of clinical and research settings.

1. Introduction:

The hypothalamic-pituitary-adrenal (HPA) axis plays a central role in transducing the subjective experience of an environmental or psychological stressor into physiological responses. Although adaptive in the short-term, extreme, chronic or frequent activation of the HPA system can have adverse health effects (Sapolsky, 1992; McEwen, 2008). As the principal end product of HPA axis activation, cortisol is widely used as an expression of HPA reactivity and adaptation to naturalistic stressors and to connect these changes to the development of a variety of disease phenotypes including depression, PTSD, cognitive decline, and cardiovascular disease (Hellhammer et al., 2009).

Even though blood and urine are most commonly utilized, salivary sampling is the preferred method for determining cortisol levels. Not only do salivary cortisol levels have a strong positive correlation with blood cortisol levels (Gallagher et al., 2006), measurement of salivary cortisol is more physiologically relevant because it closely reflects the levels of unbound cortisol in blood (Kirschbaum et al., 2008; Jessop and Turner-Cobb, 2008). For subjects, supplying a saliva sample evokes less anxiety than providing a blood sample, and less embarrassment than producing a urine specimen. The non-intrusive nature of salivary sampling renders saliva particularly attractive in population-based salivary cortisol studies or in studies involving children or individuals with physical or mental handicaps. The ease of saliva collection facilitates subject compliance and allows repeat, self-administered sampling in naturalistic settings without the need for specialized personnel or equipment.

Analytical methods for detection of cortisol in saliva include traditional immunoassays such as radio-immunoassay (RIA), enzyme-linked immunosorbent assay (ELISA) and fluoro-immunoassay (FIA). The limit of detection (LOD) for these assays range from 200 pg/ml for RIA (Kirschbaum et al., 1999) to 192 pg/ml for FIA (Höferl et al., 2005) and 30 pg/ml for ELISA (http://www.salimetrics.com). While sensitive and well below the normal value of 1 ng/ml in healthy persons (Aardal and Holm, 1995), these methods are tedious and time consuming, involve multiple step reactions and washing processes for subsequent analyses, require elaborate hardware that does not lend itself to miniaturization and portability and require labeled analogs of cortisol for the competitive immunoassay. Immunosensors/affinity-biosensors using anti-cortisol antibodies in conjunction with surface plasmon resonance (SPR) transducer were recently reported. An LOD of 10 ng/ml in saliva and urine, significantly greater than the diagnostic range, was reported by Frasconi et al. (2009). This high limit of detection is attributed to

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the low mass of the target cortisol producing a small resonance angle modulation (Mitchell et al., 2009). More recently, Mitchell et al. (2009) reported a markedly improved LOD of 46 pg/ml cortisol by SPR. However, the ultra-low resolution requires the use of the competitive immunoassays followed by incubation with secondary antibodies against the anti-cortisol primary antibody to increase the bound mass on the gold sensor layer. As an alternate strategy, electrochemical immunoensors have been also utilized for detection of cortisol in saliva. Sun et al. (2008) reported a sandwich immunoassay format based amperometric immunosensor using an anti-cortisol antibody as capture antibody immobilized on gold working electrode and alkaline phosphatase labeled secondary/detection antibody to achieve an LOD of 275 pg/ml. Using electrochemical impedance spectroscopy (EIS) technique, Arya et al. (2010) reported a label-free immunosensor that could detect 0.36 pg/ml to 3.6 ng/ml cortisol in saliva with a 40 min incubation time.

Recent advances in label-free, nanosensing systems create some exciting opportunities to readily measure salivary analytes such as cortisol with a speed, precision and resolution that exceeds the capabilities of elaborate laboratory-based assays (Tlili et al., 2010). In particular, nano-sensors fabricated using single-walled carbon nanotubes (SWNTs) are promising building blocks for point-of-use, label-free biosensors because of their high sensitivity, good selectivity, and capacity to suppress electrical ($1/f$) noise (Star et al., 2006). Previously, we had successfully applied this technology to the measurement of a salivary surrogate of the sympathetic response to stress. Here, we describe the development and proof-of-concept testing of an ultrasensitive, label-free immunosensor based on a single-walled carbon nanotube-based chemiresistive transducer for highly sensitive, selective and rapid detection of cortisol in saliva for point-of-care/bed-side diagnostics.

