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Paper-Based Analytical Biosensor Chip Designed from Graphene-Nanoplatelet-Amphiphilic-di-block-co-Polymer Composite for Cortisol Detection in Human Saliva

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Abstract

Cortisol has been identified as a biomarker in saliva to monitor psychological stress. In this work, we report label-free paper-based electrical biosensor chip to quantify salivary cortisol at a point-of-care (POC) level. A high specificity of the sensor chip to detect cortisol with detection limit of 3 pg/mL was achieved by conjugating anti-cortisol antibody (anti-CAB) on top of gold (Au) microelectrodes using 3,3′-Dithiodipropionic acid di(N-hydroxysuccinimide ester (DTSP) – as self-assembled monolayer (SAM) agent. The electrode design utilized poly(styrene)-block-poly(acrylic acid) (PS_{67}-b-PAA_{27}) polymer and graphene nanoplatelets (GP) suspension coated on filter paper to increase the sensitivity of immune response. Biosensor chip was then integrated with lab-built low-cost miniaturized printed circuit board (PCB) to provide electrical connection and to wirelessly transmit/receive electrical signals using MATLAB. This fully integrated proposed handheld device successfully exhibited a wide cortisol-detection range from 3 pg/mL to 10 μg/mL with a sensitivity of 50 Ω $(\text{pg mL}^{-1})^{-1}$. The performance of the proposed cortisol sensor chip was validated using an enzyme-linked immunosorbent assay (ELISA) technique with a regression value of 0.9951. The advantages of the newly developed cortisol immune biosensor over previously reported chips include an improved limit of detection, no need of additional redox medium for electron exchange, faster response to achieve stable data, excellent shelf life and its economical production.

1. Introduction

Psychological stress affects every part of life [1]. Chronic acute stress is harmful and ultimately could lead towards more serious diseases e.g. cardiovascular disease [2], long-term depression and anxiety [3–5], diabetes [6], Alzheimer [7,8], obesity [9,10], other disorders [11,12,13]. Most Americans are suffering from moderate to high stress, with approximately 44 % reporting that their stress levels have increased to a significant level over the past five years [14]. Top four sources of stress include financial status, unemployment, work-related stress, lack of work-life balance, family responsibilities etc [15]. The most common symptoms of stress are irritability (angry), feeling of anxiousness or nervousness, fatigue, depression (sadness) and lack of interest (motivation/energy) [14,15]. Current methods to diagnose stress include interviews and counseling [16,17], an electroencephalogram [18,19], an electrocardiogram (electrical signals) [20,21], and/or self-questionnaires [22,23]. However, these methods are purely qualitative and can introduce bias in the diagnosis. Moreover, these techniques require longer times to process and analyze the data and need access to bulky equipment to perform the tests.

Cortisol, a hormone commonly released upon stress, is a clinically proven biomarker to play an important role in the regulation of various physiological processes such as blood pressure, glucose levels, and carbohydrate metabolism [24]. While excess cortisol levels have been shown to contribute to the development of Cushing's disease with the symptoms of obesity, fatigue and bone fragility [25], decreased cortisol levels lead to Addison's disease which is manifested by weight loss, fatigue, and darkening of skin folds and scars [26]. The most dominating effect on cortisol variation comes from psychological/emotional stress, which is why cortisol is popularly called the "stress-hormone" [27]. Several clinical studies indicated day-to-day variation in cortisol among healthy individuals and its relation to the time of saliva sampling,

stress and fatigue [28]. Results showed a variation in cortisol values between participants but also within individuals correlated with various factors. Generally low cortisol levels in the morning indicated sleepiness at awakening and anxiety, exhaustion, and poor health the day before. High evening levels of cortisol were associated with symptoms of stress and related psychological disorders [29].

The most current strategies for the estimation of free cortisol are limited to laboratory techniques that are laborious, time-consuming, require large sample volume, expensive, and cannot be implemented at POC level. While the link between elevated cortisol levels in bodily fluids such as saliva, and anxiety have been identified, an effective measurement method for cortisol level detection involves enzyme-linked immunosorbent assay (ELISA) [30,31], electrochemiluminescence immunoassay (ECLIA) [32,33], chromatography [34,35] and surface plasmon resonance (SPR) [36,37]. However these methods require laboratory equipment, space and expertise as well as include extensive delays during sample preparation and results processing. Moreover, current techniques only provide a snapshot of the cortisol levels of samples submitted in a diagnostic lab and do not provide a true representation of the cortisol variations that a specimen undergoes in an environment that triggers cortisol generation or suppression. Hence, real-time and continuous monitoring of cortisol levels is required to obtain valuable information that could assist doctors in better diagnosis and treatment of cortisol-related conditions.

Electrochemical immunosensing is based on the principle of measuring the changes in electrical properties of a conductive material due to the adsorption of an analyte on the surface functionalized with antibodies. In recent years there have been reports of electrochemical sensing of cortisol using electrochemical impedance spectroscopy [38–40] and cyclic voltammetry (CV) [41,42] based electrochemical immunosensor. Immunosensor have also been integrated with microfluidic

systems for POC sensing [41,43]. To demonstrate electrochemical procedure, additional electrolyte solution (PBS (10 mM, pH 7.4) containing 5 mM $Fe(CN)_6^{3-/4-}$) has been used as redox medium. Reported techniques required incubation of saliva sample for about 25-35 min with anti-cortisol antibody (anti-CAB) covalently attached to DTSP (3,3′-Dithiodipropionic acid di(N-hydroxysuccinimide ester) - SAM (self-assembled monolayer) on Au-IDμEs (gold based interdigited micro-electrodes). A low detection limit of 0.36 pg/mL [38–40] and 10 pg/mL [41,42] were reported for EIS and CV techniques, respectively.

Herein, we present a paper based, low-cost, fast and efficient POC biosensor which operates in real-time fashion wirelessly. In our approach, we integrated the sensor chip with handheld miniaturized electronics printed circuit board (PCB) and without utilizing any additional electrolyte solution after incubating the saliva sample with sensor chip. Moreover, we introduced a new dynamic sensing capability of Au electrodes based on square rings which produced high electrical field within neighboring electrodes. Biosensor strip is comprised of a layer-by-layer assembly process. Six standard samples with known cortisol concentration (0.00003, 0.03, 0.06, 0.2, 0.6, 1.5, and 4 μg/mL) and eight saliva samples collected voluntarily from 8 healthy subjects with unknown cortisol concentration have been utilized to determine the potential usefulness of the proposed paper-based electrical biosensor chip for estimation of cortisol detection range, detection limit, sensitivity and accuracy.

