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Single-walled carbon nanotube chemoresistive label-free immunosensor for salivary stress biomarkers

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Abstract

The present work is focused on the development, analytical characterization and evaluation of selective and sensitive SWNT-chemiresistor immunosensor for the label-free detection of salivary α -amylase (SAA). SWNTs were aligned to bridge lithographically patterned gold microelectrodes using AC dielectrophoresis followed by functionalization with anti-SAA antibodies. The nano-immunosensors exhibited excellent sensitivity over the clinically relevant range (19 to 308 U ml⁻¹) with a limit of detection (LOD) of 6 μ g ml⁻¹ (0.6 U ml⁻¹) and 7.8 μ g ml⁻¹ (0.78 U ml⁻¹) in phosphate buffer and artificial saliva, respectively, and no interference from other components of saliva. This label-free nano-immunosensor technology has potential application in clinical diagnosis for stress biomarkers expressed in human saliva at the point-of-care.

Introduction

Exposure to stressful conditions and life events is increasingly recognized to render the individual at risk for a range of mental and physical health problems.^{1,2} Although initially adaptive, the complex biological processes that help the body respond to the stressor can be pernicious if sustained for extended periods of time. Stress has been implicated as a precipitating variable in the onset of conditions as varied as cardiovascular disease, diabetes, depression,^{3,4} gastrointestinal disorders, and total premature mortality. Correspondingly, understanding how stress affects general health requires greater elucidation of the psychobiological processes that contribute to the development of these physical and behavioral disorders.

Driven by the recognition that compensatory mechanisms of adaptation to psychosocial stressors are expressed as discernible physiologic changes, stress researchers have focused on identifying and measuring corresponding biological signatures. Of particular interest in establishing a relationship between stress and disease have been the patterns of activation of the sympathetic-adrenal medullary system and its attendant physiological effects. The sympathetic-adrenal medullary system is a critical neuroendocrine effector system that is called into play when an individual is exposed to physical or psychological stressors.⁵ Activation of the sympathetic-adrenal medullary system manifests as concomitant increases in heart rate, blood pressure and respiration, alterations in blood flow to tissues, increases in blood glucose, and decreases in gut motility.^{6,7} Thus, assessment of sympathetic-adrenal medullary activity

by measurement of circulating levels of catecholamines provides a convenient means of quantifying an individual's graded physiological response to a given stressor.⁸

Although blood and urine are the most commonly used biofluids for assessing catecholamines, the intrusiveness and logistical limitations inherent to sample collection and processing have led to the growing interest in the use of saliva as an alternate.⁹ Saliva can be easily and non-invasively collected in a variety of settings. Because salivary catecholamines are poorly correlated with plasma concentrations, they are not considered as useful index of general sympathetic tone.¹⁰ An attractive surrogate biomarker of adrenergic stimulation is salivary α -amylase (SAA). Mostly synthesized by the serous acinar cells of the parotid gland, α -amylase is one of the principal salivary enzymes secreted in response to neurotransmitter stimulation.¹¹ Several investigators^{12,13} have demonstrated that SAA concentrations are closely associated with plasma catecholamine levels, particularly norepinephrine (NE), and are highly correlated with NE changes in response to stress. Numerous other studies^{14–17} have shown that SAA levels increase under a variety of physical and psychological stressors, thus suggesting that SAA may be a useful indicator for activity of the sympathetic nervous system.¹⁸

The promise of SAA notwithstanding 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 way of processing saliva samples in distant, centralized laboratories results in extended reporting times and is fraught with several potential quality failure points related to samples acquisition, storage, transport, processing and reporting.