2. Materials and methods

2.1. Materials

Single-walled carbon nanotubes (SWNT) with high carboxylated functionality, sold under the trade name of P3-SWNT, were purchased from Carbon Solutions, Inc. (Riverside, CA, USA). 1-Pyrenemethylamine hydrochloride (PMA), dimethyl formamide (DMF), mercaptohexanol (MCH), cortisol, and tween 20 were obtained from Sigma Aldrich (St. Loius, Mo, USA). Cortisol-3-CMO-NHS ester (cortisol-NHS) and anti-cortisol monoclonal antibody were obtained from U.S. Biological (USA). Distilled water purified through a Milli-Q plus (Millipore Inc.) ultrapure water system was used to prepare solutions. Artificial saliva was prepared by dissolving 0.6 g/l Na_2HPO_4, 0.6 g/l anhydrous $CaCl_2$, 0.4 g/l KCl, 0.4 g/l NaCl, 4 g/l mucin and 4 g/l urea in deionized water, adjusted to pH 7.2, sterilized by autoclaving and stored in the refrigerator until use (Tlili et al., 2010).

2.2. Sensor fabrication

Gold microelectrodes were fabricated using the standard photolithography and lift-off process in the cleanroom of Center for Nanoscale Science and Engineering, University of California, Riverside. In brief, 100 nm of SiO_2 was initially deposited on the highly doped p-type semiconductor Si wafer using chemical vapor deposition (CVD) followed by defining the 200 μm × 200 μm electrode area by photolithography, e-beam evaporation of 20 nm thick Cr adhesion layer and 180 nm thick Au layer and finally lift-off to define the electrodes (Mubeen et al., 2010). Before use, the electrodes were cleaned with piranha solution (70 vol.% H_2SO_4/30 vol.% H_2O_2) for 2 min, rinsed with water and then dried under a stream of nitrogen.

A uniform suspension of SWNT (0. 1 mg/ml) in DMF was prepared by ultrasonication (power level 9, Model 50D sonicator, VWR International, Westchester, PA, US) for 90 min followed by centrifugation (31,000 × g, Model J2-HS centrifuge, Beckman, MD, US) for 90 min. The suspended SWNTs were aligned across a pair of gold microelectrodes using AC dielectrophoresis (DEP) as described. A 0.1 μL drop of the dispersed SWNT was dispensed in the gap between the pre-cleaned gold microelectrodes pair and subjected to a 4 MHz (amplitude 3 Vp-p) AC field across the electrodes for a few seconds using a function generator (Model 188-S-1257, Wavetek, Alpharetta, GA, USA) followed by annealing at 300 °C for an hour under a continuous flow of 95% nitrogen plus 5% hydrogen in order to fix the SWNT in place and make a good contact with the gold electrodes. The aligned SWNT were incubated for 1 h at room temperature in a 6 mM solution of PMA in DMF, washed extensively with DMF to remove excess reagent followed by incubation with a 1 mg/ml of cortisol-NHS solution in bicarbonate buffer (100 mM, pH 9) for 3 h and rinsing with water and PB (pH 7.2, 10 mM phosphate buffer) to remove residual and physisorbed cortisol. The devices were then incubated with 0.1% tween 20 solution (to reduce the non-specific adsorption of biomolecules), washed with water and PB followed by overnight incubation in 50 μg/ml of anti-cortisol antibody in PB solution and then washed extensively with PB and then water to remove unbounded antibody. To block non-specific response from the mucin component of artificial saliva, the sensor device was incubated with 2 mM MCH in PB for 30 min followed by washing with PB (Tlili et al., 2010).