2. Results and Discussion

2.1. Development of Layer-by-Layer Electrical Biosensor Chip

Base material for the cortisol sensor was developed by spin coating a composite of graphene nanoplatelets (GP) and poly(styrene)-block-poly(acrylic acid) polymer (PS_{67}-b-PAA_{27}) over Whatman filter paper (3 cm diameter). Spin coating technique was selected over conventional drop cast method to produce a thin and uniform layer of graphene-polymer composite with minimum surface defects. GP are carbon materials having "platelet" morphology and multifunctional properties. Each nanoplatelet of GP has a width of about 6-8 nm and length of ~25 μm. This morphology makes these platelets effective at providing connecting barrier properties. In addition, their pure graphitic composition provides significantly high electrical and thermal conductivity. GP has been used to infer differential electrical conductivity due to zero-overlap semimetal nature with both holes and electrons as charge carriers while PS_{67}-b-PAA_{27} makes an amphiphilic bridge between GP units and allows better spin coating on filter paper. Use of conductive polymers have been field changing in designing electrical sensors due to presence of free mobile electrons. But excess of such base material could create sensitivity problems. Attempts were made to minimize this by doping small amount of conductive polymer in nonconductive polymer. Doping of graphene nanoplatelets in polymeric matrix was used as an alternative and sophisticated method on improving the electron mobility along the sensor base with gold deposition. The non-covalent π-π stacking interaction was utilized as mode of composite formation between the two dimensional graphene platelets and multiple repeat units of poly(1-phenylethylene) functionalities of the diblock-copolymer as presented in Fig. 1A. Without having graphene-polymer composite as base layer, the electrode design system coated

directly on filter paper was supposed to produce average results with longer time of response (20 min) to identify the cortisol concentration in saliva. Addition of polymer made sure to provide a composite medium for improved edge-over-edge arrangements for graphene nanoplatelets and additional benefit of smoother spin coated surfaces. Spin-coating technique using polymer-GP composite could bring more uniformity on paper which helped the deposition of Au electrodes (40nm thickness) with minimum chances of electrode dis-connect due to un-even surfaces otherwise. Uniformity optimization was done by varying the speed (RPM) to achieve a smoother and uniform layer of graphene-polymer composite. Graphene-polymer composite was coated on filter paper for making it suitable to absorb water component of saliva samples. Strategy of using graphene nanoplatelets was chosen specifically for the purpose of not allowing it to make a water-tight composite with PS-b-PAA polymer, rather keeping it edge-over-edge arrangement after spin coating. Polymer-graphene platelet with such arrangements would be channeling water component of the samples but restricting the percolation of other components including cortisol.

Square rings based on Au material were fabricated as innovative design electrode for cortisol detection to achieve better accuracy and sensitivity. The small electrode setup is a key step to the fabrication of the sensor chip. This new design simply based on square rings which increased the sensitivity and introduced dynamic sensing mechanism with actual sensing area of 1 x 1 mm². Optimized structural dimensions of sensor were verified using SEM studies. The inter electrode spacing between square electrodes was 45 μm along with individual electrode width of 45 μm. The total area covered by electrodes was 495 μm^2 (11 x 45 μm), total interspacing between electrodes of 450 μm^2 (45 x 10) and total covered sensing area of approximately 1 mm^2. This configuration ensures high conductivity across the rings and provides stable electrical field across the cortisol sample. Electron beam evaporator was used to produce highly sensitive Au

electrodes directly on graphene-polymer thin layer which was intelligently grafted on filter paper using spin-coating technique (Fig. S1). A smaller electrode size was used to provide only enough space to conjugate DTSP-SAM/anti-CAB for the complete attachment to Au electrode leaving smaller areas of no anti-body attachment which may create non-specific cortisol binding. On the other hand, reducing the size and thickness of Au electrode decreases the electrical field on the Au surface which finally limits the detection procedure. To improve upon it, one way was to increase either the concentration or volume of anti-CAB/DTSP-SAM or to develop a dynamic sensing electrode platform with high electrical field surrounded by neighboring Au electrodes. Therefore, we introduced Au electrode of thickness 40 nm with interspacing distance of 45 μm and fabricated in square ring shape which enhanced the electrical field area and to provide enough space to anti-CAB for covalent attachment with DTSP-SAM conjugation. Scanning electron microscopy (SEM) was employed to characterize the surface characteristics of the covalently conjugated anti-CAB on biosensor chip as shown in Fig. 2b. High resolution SEM image revealed the sensing area of the biosensor utilized during measurements. Anti-CAB was covalently linked on Au electrode *via* DTSP/SAM (Fig. S1b). Furthermore, atomic force microscopy (AFM) was used to image the clusters formed after salivary cortisol solution interacted with anti-CAB/DTSP-SAM/Au electrode (Fig. 1c and Fig. S1c).

2.2. Raman Spectroscopic Analysis of Anti-CAB with Cortisol and Saliva Sample

Success of anti-body conjugation on gold electrodes could be characterized by chemical evaluation of sensor surface before and after antibody conjugation and post addition of cortisol using Raman spectroscopy and imaging. The location of antibody accumulation onto the biosensor chip must be highly specific to initiate a proper change in electrical signatures for

cortisol recognition. Since antibody samples are expected to collect and conjugate onto the deposited gold electrode, we can assess the deposition *via* spectral means. Given that most proteins generate a fluorescent signal, and the cortisol structure has intrinsic wavenumber peaks, a Raman analysis was performed on varying formulations of the sensor. Raman micrographs of the cortisol biosensor were measured at 1600 cm^{-1} (the characteristic graphene wavenumber). Fig. 2c, Fig. 2d and Fig. 2e show biosensors treated with antibody cortisol on Au electrodes, with known standard cortisol concentration (0.03 μg/mL) and with human saliva having unknown cortisol concentration (sample 2) respectively. White clusters (indicated by a white arrow) on each sensor variation are indicative of the chromophoric amino acids, i.e. phenylalanine, tyrosine, and tryptophan present within the antibodies (Fig. 2c). As expected, these clusters aggregate on the deposited Au electrode due to covalent conjugation. When we compare this image with biosensor chip tested with standard cortisol sample, it was found that gold electrode (Fig. 2d) had a much higher Raman intensity signal independent from antibody signatures. This strong intensity is presumably due to the extensive SP^2 carbon hybridization from cortisol's native structure. Fig. 2e displays a Raman micrograph treated with human saliva which shows a surface contaminated with human salivary proteins, as indicated by an even spread of fluorescent protein clusters. To distinguish the antibody deposition and treatment of human saliva onto sensor chip, the surface chemistry of the graphene background and deposited Au electrode were spectrally compared and electrodes clearly generated a higher overall intensity signature due to the presence of chromophoric amino acids in the antibody clusters and salivary proteins. In addition to intensity, the Diamond (D band) and Graphitic peak (G band)[44], sp^3 and sp^2 carbon hybridization respectively, were clearly distinguishable in both the measurements (Fig. 2f). Here, the G band in the electrode spectrum had a lower wavenumber while maintaining a higher G and

D band intensity ratio (I_G/I_D ratio) than the graphite background. This difference is a known trait indicating an increased amount of π-π interactions presumably caused by cortisol complementing amino-acid residues on the antibody's innate structure [44].

2.3. ELISA and Analytical Performance of Cortisol Biosensor Chip

Cortisol samples were stored at -20 °C to maintain their biological integrity. All the samples were thawed to room temperature for further use to detect cortisol concentration using both ELISA and electrical biosensor chip. ELISA technique was performed using a 96-well plate with respect to six known standard concentrations (0.00003, 0.03, 0.06, 0.2, 0.6, 1.5, and 4 μg/mL) and results are presented in Fig 3a. Sample concentrations were also tested on biosensor chip. Gold electrodes functionalized with a dithiobis (succinimidyl propionate) (DTSP) self-assembled monolayer (SAM) was used to develop an ultrasensitive electrical cortisol biosensor chip. Both standard cortisol samples and human saliva samples were analyzed using functionalized biosensor chip at RT. For each sample, the anti-CAB/DTSP-SAM / Au electrode was initially incubated with a sample solution for 12 min. A washing step using 25 μL of PBS was performed to remove the unbound cortisol from the sensor surface. All the measurements were performed in triplets and recorded in real-time fashion. With incubation time of 12 min, the data achieved its steady state response. This is a significant improvement over the previously reported studies, where sensors were generally incubated for 25-35 min [38–42] to achieve the steady state response. This makes the present strategy at least two fold quicker in achieving optimum cortisol detection. Data presented in Fig. 3b revealed that resistance increased with increase in cortisol concentration which further confirmed the presence of higher concentration of cortisol in the standard sample. A covalently immobilized cortisol antibody based DTSP-SAM/Au electrode

exhibited linear behavior in the cortisol concentration range of 3 pg/mL to 10 µg/mL. Fig. 3b showed the linearity curve generated after data normalization. In Fig. 3b, commercial standard of cortisol samples were tested. This does not include other constituents present in real human saliva. Electrical resistance of each sample represents the change of resistance before and after treating the cortisol sample with sensor chip.