Recent advances in label-free, nanosensing systems^{19–21} can offer some unique capabilities for extracting biometric information from readily accessible saliva and are creating exciting opportunities to study the psychobiology of the stress response with a precision, temporal and spatial resolution and convenience not afforded by laboratory-based assays or clinical interviews. In particular, nano-sensors fabricated using single-walled carbon nanotubes (SWNTs) are becoming very promising candidates for the development of label-free biosensors^{22–24} since they have shown high sensitivity, and good selectivity with significant suppression of electrical ($1/f$) noise,²⁴ and real-time detection by monitoring the conductance of CNT-FETs. Their large surface area to volume ratio, the low charge-carrier density that is directly comparable to the surface charge density of biomolecules, and the delocalised π orbitals that extend outside of their surface make SWNTs an ideal material for label-free 1-D nanostructure-based biosensors.^{25,26} In recent years, their unique capabilities have been exploited for label-free electrical detection of biomolecules such as proteins,²⁷ antibody–antigen interaction,^{22,28–31} DNA or PNA hybridization,^{24,32–34} aptamers,^{35,36} carbohydrate,³⁷ cells,³⁸ and bacteria.^{39,40}

Here we report the development of a SWNT-based immunosensor for the detection and quantification of SAA. The electrical measurement was applied as a probing method to follow the different steps of the immunosensor processing and as an electronic transduction for the detection of the SAA in phosphate buffer and artificial saliva. This will open new window for justifying people's stressful condition.

Experimental

2.1. Materials

Carboxylated single-walled nanotubes (SWNT-COOH, 80–90% purity and 1.5 to 3 atomic%–COOH content) were obtained from Carbon Solution Inc (Riverside, CA, USA). 1-Pyrenebutanoic acid succinimidyl ester was purchased from Molecular Probes (Carlsbad, CA, USA). Dimethylformamide (DMF), ethanolamine, mercaptohexanol (MCH), SAA (activity:

100 U mg⁻¹), and Tween 20 were obtained from Sigma Aldrich (St Louis, Mo, USA). Anti-SAA monoclonal antibody was obtained from Lee Biosolutions (St Louis, Mo, USA). Solutions were prepared in deionized water prepared by purifying distilled water through a Milli-Q plus (Millipore Inc) ultrapure water system.

2.2. Artificial saliva

Artificial saliva was made by a slight modification of the composition reported by Vahed *et al.*⁴¹ It consisted of 0.6 g l⁻¹ Na₂HPO₄, 0.6 g l⁻¹ anhydrous CaCl₂, 0.4 g l⁻¹ KCl, 0.4 g l⁻¹ NaCl, 4 g l⁻¹ mucin and 4 g l⁻¹ urea dissolved in deionized water, adjusted to pH 7.2, sterilized by autoclaving and stored in the refrigerator until use.

2.3. Immunosensor fabrication

2.3.1. Microelectrodes—An array of 16 pairs of 200 µm by 200 µm gold microelectrodes separated by 3 µm gap were microfabricated on a 1 cm by 1 cm highly doped p-type silicon (100 oriented) wafer using standard lithography and lift-off process as described previously.⁴² In brief, 100 nm of SiO₂ were initially deposited on the Si wafer using chemical vapor deposition (CVD) and the electrode area was defined by photolithography followed by e-beam evaporation of 20 nm thick Cr adhesion layer and 180 nm thick Au layer and finally lift-off to define the electrodes (Fig. 1(a)).

2.3.2. SWNTs solubilization and alignment—A uniform suspension of SWNT-COOH in DMF was prepared by sonicating a 0.1 mg ml⁻¹ dispersion for 90 min followed by centrifugation at 31 000g for 90 min and collecting the supernatant. The suspended SWNTs were aligned across a pair of gold microelectrodes separated by 3 µm on Si/SiO₂ substrate, micro-fabricated as described above using AC dielectrophoresis (DEP) as follows. The electrode pattern was cleaned with piranha solution (70 vol% H₂SO₄/30 vol% H₂O₂) for 5 minutes, rinsed with water and then dried under a stream of nitrogen. A 1 µl drop of the carbon suspension was placed between the gap and an AC electrical field of 3 V_{p-p} at a frequency of 4 MHz was applied for a few seconds, washed with deionized water and then blow-dried with nitrogen. The aligned SWNTs were then annealed at 300 °C for 1 h under 95% of N₂ and 5% of H₂ atmosphere to remove DMF residues and improve the contact resistance between the gold electrodes and SWNTs.

