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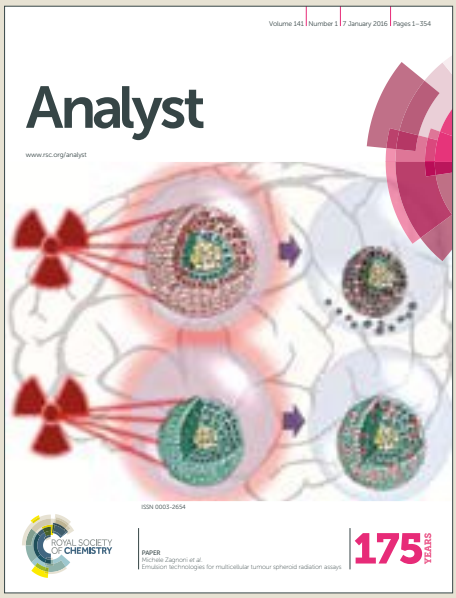

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ARTICLE

Detection of Prostate Specific Antigen (PSA) in Human Saliva Using an Ultra-Sensitive Nanocomposite of Graphene Nanoplatelet with Diblock-co-Polymer and Au Electrodes

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Prostate-specific antigen (PSA) is a commonly used biomarker for the detection of prostate cancer (PCa) and there are numerous data available for its invasive detection in serum and whole blood. In this work, an electrochemical sensing method was devised to detect the traces of PSA in human saliva using a hybrid nanocomposite of graphene nanoplatelets with diblock co-polymer and Au electrodes (GRP-PS_{67}-*b*-PAA_{27}-Au). The pure graphitic composition on filter paper provides significantly high electrical and thermal conductivity while PS_{67}-*b*-PAA_{27} makes an amphiphilic bridge between GRP units. The sensor utilizes binding of anti-PSA antibody with antigen-PSA to act as a resistor in a circuit providing impedance change that in turn allows for the detection and quantification of PSA in saliva samples. A miniaturized electrical impedance analyzer was interfaced with a sensor chip and the data was recorded in real-time using a Bluetooth-enabled module. This fully integrated and optimized sensing device exhibited a wide PSA range of detection from 0.1 pg/mL – 100 ng/mL (R^2 = 0.963) with a lower limit of detection 40 fg/mL. The performance of the biosensor chip was validated with an enzyme-linked immunosorbent assay technique with a regression coefficient of as high as 0.940. The advantages of the newly developed saliva-PSA electrical biosensor over previously reported serum-PSA electrochemical biosensors include a faster response time (3 – 5 min) to achieve a stable electrical signal for PSA detection, high selectivity, improved sensitivity, no additional requirement of redox electrolyte for electron exchange and its excellent shelf life. The presented sensor is aimed for clinical commercialization to detect PSA in human saliva.

Introduction

Prostate cancer (PCa) is one of the most widespread malignancies amongst the male population in the U.S [1]. It represents 9.6% of all new cancer cases in the U.S. [2] and it is the third most common cancer in men [3]. At present, there are no effective cures for the treatment of PCa in the advanced metastatic stage [4], making early detection critical for this population. Prostate specific antigen (PSA), a valuable biomarker, is not only used for the early detection of PCa [2] but also to monitor therapeutic response [1]. In this case, detectable level of PSA indicates the recurrence of the disease [5]. Currently, testing to trace the level of PSA in serum occurs at dedicated centralized laboratories using large automated analyzer. This centralized testing requires additional sample transportation, waiting times, administration, of which all increase total costs [6].

During the past few years, a lot of efficient methods have been developed for the detection of PSA based on immunoreaction such as enzyme-linked immunosorbent assay (ELISA) [7], time-resolved fluorescence assay [8], chemiluminescence immunoassay [9], bioluminescent immunoassay [10], and electrochemical immunoassay [11] to detect in a tedious and expensive way. PCa tumor growth usually leads to the release of high concentration of PSA into the circulatory system [12]. Approximately 75% of the cancers detected when serum-PSA is in the range 2 – 10 ng/mL [5], [13], [14]. Furthermore approximately 13-22% of men with a serum PSA in the range 2.5 – 4 ng/mL have a PCa detectable by biopsy and 80-85% of these are organ confined [5].