2.3. Device characterization and sensing

The sensing protocol consisted of monitoring the initial resistance (R_0) of the immunosensor fabricated above by measuring the source–drain current (I_{DS}) as a function of source–drain voltage (V_{DS}) from −0.5 V to +0.5 V using semiconductor parameter analyzer (HP 4155A, Agilent technologies Inc., CA, USA) and taking the inverse of the slope of the I–V curve from −0.1 V to +0.1 V followed by incubation for 10 min at room temperature with different concentrations of cortisol and derivatives in buffer or artificial saliva followed by washing 3-times with PB and recording the new resistance as before.

2.4. Fluorescence imaging

A thick-film of SWNT was deposited on a microscope cover slip by spray-coating several layers of SWNTs suspension in DMF followed by heating in the oven to dry. SWNT coating was treated sequentially with PMA, cortisol-NHS, tween 20, biotinylated anti-cortisol antibody and Avidin-Texas red (Av-TxR), washed 3-times with PB and imaged for fluorescence. The cortisol-antibody functionalized SWNT were incubated with PB and 2 μg/ml cortisol at room temperature for 30 min, washed with PB and imaged once again for fluorescence. For a control experiment, SWNTs coating modified sequentially with PMA, cortisol-NHS, and tween 20 were incubated with Av-TxR, washed 3-times with phosphate buffer and imaged for fluorescence. Fluorescence images were captured using Olympus BX51 fluorescence microscope and analyzed by QCapture Pro™ software.

3. Results and discussion

One-dimensional nanostructures-based field effect transistors (FET)/chemiresistors have been applied as transducers for label-free immunosensors. In the conventional format of these immunosensors, the nanostructure is functionalized with

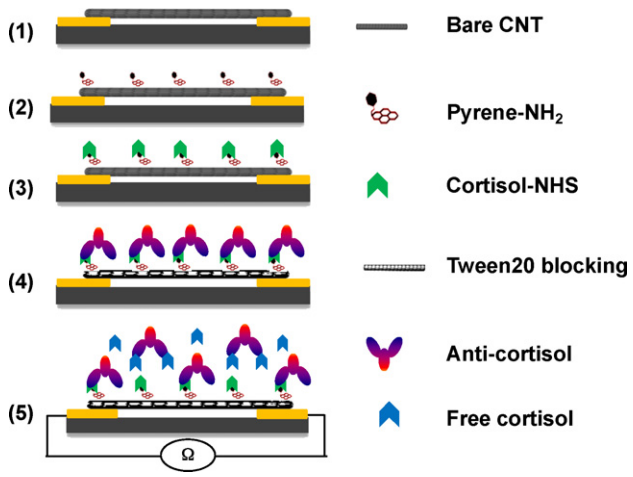

Fig. 1. Schematic of SWNTs chemiresistor transducer-based cortisol immunosensor fabrication steps and sensing by displacement. (1) Aligned SWNTs; (2) non-covalent functionalization with PMA; (3) covalent attachment of cortisol-NHS; (4) ligation of anti-cortisol antibodies to cortisol and blocking with tween 20; (5) displacement of anti-cortisol by free-cortisol in analyte sample.

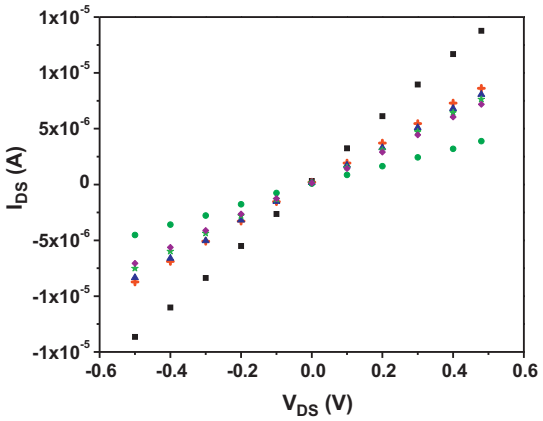

Fig. 2. Source–drain current vs. source–drain voltage of SWNTs device at various stages of fabrication and sensing; (■) Bare SWNTs; (+) after PMA treatment; (▲) after cortisol functionalization; (*) after blocking with tween 20; (●) post anti-cortisol antibody ligation; and (♦) incubation with free-cortisol.