2.3.3. Antibody immobilization—The SWNTs were functionalized with anti-SAA monoclonal antibodies by the protocol described previously.⁴³ In brief, the process comprised of incubating the sensor device prepared in Section 2.3.2. with 6 mM solution of 1-pyrenebutanoic acid succinimidyl ester (PASE) in dry DMF for 1 h at room temperature followed by extensive rinsing with DMF to remove excess PASE and then water to remove residual DMF. The PASE-functionalized SWNTs were then incubated with anti-SAA monoclonal antibody (50 µg ml⁻¹ in 10 mM, pH 7.2 phosphate buffer, designated as PB from here on) for 3 h at room temperature, rinsed three times with phosphate buffer to wash away weakly bound antibodies, incubated with ethanolamine (100 mM, in pH 8.5 buffer) for 30 min to passivate the unreacted succinimidyl ester groups followed by 0.01% Tween 20 to prevent any non-specific binding sites on SWNTs, and subsequently washed with deionized water followed by PB. For the detection of SAA in artificial saliva, the device was incubated with 2 mM mercaptohexanol (MCH) in buffer for 30 min and then washed several times with water and phosphate buffer.

2.4. Device characterization and sensing

Sensors were characterized in terms of their current–voltage (*I*–*V*) response measured using a potentiostat (Model 1202A, CH Instruments, Inc., TX, USA) with 20 µl drop of PB on the

sensor. The voltage was swept from -1 V to $+1$ V and the current was recorded. The device resistance was measured as the slope of I - V near zero voltage in the linear range of ± 100 mV. Resistance changes of the sensor upon exposure to different SAA concentrations in either 10 mM PB or spiked artificial saliva were measured in wet condition (with 10 μ l drop of PB on the sensor) after the sensor was incubated in respective analyte solution for 10 minute and washed three times with PB.

Results and discussion

Because of their superior electrical properties and extreme sensitivity to environments involving physical/chemical adsorption, as a result of surface arranged carbon atoms and the delocalised π orbitals that extend outside of their surface, SWNT is an excellent candidate for 1-D semiconductor conductance channel of an FET sensor. Typically, SWNT-based sensor devices have utilized either a single SWNT or a network of SWNTs as the gate material between the source and the drain. The techniques used for fabricating single SWNT-based devices ranged from manipulating carbon nanotubes onto pre-patterned electrodes by an atomic force microscope,⁴⁴ random dispersion of suspended carbon nanotubes onto pre-patterned electrodes^{45,46} and lithographically patterning catalyst (as carbon nanotube nucleation sites) on electrode.^{47,48} These methods while adequate for demonstrating the operational characteristics of individual devices have low throughput and limited controllability and hence are unattractive for scaling up to high-density sensor arrays and for mass-production. Sensor devices based on SWNTs network offer a simple, cost-effective and scalable alternative to single SWNTs as they can be prepared by drop casting of SWNTs solution or suspension on prefabricated microelectrodes. However, the inability to control the nanotubes positioning, reduced sensitivity, and slower response and recovery times for the sensors are the limitations of the devices fabricated by drop-casting alone. Alternating current dielectrophoresis (DEP) in conjunction with drop-casting mitigates these problems aligning nanotubes nearly perpendicular to the electrodes, and depending on the frequency, even detangling them into smaller bundles and improving the device on-off characteristic.⁴⁹ In this work, carboxylated SWNTs (60% semiconducting and 40% metallic) were suspended in DMF and AC dielectrophoretically aligned across a pair of gold electrodes 3 μ m apart using 3 V_{p-p} and 4 MHz ac field for less than 5 s followed by annealing under 5% H₂ and 95% N₂ environment at 300 °C for 1 h. SEM and AFM images of the assembled device (Fig. 1(b) and 3(a)) showed that the suspended nanotubes were aligned parallel to one another in the electrical field direction with good overlap on the gold electrodes and had an average diameter of 2.3 nm (equivalent of 2–3 SWNTs). Current vs. voltage (I_{SD} vs. V_{SD}) measurement of SWNTs aligned on gold electrodes before and after annealing revealed that annealing was essential to have the device with a good Ohmic contact (Fig. 1(c)). Measuring the source-drain current (I_{SD}) as a function of the gate voltage (V_{GS}) revealed the SWNTs connecting the source and drain to be p-type with a modest on-off ratio of between 2 and 3 (Fig. 1(d)). The low on-off ratio can be attributed to the presence of bundled and metallic nanotubes in the device.⁵⁰