The availability of a handheld point-of-care testing (POCT) device could help to reduce the number of clinic visits, decrease costs to the patient and the healthcare providers, improve health care standard and acceptable clinical outcome [15]–[22]. Developing an electrical biosensor chip for PSA detection offers a great possibility to create a low-cost and highly sensitive point-of-care (POC) sensor to detect and quantify the PSA information in real-time. This would also drastically decrease the need for a sample transport to the main laboratories and helpful in building new economical outcome in health care management. The previously developed biosensor chips [23]–[47] utilized an invasive approach and required serum/whole blood as a sample to detect the level of PSA. The most common techniques previously reported

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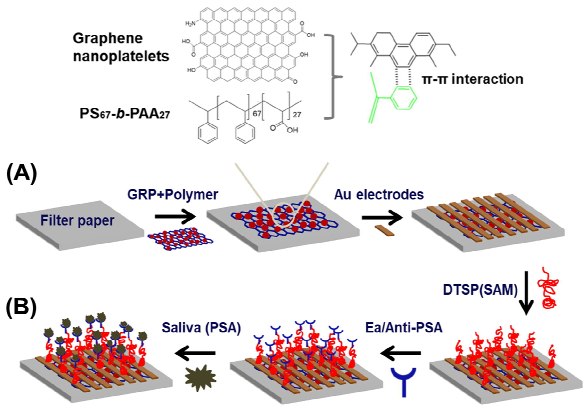
to detect PSA at POC level include surface plasmon resonance (SPR) [24], [40], surface enhanced raman scattering (SERS) [41] fluorescent assay [31], [35], [37], [38], [46], electrochemical impedance spectroscopy (EIS) [23], [26], [28], [30], [32], [36], [43], [44], cyclic voltammetry (CV) [25]–[27], [29], [32], [36], [43], electrochemiluminescent (ECL) [38], amperometric [29], capacitance [34] and photo-electrochemical (PEC) [45], [47]. Details of serum-PSA and whole blood-PSA biosensors with their sensor preparation materials, sample analysing technique, RoD, LoD and immobilization time are highlighted in Table S1.

PSA is highly abundant in human semen; however, a small amount is also found in the blood with a concentration below 4 ng/mL [5]. Originally approved in 1986, current FDA guidelines recommend PSA detection to assess the progression of prostate cancer in men who had already been diagnosed with the disease. Numerous studies published point of care detection of PSA utilizing serum/whole blood samples (Table S1). In comparison to blood, saliva sample collection is less invasive and therefore requires lower cost making the detection of PSA truly point of care. Saliva sample can easily be collected at lower cost putting lesser burden on patients as compared to the collection of blood. Approximately 1400–2000 proteins have already been identified in saliva [48], [49] including PSA [50]–[52]. The number of proteins in saliva shows its diversity, and each of these proteins can be used as a simple tool to assess toxicity, infectious, immunological and hormonal levels [53]. Several independent studies have shown that saliva PSA is directly correlated with blood PSA in patients with recurrent or metastatic prostate cancer and may, therefore, be a useful PA biomarker. Range of PSA in saliva sample is 0.01-1.74 ng/mL as previously measured using micro-particle enzyme immunoassay technology [51]. The same group also studied the saliva-PSA in low-serum PSA concentration group and results were reported to be 0.010 ± 0.011 ng/mL (range 0.000-0.039 ng/mL) using RT-PCR [51]. We have also found interesting articles [50-52] which show co-relation between serum-PSA and saliva-PSA and they utilized standard techniques in their work. Furthermore, a previously reported study confirms that PSA transits from blood to the salivary gland in animal models [51]. Additionally, a significant correlation has also been identified between saliva PSA and serum PSA levels in conditions with prostate adenocarcinoma [51]. From the best of our knowledge, a point of care biosensor for detection of PSA in human saliva is not known. The success of this research will help to make PSA detection point of care in a true sense.