The collected human saliva samples were either freshly used or stored at -20 ºC to maintain its biological characteristics. Before sensing, the saliva samples were maintained to room temperature and centrifuged at 3500 rpm for 15 min to extract saliva from salivettes. The separated saliva was then pipette out and kept at -20 ºC when not in use. Eight collected samples of saliva were analyzed in similar fashion as standard cortisol and results were measured in terms of change in electrical resistance (Fig. 4) after treating 5 µL of each sample (sample # 1, 2, 3, 4, 5, 6, 7 and 8) on covalently conjugated anti-CAB/DTSP-SAM/Au electrode. Data was normalized and cortisol concentration was extracted after comparison with resistance of known cortisol concentration samples (0.00003, 0.03, 0.06, 0.2, 0.6, 1.5 and 4 µg/mL). Normalized data for cortisol in saliva (5 µL) is tabulated in Table 1. Variation of data has been observed based on saliva samples used at the time of measurement (Fig. 5). Data presented in Fig. 5a is finally calibrated as a function of cortisol concentration in eight saliva samples in logarithmic scale. Sample information is reported in terms of biosensor resistance after treating saliva with sensor chip and the estimated amount of cortisol in the analyzed sample. Electrical resistance of Fig. 5b represents the biosensor chip resistance with saliva on it. Biosensor reveals the estimated amount of cortisol present in Saliva. This high value of resistance also included the base resistance (140-150 Ω) of the biosensor chip coated with DTSP-SAM/Anti-CAB on Au electrodes. Electrical biosensor chip exhibited the detection range of 0.018-4.552 ng/mL based on eight tested samples

with regression value of 0.993. These values fit well in the previously determined detection range of 3 to 10 μg/mL (R^2 0.995) for known cortisol concentration sample. These measurements were made in triplicate and an average of these was used to estimate the cortisol concentration in saliva. We successfully achieved to assess the cortisol level down to 3 pg/mL based on 5 μL sample volume of saliva. It is noted that data presented in this study required total of 12 min incubation time of sample treatment with anti-CAB/DTSP-SAM/Au electrode to gain the optimum binding of the antibody-cortisol and cortisol biomarker present in saliva.

2.4. Electrical Measurements and Assembling of Low-Cost PCB based Electronics

Electrochemical impedance spectroscopy (EIS) and cyclic voltammetry (CV) are the standard techniques utilized to detect a bioanalyte on the sensor surface. A low-cost, easy-to-use, simple and re-configurable miniaturized electronics printed circuit board (PCB) was developed to quantify the biosensor chip before and after treating saliva sample with immobilized antibody cortisol surface. DC voltage of 5V was applied directly to the microcontroller embedded in PCB which then transferred signals to the Bluetooth device and LCD. Electrical signals were monitored on Matlab in real-time fashion. Readings were recorded in in terms of electrical resistance. Electrical resistance can easily be displayed into current and voltage gain if required by reconfiguring the program for microcontroller. It was different from previous reports where generally EIS and CV were utilized to detect the concentration of salivary cortisol and electrolyte suspensions used to create the redox medium configuration at Au electrode to monitor the change. Herein, without adding any electrolyte, saliva was directly added to the biosensor chip and the change of resistance was recorded after cortisol interacting with antibody-cortisol attached to DTSP/SAM/Au electrode.

We anticipate that this low-cost PCB based electronics with covered area of 2 × 3 inch² can easily be assembled in handheld device with on-screen display and readout data can easily be shared wirelessly with clinicians and psychiatrists using portable devices such as tablets, smart phones and laptops.

2.5. Validation of Results and Shelf Life

To validate the results obtained from the electrical biosensor chip, saliva samples were tested for cortisol estimation using ELISA with Cortisol Luminescence Immunoassay (RE62011) kit. All experiment was carried out at RT. Saliva samples from eight subjects were tested in set of triplicate and the average value was then utilized to determine the final cortisol concentration as shown in Fig. 6. Since both methods have different transduction techniques to detect cortisol concentration, the outcome was in different magnitudes. To compare and correlate these responses, one had to be scaled to the other. All values of human salivary cortisol concentration obtained from the electrical biosensor calibration curve were adjusted by a factor of 4.1 to compare them with values obtained using ELISA assay. A good correlation was achieved between the two sets of data, thus enabling quantitative validation of the cortisol biosensor as shown in Fig 6a. Both techniques (ELISA and Biosensor) showed comparable results with regression value of 0.9951 (Fig 6b), thus endorsing the authentication of the POC cortisol-immunosensing sensor. Finally, shelf life of the sensor conjugated with Anti-CAB before and after testing with standard cortisol sample of 0.2 ng/mL was studied. Measurements were taken at intervals of 1 week at 4 ºC as shown in Fig 7. The results of the study showed that Au electrodes with anti-CAB/DTSP-SAM (Fig. S3) were stable for almost 4 weeks and beyond that

time the electrochemical response reduced. Also, there was very less variation even after 6 weeks without conjugation of antibody cortisol on the sensor.

3. Experimental

Electrochemical immunosensing has emerged as a promising label-free detection technology alternative to optical detection. It is based on the principle of measuring the changes in the electrical properties of a conductive substrate due to the adsorption of an analyte on the surface functionalized with antibodies sensitive to the analyte. The high specificity of the biosensor strip to detect cortisol level was achieved by coating the filter paper with anti-cortisol antibody conjugated using 3,3′-Dithiodipropionic acid di(N-hydroxysuccinimide ester (DTSP)- self-assembled-monolayer (SAM) to gold electrodes deposited on top of multiple layers of composite containing, poly(styrene)-block-poly(acrylic acid) (PS_{67}-b-PAA_{27}) and graphene nanoplatelets (GP).

3.1. Preparation Graphene-Polymer Layer on Filter Paper

GP (10 mg/mL) was mixed with PS_{67}-b-PAA_{27} amphiphilic polymer suspension (1.0 mg/mL) in 10 mL of water. GP and PS_{67}-b-PAA_{27} were purchased from STERN Chemicals, USA and Sigma-Aldrich, Saint Louis, MO, USA, respectively. Mixture of graphene-polymer composite was probe sonicated at amplitude 4, with pulse rate of 5 sec on and 1 sec off for 4 h with intermittent cycles of 30 min. Process was completed at controlled temperature of 37 °C. To increase the uniformity of graphene-polymer suspension over filter paper, a spin coating technique was utilized at 200 rpm for 45 sec followed by 500 rpm for 180 sec (Fig. S2). Samples

were dried using nitrogen spray gun for 10 min and were further dried overnight under biosafety hood at room temperature to maintain sterility.

3.2. Fabrication of Au-Electrodes and Surface Characterization

Micro-electrodes with thickness of 40 nm were fabricated to increase the sensitivity and dynamic range of sensing mechanism. Au material (Kurt J. Lesker Company, Jefferson Hills PA) was utilized during evaporation. This configuration ensures high conductivity and mechanical reliability for stable electrical signal transfer as well as an actual sensing covered area of 1 × 1 mm^2. The design was initially created in AutoCAD 2014 and shadow mask (Photo Sciences Inc., Torrance CA) was then developed on nickel brass with thickness of 0.025 mm. Electron beam evaporator was finally used for the fabricating Au electrodes using shadow mask directly on thin layer of graphene-polymer.