Covalent and non-covalent methods are the two common techniques for biofunctionalization of SWNTs. Amide bonding between the amine groups of biomolecules and carboxylic groups on SWNT sidewalls is the most common method for covalent binding of biomolecules onto SWNTs. However, this technique can have adverse effect on both SWNTs and biomolecules. The oxidation of SWNTs to form carboxylic acid groups on the sidewalls and ends can change SWNTs geometric and electronic properties while the use of harsh reagent carbodiimide can affect the biomolecule activity.^{19,51} Non-covalent biofunctionalization of SWNTs can be achieved by either direct adsorption of biomolecules onto SWNTs or to an adsorbed bifunctional molecule to which the biomolecule is then conjugated with high degree of control and specificity. In this work we used a bifunctional linker, PASE, to first modify the SWNTs non-covalently and then linked the biomolecule/antibody covalently by the reaction between

the *N*-hydroxysuccinimide of PASE and amine of the protein. Fig. 2 shows the schematic of the various steps in the anti-SAA modified SWNT SAA immunosensor fabrication and corresponding *I*–*V* characteristics of the SWNTs device after each modification step. As shown in the figure the current decreased or resistance increased significantly after the PASE coating and antibody conjugation steps. This was in accordance with the literature⁵² and attributed to scattering potential generated by the immobilization of the molecules and thereby decreasing the hole mobility⁵² and/or electron donation from these molecules to the nanotubes resulting in charge carrier reduction in the p-type SWNTs.

As an additional confirmation of the successful immobilization of anti-SAA antibodies, AFM studies were performed on unfunctionalized/bare and anti-SAA functionalized SWNTs (Fig. 3). The images revealed an increase in the SWNT height from the initial value of ~2.3 nm for bare to 12 nm after modification with antibodies, confirming successful immobilization of anti-SAA.

Fig. 4 shows the response $[(R - R_0)/R_0]$, where *R* is the resistance after exposure to SAA and *R*₀ is the initial sensor resistance] of the immunosensor as a function of SAA concentration in phosphate buffer. As shown, the immunosensor had a wide dynamic range (up to 1000 μg ml⁻¹) that was linear from 10 to 750 μg ml⁻¹. The sensitivity (slope) in the linear region was $3 \times 10^{-4} \mu\text{g}^{-1} \text{ ml}$ ($r^2 = 0.968$). The estimated limit of detection (defined as $\text{LOD} = 3S_D/m$, where *m* is the slope of the linear part of the calibration curve and *S*_D is standard deviation of the blank measurement) was ~6 μg ml⁻¹.