the capture/detection element such as antibody, DNA probe, aptamers, binding protein, etc. and the modulation of the resistance/conductance of the device upon the binding of the target is measured and correlated to the concentration. We initially investigated the sensing of cortisol using this traditional format. The SWNTs gate of the transducer was functionalized with anti-cortisol antibody through the interaction of non-covalently attached 1-pyrene butanoic acid succinimide ester (PBASE) followed by covalent conjugation of the antibody by the amide bond between the succinimide and amine groups of the antibody (Tlili et al., 2010; Cella et al., 2010). Upon exposure to different concentrations of cortisol, the immunosensor response, normalized by resistance change, for the concentration range investigated was a mere $0.75 \pm 3\%$ without any concentration dependence. The weak response, similar to blank (no cortisol), is attributed to the small size and charge of cortisol when compared to the successfully detected targets (proteins, DNA, viruses and bacteria) using 1-D nanostructure-based chemiresistor/FET immunosensors, since the magnitude of the 1-D chemiresistor/FET transducer response is dependent on the magnitude of the gate potential which in this case is the target charge. To detect cortisol using chemiresistor/FET transducer, we changed the sensing format from the traditional binding/capture of the antigen by the antibody to the displacement format. In this format, the SWNT network bridging the source and drain electrodes is first modified with a cortisol analog, cortisol-NHS, followed by ligation with anti-cortisol antibody, a large size and charge protein, to saturate all the cortisol sites. On addition of cortisol to the immunosensor, the antibody bound to the cortisol functionalized SWNTs is stripped/displaced from the bound antigen analog and triggers a large change of the device resistance/conductance, thus resulting in an excellent sensitivity and limit of detection. Fig. 1 shows the fabrication steps and Fig. 2 shows the current vs. voltage plot for the different steps registered to track the fabrication process. The change in I_{DS}–V_{DS} characteristic of the device is a measure of the change of the conductance (inverse of resistance) brought about by the modulation of the surface properties of SWNTs. The change, increase or decrease, in the device conductance from the previous measurement upon modification is an indication of alteration of the surface properties of SWNTs. A linear relationship between I_{DS}–V_{DS} in the investigated voltage window is an indicator of good ohmic contact between SWNTs and gold electrodes. As shown in the figure (Fig. 2), the resistance (inverse of the slope of I–V plot between −0.1 and +0.1 V) of the SWNT network increased upon modification with PMA, and anti-cortisol antibody. These are attributed to 1) the charge transfer from pyrene linker to the carbon nanotube via π-π stacking causing a decrease in the carrier density (hole) in the SWNTs and 2) the effect of scattering potential generated by the antibody interaction with the cortisol molecules functionalized carbon nanotubes which decreases the mobility of holes in SWCNTs (Gruner, 2006), and/or to the Schottky barrier mechanism due to the modification of the metal work-function (Chen et al., 2004). Upon incubation of the final device with 1 μg/ml of cortisol, the resistance reverted back to the value after the blocking with tween 20, an evidence of the successful detection of cortisol. In order to confirm that the observed resistance increase and decrease was due to the binding of anti-cortisol to the cortisol analog tethered to SWNT and antibody displacement, respectively, fluorescence imaging studies were conducted. As shown in Fig. 3, there was an intense well-distributed fluorescence after functionalization (Fig. 3, panel/bar 2) compared to control (SWNT-functionalized with cortisol-NHS followed by reaction with Av-TxR) (Fig. 3, panel/bar 1), confirming specific-biding/ligation of anti-cortisol to immobilized cortisol. When incubated with 2 μg/ml of cortisol there was a 50% decrease of fluorescence (Fig. 3, panel/bar 4) compared to the small decrease observed upon incubation with PB (Fig. 3, panel/bar 3), confirming the displacement of anti-cortisol from the surface.