3.2.1 Scanning Electron Microscopy: For SEM imaging, the developed biosensors were mounted on SEM sample holder using a piece of carbon tape. The images were obtained using a Hitachi (Schaumburg, Illinois) S-4700 SEM with Oxford Instruments (Abingdon, Oxfordshire). Imaging was performed in a long working distance mode, with an accelerating voltage of 10kV, extracting current of 10 μA, and working distance of 25 mm for scanning.

3.2.2 Atomic Force Microscopy: For AFM analysis, a square of biosensor strip was pasted on a glass slide using a double-sided tape. AFM images were obtained using an Asylum Cypher (Santa Barbara, CA, USA) with tapping mode and phase mode. The surface of strip was scanned in air using OMCL-AC160TS cantilevers at a set point of 0.63 V, 1Hz scan rate, and drive frequency of 336.3 kHz.

3.3. Antibody Conjugation on Gold Electrode

DTSP, sodium borohydride ($NaBH_4$), monoclonal anti-cortisol antibody (anti-Cab), Ethanolamine (EA) and Glycine were purchased from Sigma Aldrich. Phosphate-buffered saline (PBS) solution (10 mM, pH 7.4) was prepared by dissolving one PBS tablet in 200 mL. 2 mg/mL of DTSP solution was prepared in acetone and mixed with $NaBH_4$ (10 mg/mL in DI water) and pipette onto sensor surface. Biosensor chip with DTSP + acetone solution was incubated for 1.5 h for formation of SAM. Sensor was rinse with acetone to remove unbound DTSP followed by second rinse with PBS buffer for 3 times to remove any acetone. 65 µL of 1 µg/mL antibody was prepared in PBS (10 mM, pH 7.4). SAM (DTSP) modified sensor was then incubated with the antibody for another 1 h. This makes covalent bonding of amine group of antibody with the succinimidyl group of DTSP. The sensor was again washed with PBS (10 mM, pH 7.4). Finally, 20 µL of ethanolamine (1%) + glycine (1%) solution in PBS (v/v 9:1) was prepared and used for 10 min to block unreacted succinimidyl group. The sensor was finally dried using nitrogen spray gun and stored in air tight container at 4 ºC for further use.

3.4. Raman Spectroscopy

All Raman measurements were taken on a Nanophoton Raman instrument with a 532nm wavelength laser. For each spectrum, a grating scan was taken over the range of 120-2700 cm^{-1}. Point scans were performed at 0.2% laser power for one minute using a 20× objective. An average of 20 spectra was recorded and averaged per sample. For Raman micrographs, samples were imaged using line scans at 50% laser power and a 5× objective for 20 seconds per scan.

3.5. Saliva Collection Procedure

Saliva sample of each participant was unstimulated which revealed the participants' real metabolic conditions and increased the test accuracy. Initially subjects were asked to rinse mouth with water for 30 sec followed by holding saliva in mouth for 20 sec. They were asked to spit saliva directly into sterilized vial (10 mL). PVDF membrane filter (0.22 μm) from Sigma Aldrich was used to filter the saliva being attached to a syringe (10 mL from Sigma Aldrich) into eppendorf tubes (1 mL) gently and stored at -20 °C to avoid any chemical loss. Before sensing process, the saliva samples were de-freezed to room temperature and centrifuged at 3500 rpm for 15 min to extract saliva from salivettes. The separated saliva was then pipette out and kept at −20 ℃ when not in use. These samples were further used to detect cortisol using electrical biosensor chip and compared with commercial product by ELISA. For each measurement, 5 μL of sample was dropped on sensing area of biosensor strip using pipette. Electrical measurements were demonstrated using a lab-built electronics and real time monitoring of salivary cortisol. Finally, used biosensor strip, syringe, vial and pipette tips were disposed into biohazard waste.

3.6. Miniaturized Printed Circuit Board

Microcontroller (ATmega328P) was reconfigured to control the input voltage of 5V, Bluetooth module (HC-05) and LCD to display the output resistance across biosensor chip. Matlab was used to run the Bluetooth data and recorded it in real-time monitoring. The Bluetooth device used in this study was a commonly used Bluetooth module that has a standard baud rate of 9600 and operates at 2.4 GHz for its transmission with input voltage of 3.3V. To achieve the layer optimization and selecting appropriate electrical connection with respect to design rule checking (DRC), prototype was designed in standard Eagle software. Finally, layout design was printed and integrated with battery and biosensor chip.

3.7. Cortisol Detection using Commercial ELISA Kit

The ELISA technique was performed using a 96-well plate. Six known concentrations (0.0003, 0.03, 0.06, 0.2, 1.5, 4 μg/mL) of cortisol and eight human saliva samples (1, 2, 3, 4, 5, 6, 7 and 8) with unknown cortisol concentration were pipetted into the microliter plate wells with volume of 20 μL of each sample. After 3h incubation at RT (18-25°C) with 100 μL of competition enzyme, wells were emptied, washed with diluted wash buffer and then added with given substrate solution mixture (20 μL). As precautionary measures, well were washed properly, as improperly washed wells would give erroneous results. After 10 min, each well was then measured for 2 sec in Luminometer with Gen 5.0 software. Measurements were tested in triplicate sets and the average value was then utilized to determine the final cortisol concentration.

4. Conclusion

A highly sensitive electrochemical immunosensor has been presented which measure the change in electrical resistance of Au surface electrode functionalized with antibody under influence of different concentration of cortisol present in human saliva. This electrochemical immunosensing platform has been utilized for a detection of cortisol *via* covalent conjugation of Anti-CAB onto DTSP-SAM modified Au electrode. The fabricated electrical biosensor chip successfully exhibited detection range of 3 pg/mL to 10 μg/mL, detection limit of 3 pg/mL and sensitivity of 50 Ω $(pg/mL^{-1})^{-1}$ with the regression coefficient of 0.9951. Results obtained using both methodologies (ELISA and biosensor chip) were comparable within 1.5-3.7% variation. This electrochemical immunosensor could detect cortisol within 12 min and the results correlated well

with ELISA assay. The proposed system has great potential to transfer cortisol data obtained from electrochemical biosensor using Bluetooth and other existing wide-area networks to enable real-time remote monitoring of subjects. The area of simple miniaturized printed circuit board electronics integrated with biosensor chip has great significance for on-site diagnostics and has great prospects of commercialization at low-cost.

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Supporting information

Layer-by-layer schematic description of the fabricated cortisol biosensor chip (Fig. S1), SEM image of electrodes, graphene-polymer (Fig. S2) and AFM image of single Au electrode conjugate with DTSP/SAM/Anti-CAB (Fig. S3) are available as supporting information and free of charge via the Internet at http://pubs.acs.org.

References

(1) Zora, D.; Chloe, E. B.; Alice F-D.; Garth, H. R.; Mack, T. R.; Raymond P. S.; Katherine L. T.; Christopher, M. M.; *J. Open Biomark.* **2008**, *1*, 7–19.

(2) Dimsdale, J. *J. of the American College of Cardiology*. **2008**, *51* (13) 1237–1246.

(3) Chaby, L. E.; Cavigelli, S. A.; Hirrlinger, A. M.; Caruso, M. J.; Braithwaite, V. A. *Behav. Brain Res.* **2015**, *278*, 492–495.

(4) Lovibond, P. F. *J. Abnorm. Psychol.* **1998**, *107* (3), 520–526.

(5) Dass, T.; Foundation, P. *Depression.* **2005**, 1–3.