A series of experiments were performed to investigate the selectivity/specificity of the sensor. The response of negative control, sensors in which SWNTs were not modified with antibodies but were coated by blocking agent Tween 20 by incubation with 0.01% Tween 20 for 30 min, to SAA was evaluated to confirm the response was not a result of SAA binding to SWNTs as proteins have a high binding affinity to bare SWNTs that are hydrophobic. A $1.4 \pm 1.1\%$ normalized resistance change over 10 to 750 μg ml⁻¹ SAA concentration confirmed the response to be the result of binding of SAA antigen to anti-SAA antibody (data not shown). Next the specificity of antibody was investigated by checking the response of the immunosensor (with anti-SAA antibody) to human serum albumin. Once again, a response of only $3.9 \pm 1.5\%$ for 1 mg ml⁻¹ human serum albumin when compared to $25.8 \pm 3.1\%$ for the same concentration of SAA (Fig. 5) demonstrated the sensor selectivity against one of the most abundant protein in body. However, the sensor failed the matrix selectivity test. When exposed to artificial saliva, the matrix in which SAA has to be measured, a response of $17 \pm 4\%$ was observed (Fig. 5). Upon systematic experimental investigation and the literature search the cause for this response was identified to be emanating from the non-specific interaction of mucins in the artificial saliva with gold electrodes. Mucins are a large group of glycoprotein molecules that are reported⁵³ to adsorb extensively on nonpolar/hydrophobic and negatively charged, such as –COOH, hydrophilic surfaces, even though it is negatively charged. On the other hand, the adsorption is low on uncharged polar, such as –OH, group. Mercaptohexanol is widely used in the field of biosensors to alleviate non-specific binding of proteins on gold⁵⁴ and has been shown to play a critical role in sensitizing and stabilizing of CNTs-based sensor in aqueous medium.³³ The latter was attributed to MCH adsorption on Au shifting the Au Fermi level towards valence band of CNTs and therefore decreases the carrier injection barrier.³³ Upon incorporation of MCH treatment as the final step in immunosensor preparation, the response of the sensor to artificial saliva decreased to only 1.4% (data not shown). Fig. 6 shows the calibration plot for the modified device, *i.e.* with MCH blocking of gold electrodes. As shown, the sensor exhibited a wide dynamic range (up to 1000 μg ml⁻¹) that was linear from 10 to 750 μg ml⁻¹ (1 to 75 U ml⁻¹). The sensitivity (slope) in the linear region was $2.4 \times 10^{-4} \mu\text{g}^{-1} \text{ ml}$ ($r^2 = 0.975$). The estimated limit of detection (defined as $\text{LOD} = 3S_D/m$, where *m* is the slope of the linear part of the calibration curve and *S*_D is standard deviation of the

blank measurement) was $\sim 7.8 \mu\text{g ml}^{-1}$. The limit of detection and the dynamic range of the SWNT-FET devices in artificial saliva were comparable to phosphate buffer.

Conclusions

In summary, we have shown that the salivary α -amylase can be easily detected by SWNT-FETs nano-sensor. The nano-sensors based on carbon nanotube were modified with whole monoclonal anti-salivary antibody that can selectively detect the salivary α -amylase either in phosphate buffer or in artificial saliva. Our immunosensor shows linear detection range from $10 \mu\text{g ml}^{-1}$ to 1 mg ml^{-1} (1 to 100 U ml^{-1}) salivary α -amylase in both phosphate buffer and artificial saliva. The limit of detection of salivary α -amylase in phosphate buffer and artificial saliva was $6 \mu\text{g ml}^{-1}$ (0.6 U ml^{-1}) and $7.8 \mu\text{g ml}^{-1}$ (0.78 U ml^{-1}), respectively. Therefore, our immunosensor based on SWNT-FETs devices is a promising candidate for the development of an integrated, high-throughput, fast response, low cost, and real-time biosensor that can provide a cost effective point-of-care assessments of this salivary stress biomarker. It could satisfy the clinical range of $19\text{--}308 \text{ U ml}^{-1}$ (ref. ⁵⁵) and can be further applied to another salivary biomarker, which should open a new window to enlighten the human stress.

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Fig. 1. (a) Optical image of the 16-electrodes array chip; (b) SEM image of AC dielectrophoretically aligned carbon nanotubes; (c) I_{DS} vs. V_{DS} of AC dielectrophoretically aligned SWNTs network before (■) and after (●) annealing; (d) I_{DS} vs. V_{GS} of SWNTs network device after annealing.

Fig. 2.
 (a) Schematic diagram showing the fabrication of the α -amylase immunosensors, (b) I_{DS} vs. V_{DS} for the bare SWNTs network (■), after functionalization with PASE (●), immobilization of the anti-SAA antibody (▲), and blocking with ethanolamine and Tween 20 (▼).

Fig. 3.
AFM image of the bare SWNT (a) and after anti-SAA immobilization (b).

Fig. 4.
Immunosensor calibration in phosphate buffer.

Fig. 5.
Immunosensor selectivity.

Fig. 6.
Immunosensor calibration in artificial saliva.