Fig. 4 shows the calibration plot for the cortisol immunosensor in phosphate buffer. The normalized resistance change $[(R - R_t)/(R_{ab} - R_t)$, where R_{ab} is the resistance of fully assembled device (SWNTs functionalized with PMA + cortisol-NHS + tween 20 + antibody); R_t is the resistance of SWNTs after functionalization with PMA + cortisol-NHS + tween 20; and R is the resistance of the fully assembled (functionalization with PMA + cortisol-NHS + tween 20 + antibody) device after exposure to the analyte] showed an inverse relationship to cortisol concentration, typical of competitive immunoassay, over a range of 1 pg/ml to 1000 ng/ml. In contrast, the normalized resistance change for negative control devices (SWNTs/tween 20/anti-cortisol) was close to zero over the entire range of cortisol concentration. The immunosensor response was linear over the range of 1 pg/ml to 10 ng/ml and had a sensitivity (slope of the linear region) of 14.9 $(ng/ml)^{-1}$ ($r^2 = 0.89$). The immunosensor had excellent intra- and inter-batch reproducibility as evidenced by the low 1.2–2.3% relative standard deviation of the data points obtained from a total of 11 electrodes prepared in two batches. The limit of detection calculated by the formula $LOD = 3S_D/m$, where m is the slope of the linear part of