(6) Magarinos, A. M.; McEwen, B. S. *Proc. Natl. Acad. Sci. U. S. A.* **2000**, *97* (20), 11056–11061.

(7) Yana, M. H.; Wang, X.; Zhu, X. *Free Radical Biology and Medicine*. **2013**, *62*, 90–101.

(8) Zhu, X.; Smith, M. A.; Honda, K.; Aliev, G.; Moreira, P. I.; Nunomura, A.; Casadesus, G.; Harris, P. L. R.; Siedlak, S. L.; Perry, G. *J. Neurol. Sci.* **2007**, *257* (1–2), 240–246.

(9) Yang, L.; Li, P.; Fu, S.; Calay, E. S.; Hotamisligil, G. S. *Cell Metab.* **2010**, *11* (6), 467–478.

(10) Dallman, M. F. *Trends in Endocrinology and Metabolism*. **2010**, *21* (3), 159–165.

(11) DeKloet, E. R.; Joels, M.; Holsboer, F. *Nat. Rev. Neurosci.* **2005**, *6* (6), 463–475.

(12) Cohen, S.; Janicki-Deverts, D.; Miller, G. E. *J. of American Medical Association.***2007**, *298* (14), 1685–1687.

(13) McEwen, B. S. *European J. of Pharmacology*. **2008**, *583* (2-3), 174–185.

(14) American Psychological Association. **2015**, 1–23. https://www.apa.org/news/press/releases/stress/2014/stress-report.pdf

(15) Anderson, N. B.; Belar, C. D.; Breckler, S. J.; Nordal, K. C.; Ballard, D.; Bufka, L. F.;

Bourdeau, T. L. *American Psychological Association*. **2011,** 1-73.

(16) Pelcovitz, D.; Van der Kolk, B. D.; Roth, S.; Mandel, F.; Kaplan, S.; Resick, P. *J. Trauma. Stress.* **1997**, *10* (1), 3–16.

(17) Bryant, R. A.; Harvey, A. G.; Dang, S. T.; Sackville, T. *Psychol. Assess.* **1998**, *10* (3), 215–220.

(18) Tian, H.; Cao, L.; Wang, J.; Xu, T.; Zhan, Y.; Liu, L. *Occup. Dis. Environ. Med.* **2013**, *1* (1), 1–3.

(19) Hosseini, S.A.; Khalilzadeh, M. . *Biomed. Eng. Comput. Sci. (ICBECS),* **2010**, 1–6.

(20) Sioni, R.; Chittaro, L. *IEEE Comput.* **2015**, *48* (10), 26–33.

(21) Bong, S. Z.; Murugappan, M.; Yaacob, S. *Communications in Computer and Information Science.* **2012,** *330*, 198–205.

(22) Harville, E. W.; Savitz, D. a; Dole, N.; Herring, A. H.; Thorp, J. M. *J. Womens Health* **2009**, *18* (9), 1425–1433.

(23) Sawatzky, R. G.; Ratner, P. a; Richardson, C. G.; Washburn, C.; Sudmant, W.; Mirwaldt, P. *Nurs. Res.* **2012**, *61* (1), 13–21.

(24) Hellhammer, D. H.; Wust, S.; Kudielka, B. M. *Psychoneuroendocrinology.* **2009**, *34* (2), 163–171.

(25) McEwen, B. S. *J. Clin. Endocrinol. Metab.* **2002**, *87* (5), 1947–1948.

(26) Edwards, O. M.; Courtenay-Evans, R. J.; Galley, J. M.; Hunter, J.; Tait, A. D. *Lancet.* **1974**, *2* (7880), 548–551.

(27) Holsboer, F.; Ising, M. *Annu. Rev. Psychol.* **2010**, *61*, 81–109, 1-11.

(28) Dahlgren, A.; Kecklund, G.; Theorell, T.; Akerstedt, T. *Biol. Psychol.* **2009**, *82* (2), 149–155.

(29) Naumova, E. A; Sandulescu, T.; Bochnig, C.; Khatib, P. Al; Lee, W.-K.; Zimmer, S.; Arnold, W. H. *Sci. Rep.* **2014**, *4*, 4884.

(30) Lewis, J. G.; Elder, P. A. *J. Steroid Biochem.* **1985**, *22* (5), 673–676.

(31) Manenschijn, L.; Koper, J. W.; Lamberts, S. W.; van Rossum, E. F. *Steroids*. **2011**, *76* (10–11), 1032–1036.

(32) Carrozza, C.; Corsello, S. M.; Paragliola, R. M.; Ingraudo, F.; Palumbo, S.; Locantore, P.; Sferrazza, A.; Pontecorvi, A.; Zuppi, C. *Ann. Clin. Biochem.* **2010**, *47* (3), 228–232.

(33) M., Y.; G., K.; S., Z. *Cent. Eur. J. Med.* **2009**, *4* (1), 59–64.

(34) Klopfenstein, B. J.; Purnell, J. Q.; Brandon, D. D.; Isabelle, L. M.; DeBarber, A. E. *Clin. Biochem.* **2011**, *44* (5–6), 430–434.

(35) Brandon, D. D.; Isabelle, L. M.; Samuels, M. H.; Kendall, J. W.; Loriaux, D. L. *Steroids.* **1999**, *64* (6), 372–378.

(36) Stevens, R. C.; Soelberg, S. D.; Near, S.; Furlong, C. E. *Anal. Chem.* **2008**, *80* (17), 6747–6751.

(37) Mitchell, J. S.; Lowe, T. E.; Ingram, J. R. *Analyst.* **2009**, *134* (2), 380–386.

(38) Arya, S. K.; Chornokur, G.; Venugopal, M.; Bhansali, S. *Biosens. Bioelectron.* **2010**, *25* (10), 2296–2301.

(39) Arya, S. K.; Chornokur, G.; Venugopal, M.; Bhansali, S. *Procedia Engineering*. **2010**; 5, 804–807.

(40) Arya, S. K.; Chornokur, G.; Venugopal, M.; Bhansali, S. *Analyst.* **2010**, *135* (8), 1941–1946.

(41) Vasudev, A.; Kaushik, A.; Tomizawa, Y.; Norena, N.; Bhansali, S. *Sensors and Actuators B: Chem.* **2013**, *182*, 139–146.

(42) Singh, A.; Kaushik, A.; Kumar, R.; Nair, M.; Bhansali, S. *Appl. Biochem. Biotechnol.* **2014**, *174* (3), 1115–1126.

(43) Yamaguchi, M.; Matsuda, Y.; Sasaki, S.; Sasaki, M.; Kadoma, Y.; Imai, Y.; Niwa, D.; Shetty, V. *Biosens. Bioelectron.* **2013**, *41* (1), 186–191.

(44) Hodkiewicz, J. *Thermo Electron Scientific Instruments.* ***2010****,* 51901.