Herein, instead of using serum as an analyte source, we report non-invasive approach and present a POC biosensor to detect PSA level in human saliva for the first time. We develop a disposable paper-based ultra-sensitive and highly-selective graphene-polymer-Au (GRP-PS_{67}-*b*-PAA_{27}-Au) biosensor to monitor PSA level in seven human saliva samples. The pure graphitic composition provides significantly high electrical conductivity while PS_{67}-*b*-PAA_{27} makes an amphiphilic bridge between GRP units. To the best of our knowledge, this is the first study utilizing human saliva as a body fluid for the detection of PSA at POC level. The change in resistance of a base layer of biosensor confirmed the chemical reaction of an analyte (PSA) with anti-PSA antibody covalently conjugated on Au electrodes via dithiobis (succinimidyl propionate), DTSP. Detected signals were monitored in term of change in electrical resistance in real-time by integrating the biosensor chip with a hand-held printed circuit board (PCB). RoD and LoD for PSA were studied with standard PSA aqueous solution (0.1 pg/mL – 100 ng/mL) to generate a calibration curve and the concentration of PSA was then quantified in six human saliva samples collected from healthy subjects. The change in electrical resistance was proportional to the amount of PSA binding, as such allowing for a highly specific and sensitive detection. Results determined using biosensor chip were also validated using standard ELISA. Selectivity of the proposed biosensor chip has been tested against beta 2-microglobuin (B2M), lactoferrin (LF) and ascorbic acid (AA) and the results confirmed the high selectivity of the developed biosensor towards PSA detection and showed negligible response towards unwanted compounds present in the test sample. Furthermore, shelf life stability of the sensor chip conjugated with anti-PSA antibody before and after testing with PSA aqueous solution was evaluated over the period of 8 weeks.



Scheme 1. Schematic layout description of electrical biosensor chip. (A) Spin coating of graphene-polymer on filter paper followed by deposition of Au electrode using e-beam evaporator. (B) Anti-PSA antibody attachment on Au electrodes via SAM layer followed by testing saliva sample to detect PSA concentration.

Results and Discussion

Schematic representation of the developed biosensor chip is shown in scheme 1. Biosensors developed on papers offer an alternative to conventional technologies (e.g. silicon wafer) for fabricating simple, low-cost, and disposable analytical devices for wide ranges application areas. The advantage of paper as a sensing platform is their ability to allow passive liquid transport and excellent compatibility with biochemical. Base material of a multilayer biosensor chip was composed of a graphene-polymer nanocomposite. The graphene-polymer composite was coated on filter paper for making it suitable to absorb the fluid 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 [17]. Polymer-

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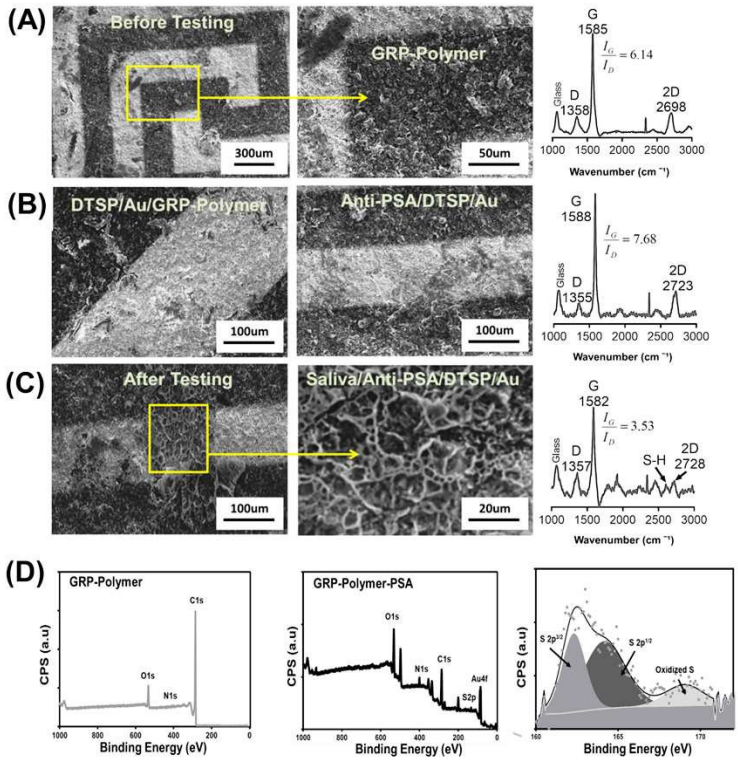

Fig. 1. Surface characetrization using SEM, Raman spectroscopy and X-ray photoelectron spectroscopy of the developed biosensor chip. (A) Graphene-polymer coated on flter pape followed by deposition of Au electrode (left). Raman micrograph of the graphene-polymer grafted on filter paper to reveal 1585 cm^{-1} as the characteristic graphene wavenumber. A single Au electrode immoblized with Anti-PSA antibody via DTSP (B) before and after testing with human saliva sample. (D) XPS spectrum for graphene-polymer coated on paper before Au electrode deposition (left). Spectral positions of C1s, N1s, O1s, Au4f and S2p features are retained even after testing of saliva sample as expected. Sp2 region is further de-convolved (right).

graphene platelet with such arrangements would be channeling water component of the samples but restricting the percolation of other components including PSA. Further, graphitic composition provides significantly high electrical and thermal conductivity while PS_{67}-*b*-PAA_{27} makes an amphiphilic bridge between GRP units and allows better spin coating on filter paper as shown in Fig. 1a (left). Fig. 1a (right) is a Raman micrograph of the graphene-polymer grafted on filter paper to reveal 1585 cm^{-1} as the characteristic graphene wavenumber.