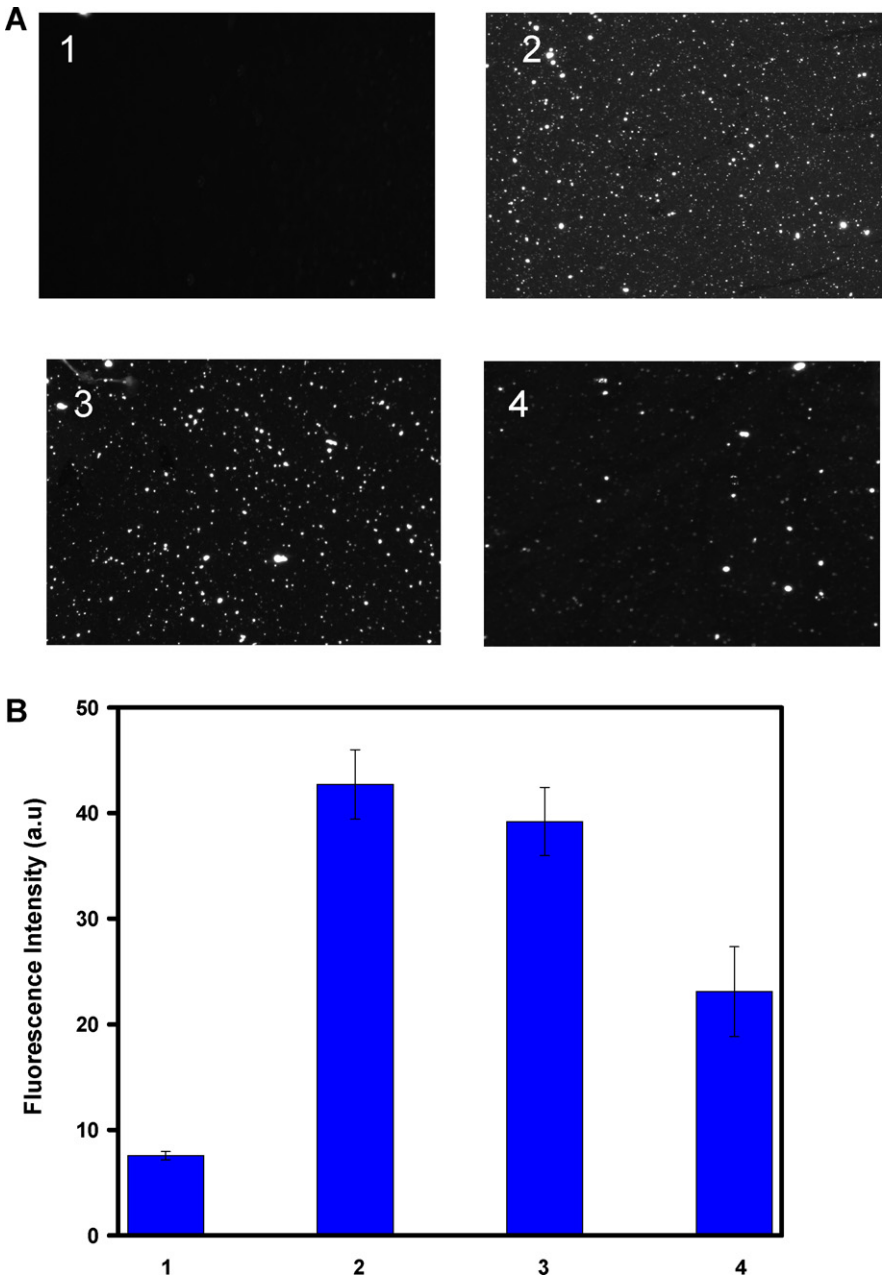

Fig. 3. Fluorescence images (A) and fluorescence intensity (average of 6 images) (B) of (1) SWNTs + PMA + cortisol-NHS + tween 20 + Av-TxR (control), (2) SWNTs + PMA + cortisol-NHS + tween 20 + biotinylated anti-cortisol + Av-TxR, (3) after exposure of (2) with PBS and (4) after exposure of (2) with 2 μg/ml cortisol.

the calibration curve and S_D is standard deviation of the blank measurement, was 0.11 pg/ml.

For successful application, a biosensor should be selective/specific for the target analyte. As shown in Fig. 4, there was no decrease of the normalized resistance of the cortisol immunosensor upon incubation with 1 pg/ml to 1 μg/ml (same concentration range as cortisol) of 21-hydroprogesterone, a glucocorticoid hormone from the cortisol family, demonstrating the high selectivity of the immunosensor. The sensor, however, failed the matrix selectivity test. When exposed to ten-fold diluted artificial saliva, the matrix in which cortisol has to be determined for clinical diagnostic applications, the sensor resistance either did not decrease or even increased. A systematic investigation for the probable cause indicated that the matrix effects resulted from the non-specific adsorption of mucin, a large group of glycoprotein present in saliva, on gold electrodes of the device which negated/screened the resistance decrease from the anti-cortisol displacement. Based on our past experience of successful blocking of non-specific adsorption of mucin on gold (Tlili et al., 2010) by mercaptohexanol (MCH), the treatment of devices with MCH as the fabrication step was introduced. Fig. 5 shows the calibration plot of the immunosensor for cortisol spiked in ten-fold diluted artificial saliva. As shown the immunosensor demonstrated a wide dynamic range from 1 pg/ml to 1000 ng/ml that was linear from 1 pg/ml to 10 ng/ml. The sensitivity in the linear range was 13.97 $(ng/ml)^{-1}$ and the estimated LOD was 1 pg/ml. Once again, the sensor had good reproducibility as evidenced by a small, 1.3–3.9%, relative standard deviation of the data points obtained from 6 electrodes. These performance characteristics are very comparable to phosphate buffer. Further, the ultra-low 1 pg/ml LOD without use of a label is superior to the various label immunoassay techniques (Kirschbaum et al., 1999; Höferl et al., 2005; http://www.salimetrics.com), SPR (Frasconi