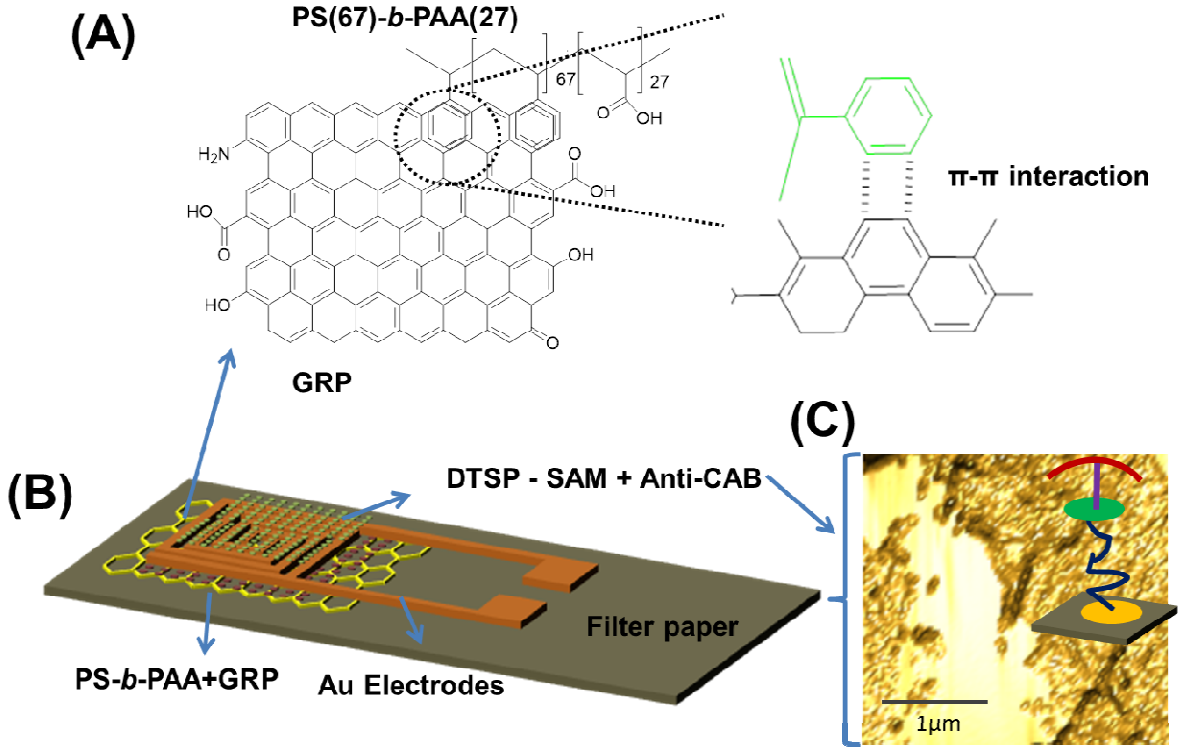


Fig. 1. Graphical representation of paper based electrical biosensor chip. (A) Graphene-Nanoplatelet (GP) and poly(styrene)-block-poly(acrylic acid) (PS_{67}-b-PAA_{27}) composite (10:1; w/w). (B) Schematic description of paper-based biosensor chip. Filter paper with anti-cortisol antibody (anti-CAB) conjugated using 3,3′-Dithiodipropionic acid di(N-hydroxysuccinimide ester) (DTSP) – self-assembled-monolayer (SAM) to gold electrodes deposited on top of multiple layers of composite containing PS_{67}-b-PAA_{27} and GP. (C) Atomic force microscopy reveals the true representation of cortisol sample linked with anti-CAB/DTSP-SAM on single Au electrode.

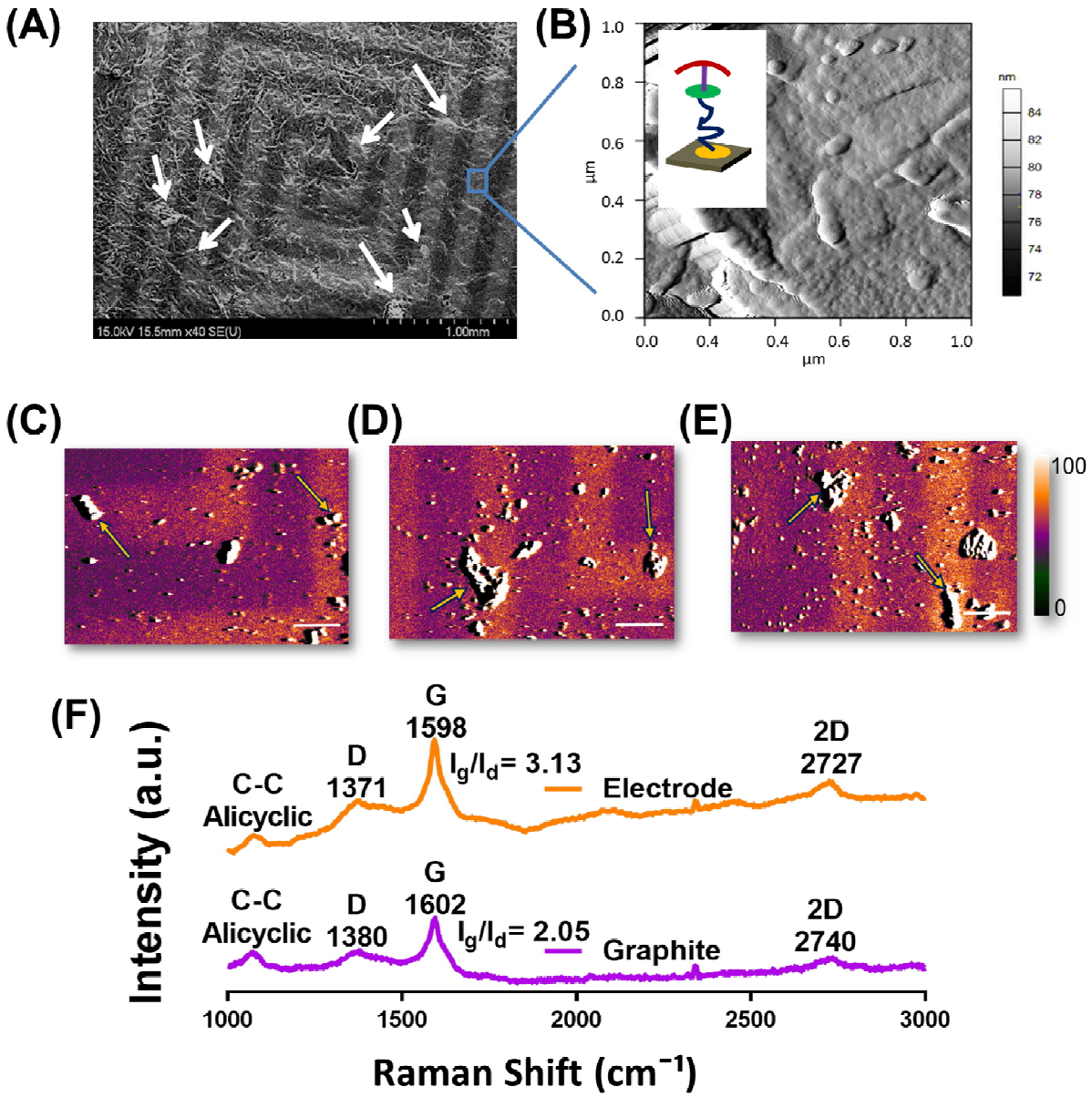


Fig. 2. Surface characterization of the biosensor chip. (A) SEM and (B) AFM images reveal the location of the conjugated anti-CAB linked with DTSP-SAM on Au electrodes. Cortisol from saliva interacts with covalently conjugated Anti-CAB/DTSP-SAM/Au electrode. White arrows indicate the site of antibody-cortisol interactions occurred at Au electrodes and clusters produced after chemical interaction of cortisol and anti-CAB as visible through AFM (B). (C-F) Raman

spectroscopic analysis of Anti-CAB coated cortisol biosensor chip. (C) A Raman generated intensity heat map of a graphene-polymer background and deposited Au electrode are decorated surface, exposed to (D) standard cortisol sample (0.03 ng/ml), and (E) original human saliva sample (unknown cortisol concentration). (F) Raman generated spectral analysis of cortisol sensors electrode and graphite surface labeled with respective chemical peak associations. (All heat maps were measured at 1600 cm^{-1}, color bar indicates intensity value per pixel, scale bar for Raman micrographs: 30 μm, 5X objective).

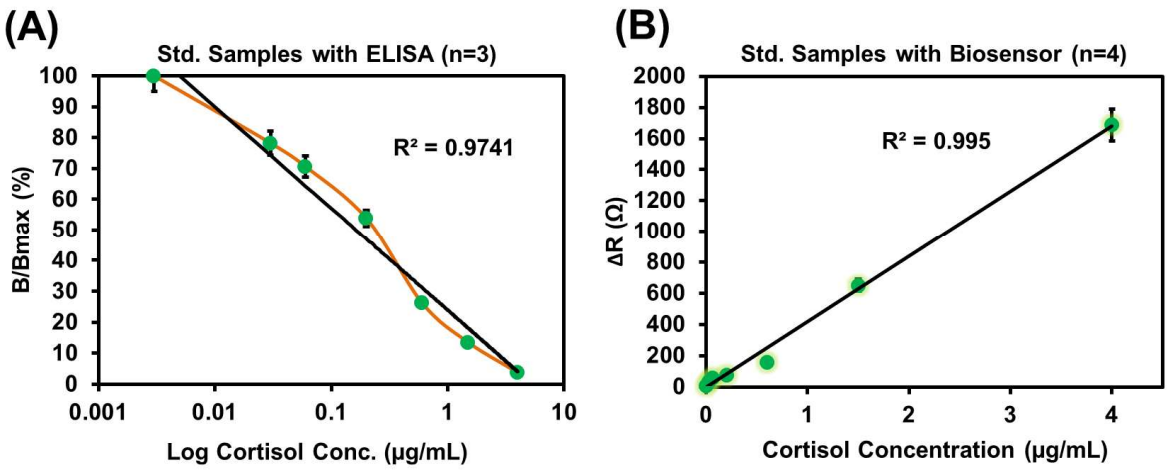


Fig. 3. Calibration curve for standard cortisol samples with known concentrations analyzed using (A) ELISA and (B) Electrical biosensor chip. Six different sets of calibration samples (0.0003, 0.03, 0.06, 0.2, 1.5, 4 µg/mL) were measured. Electrical resistance shows the net change response (value) of biosensor chip before and after treating the sample. Error bars indicate the relative standard deviation for n=3 (ELISA) and n=4 (Biosensor).

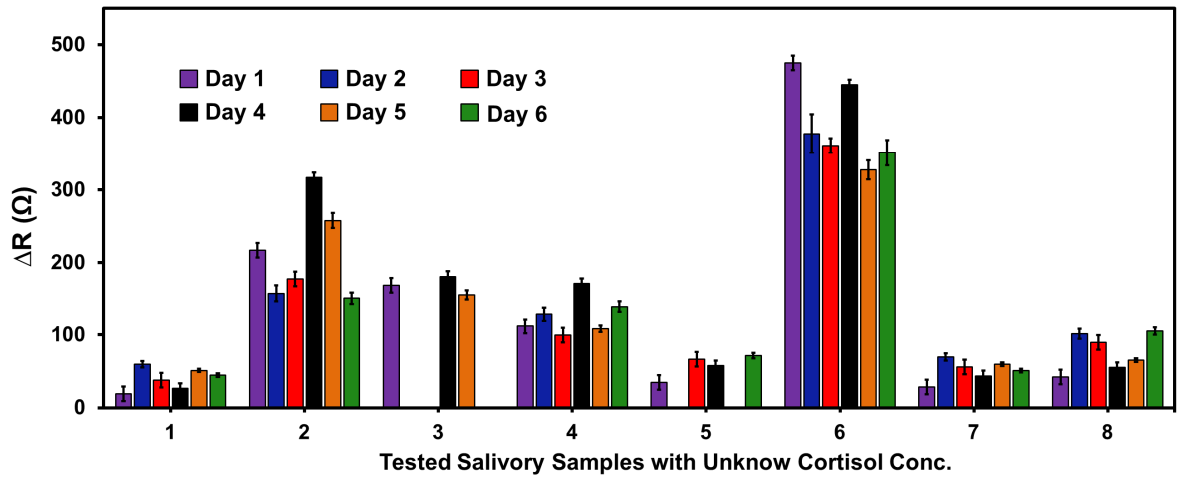


Fig. 4. Performance of the biosensor with clinical samples. Human saliva from eight subjects was analyzed using biosensor device at different days. Error bar for each day represents the mean of 3 same samples analyzed simultaneously. Success rate to readout electrical data (resistance) from biosensor chip after treating human saliva with sensor's surface was 91.12%.

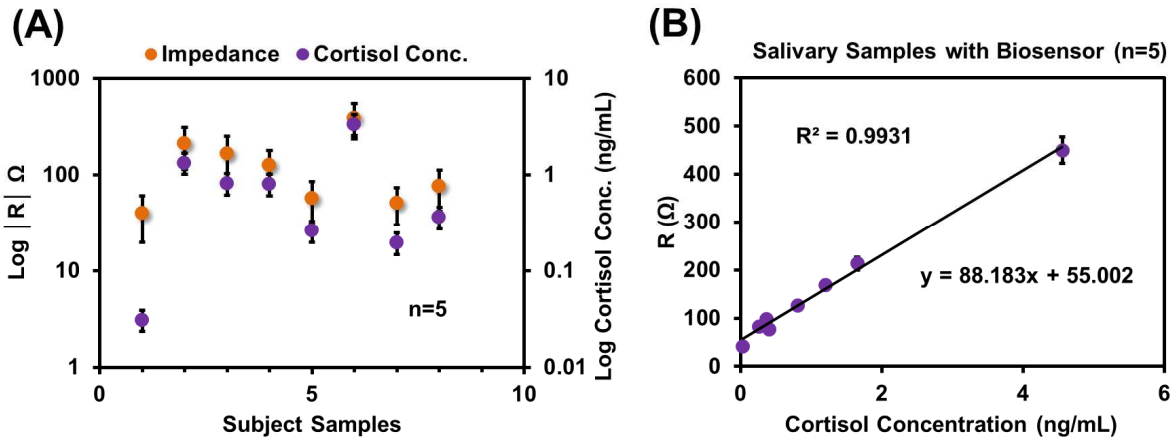


Fig. 5. Calibration results for determination of cortisol concentrations in saliva samples using biosensor chip. Estimated cortisol concentration (on logarithmic scale) was determined after comparing resistance values of subjects' saliva (Fig. 4) with resistance of known cortisol concentration (Fig. 3B). (B) Detection range of 0.018-4.552 ng/mL based on eight given samples with regression value of 0.993. These values fit well in the previously determined detection range of 3 pg/mL to 10 µg/mL (R^2 0.995) (Fig. 3B). Error bars in (A) represent the relative standard deviation for n=5 (five data points were chosen by MATLAB for every 30 sec during steady state condition). Electrical resistance in (B) shows the net value of the biosensor chip after sample treatment.