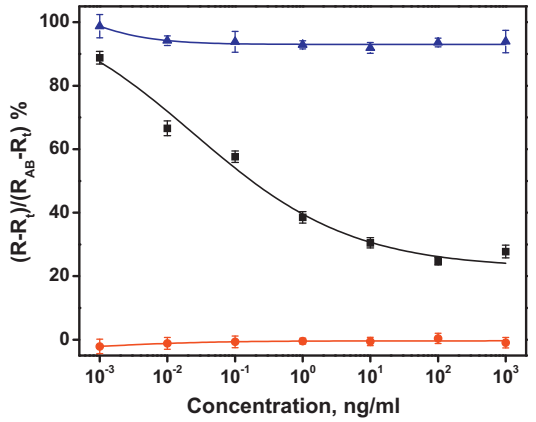

Fig. 4. Calibration plots and selectivity of cortisol immunosensor in PBS. (■) Cortisol (each data point is an average of 11 electrodes prepared in 2 batches), (▲) 21-hydroprogesterone (each data point is an average of 7 electrodes prepared in 2 batches) and (●) negative control (each data point is an average of 4 electrodes). Error bars represent ±1 S.D.

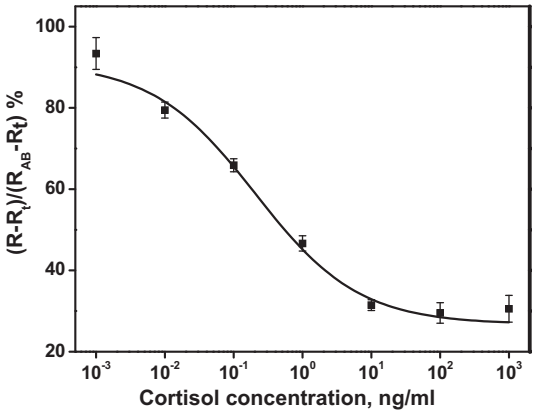

Fig. 5. Calibration plot of cortisol immunosensor in artificial saliva (each data point is an average of 6 electrodes and error bars represent ±1 S.D.).

et al., 2009; Mitchell et al., 2009) and amperometric (Sun et al., 2008) immunosensors and comparable to EIS immunosensor (Arya et al., 2010) and well below the 1 ng/ml therapeutic level in normal in healthy individuals (Aardal and Holm, 1995) even after diluting the sample to reduce matrix interference.

4. Conclusion

Despite the increasing use of salivary cortisol in psychobiological investigations, its clinical utility has been restricted by the lack of appropriate technology platforms that allow near "real-time" detection and quantification of this biological response indicator. The conventional ways of processing saliva samples in distant, centralized laboratories results in extended reporting times and are fraught with several potential quality failure points related to sample acquisition, storage, transport, processing and reporting. Also, the logistical challenges of repeat biofluid collection can be significant in epidemiologic studies involving longitudinal monitoring and the added costs of sample analysis in centralized laboratories can prove prohibitive, particularly in resource-poor settings. Technological advances that render biomarkers more field-practical and accessible to end-users will allow biomarker-based strategies to become ingrained in experimental studies and eventually, lead to better risk assessment, diagnosis and a patient-centric management of stress-related diseases. Towards this end, we have developed a highly sensitive, selective, easy-to-use and field-deployable immunosensor for the detection of stress biomarker cortisol in saliva. This label-free cortisol biosensor is the first example of chemiresistive biosensor based on the displacement of the monoclonal anti-cortisol antibody from the carbon nanotube devices, which cannot be detected by the conventional method based on the immobilization of the antibodies onto the carbon nanotube surface due to the small and uncharged molecules. The present study provides proof of concept for developing a simple, sensitive, selective, fast response, and miniaturized carbon nanotube chemiresistor platform for detecting the cortisol target in phosphate buffer as well as artificial saliva. Thus, the simple strategy described here may easily be applied to any small and uncharged molecules by using adequate displacing moieties, which can open a new window for medical diagnostics application. Our future goal will be to increase the sensitivity of the cortisol biosensor based on the conductivity change of SWNT by increasing the charge of the displacing moiety. Using the new approach described in this study we are able to detect small and uncharged molecules rapidly with high reproducibility, sensitivity and selectivity in phosphate buffer as well in complex media (artificial saliva) and also provide a promising platform for further detection of small and uncharged molecules, which can open new window in clinical diagnostics.

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