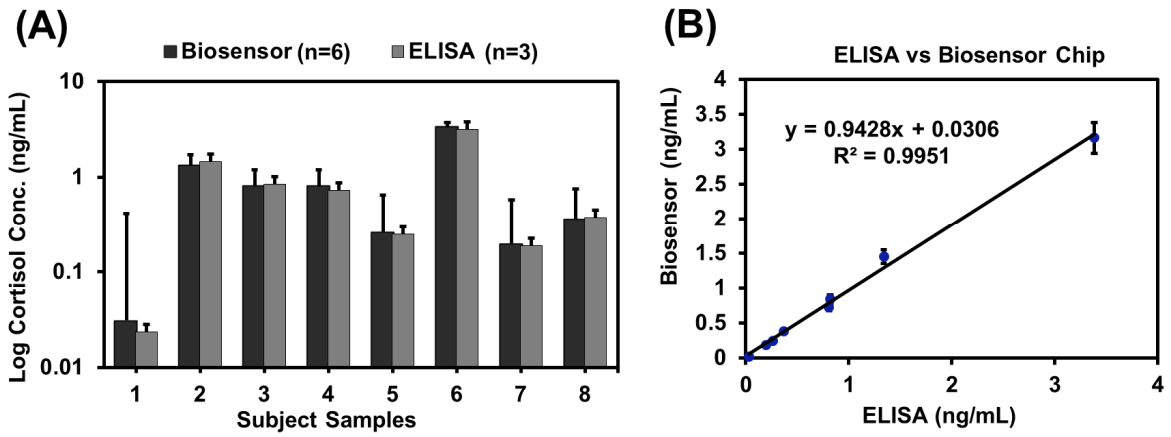


Fig. 6. A comparison of two methods to demonstrate the accuracy (% error) based on cortisol concentration. (A) Estimated error of 1.2 – 3.7 % for the real saliva sample of different subjects analyzed using biosensor device and ELISA procedure. Each error bar indicates the average of results recorded for the same individual sample (n=6 for biosensor and n=3 for ELISA). (B) Two methods (ELISA and Biosensor) co-relate with regression value (R^2 0.955). This result shows strong validity of the proposed biosensor chip for detection of cortisol concentration of human saliva.

Table 1. Quantitative analysis of cortisol concentration in human saliva for eight subjects. Data was obtained using electrical biosensor chip after comparison with calibrated results of standard cortisol samples.

Sample #	Cortisol Conc. (ng/ml)	Cortisol Conc. (ng/ml)	Cortisol Conc. (ng/ml)	Cortisol Conc. (ng/ml)	Cortisol Conc. (ng/ml)	Cortisol Conc. (ng/ml)
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
1	0.018	0.0492	0.0295	0.0125	0.0446	0.0325
2	1.876	0.186	1.354	2.589	1.864	0.1756
3	0.946	----------	----------	1.321	0.1856	----------
4	0.767	0.855	0.627	0.9754	0.732	0.895
5	0.0285	----------	0.489	0.03985	----------	0.4897
6	4.552	3.024	2.994	4.236	2.574	2.918
7	0.0216	0.464	0.569	0.0325	0.0492	0.0441
8	0.0309	0.588	0.549	0.04761	0.315	0.659

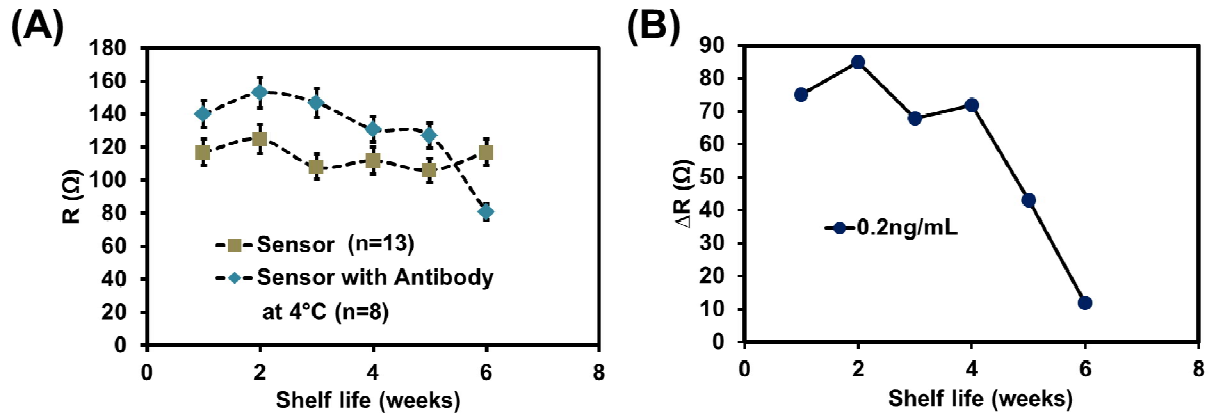
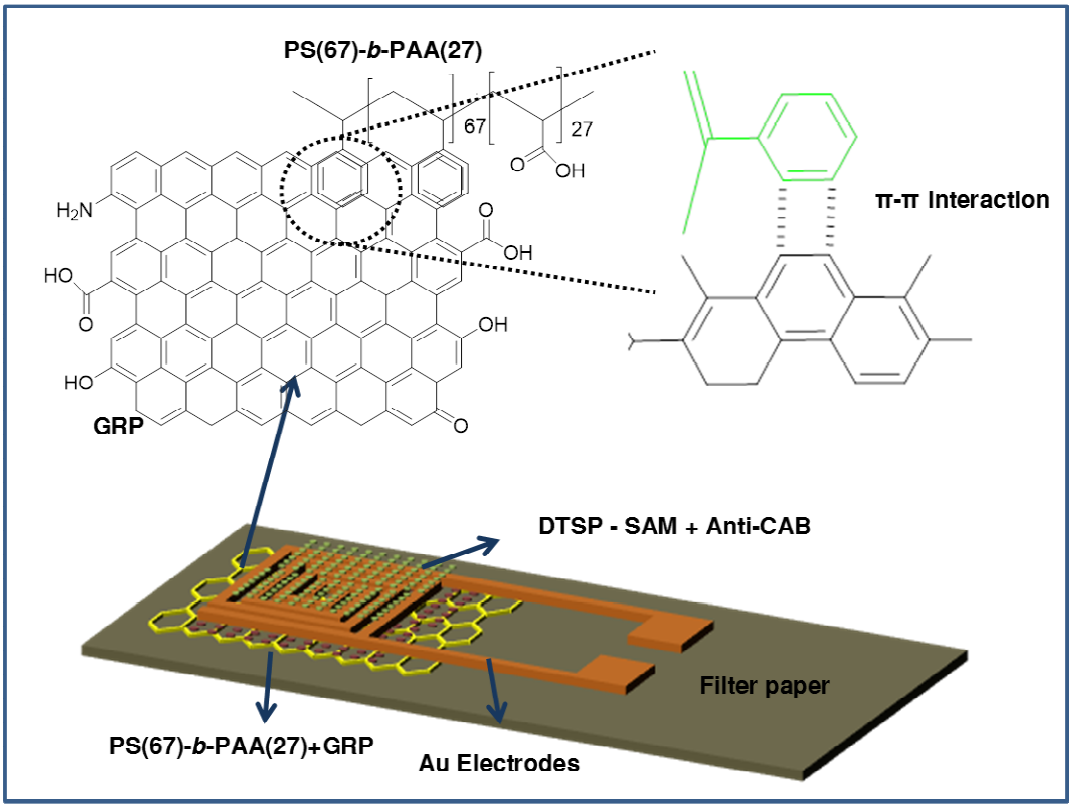


Fig. 7. The shelf life of the biosensor chip conjugated with Anti-CAB (A) before and (B) after testing with standard cortisol sample (0.2 ng/mL). Measurements were taken at intervals of 1 week at 4 ºC. Electrodes with antibody-cortisol were stable for almost 4 weeks and beyond that the immune time response reduced. Without anti-CAB, sensor exhibited very less variation even after 6 weeks.

For TOC only


PS(67)-b-PAA(27)
67
27
O
OH
H2N
HO
O
OH
HO
OH
O
GRP
π-π Interaction
DTSP - SAM + Anti-CAB
Filter paper
PS(67)-b-PAA(27)+GRP
Au Electrodes