Choice of electrode design is always crucial for the impedance based electrical biosensor chip. Square based Au electrode design was selected to provide uniform electrical impedance in the sensing area to achieve the lower concentration of PSA as shown in Fig. 1b. Anti-PSA antibody (20 µg/mL) was conjugated using the procedure described in experimental section. SEM images of the developed sensor chip before and after testing are shown in Fig. 1b and 1c respectively. Anti-PSA antibody was covalently linked on Au electrode *via* DTSP/SAM conjugation chemistry (Fig. 1b). Given that most proteins are comprised of chromophoric amino acids, like phenylalanine, tyrosine, and tryptophan, visible light Raman spectroscopic analysis was performed. As we can see from the Raman spectral analysis, the graphene-polymer base provides a graphitic (sp^2) to diamond (sp^3) [54] intensity ratio (I_G/I_D) of 6.14 as shown in Fig. 1a (right), while the sensor conjugated with anti-PSA antibody has an I_G/I_D of 7.68 as shown in Fig. 1b (right). This increased in I_G/I_D is due to the surface-enhanced Raman scattering (SERS) caused by the presence of the Au electrodes. After comparing this data with the sensor chip tested with saliva, a significant decrease in I_G/I_D from 7.68 to 3.53 was noticed (Fig. 1c, right). The graphitic wavenumber is also downshifted from 1588 to 1582 cm^{-1}. This shift is a known trait indicating an increased amount of π-π interactions from the saliva proteins complementing the amino-acid residues on the antibody's innate structure [54]. As shown in Fig. 1c, once saliva sample comes into contact with the sensor, PSA proteins bind to the anti-PSA antibody covalently attached to Au electrodes and allows for a more accurate measurement. The saliva sample resulted in an additional thiol peak found at 2610 cm^{-1} as shown in Fig. 1c (right), further supporting the abundance of saliva-PSA on the Au electrodes.

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X-ray Photoelectron Spectroscopy analysis was further carried out for GRP-polymer coated on a filter paper which revealed the presence of spectral atomic positional peaks for C1s, N1s and O1s as shown in Fig. 1d (left). They were further de-convoluted and the narrow scan regions from them revealed different bond types confirming the composition of GRP-polymer (Fig. S2). C1s narrow scans depicted sp^2 and sp^3 carbon originating from the polymer backbone and graphene platelets, whereas O1s narrow scan depicted C-O, and C=O bonds probably due to the graphene nanoplatelets [17], [55]. XPS of GRP-polymer-PSA was also performed which shows the presence of Au4f and S2p peaks, along with C1s, N1s and O1s peaks. The Au4f peak confirms the Au electrodes on the GRP-polymer composite and the increased S2p signal depicts successful interactions of proteins with Au electrodes due to the disulfide linkage with PSA. S2p narrow scan was further de-convolved as shown in Fig. 1d (right).

PSA Detection in Aqueous Solution and Human Saliva Samples

Fig. 2 presents the response of the fabricated sensor chip for PSA aqueous solution and human saliva samples. As seen from Fig. 2a, the change in resistance was increased with the increasing PSA concentration (0.1 pg/mL – 100 ng/mL). It shows the step response of the sensor at increasing concentration of PSA in the solution and inset of Fig. 2a indicates the lowest detection of 40 fg/mL. Change in resistance was recorded for each saliva sample using volume of 10 µL after testing with the sensor chip. The logarithm of PSA concentration and the increased electrical resistance shows a good linear relationship with a sensitivity of 0.875 $\Omega fg^{-1}mL$ (Fig. 2b). The change in resistance (ΔR) was calculated by plotting the change in resistance versus the logarithm of PSA concentrations (ng/mL). We recorded impedance based electrical signals for PSA detection with low detection of 40 fg/mL which is lower than 13 pg/mL [25], 2 pg/mL [26], [35], 15 pg/mL [27], 2.3 pg/mL [36], 2.7 pg/mL [44] and 1 pg/mL [28], [45] for electrochemical sensors, 91 pg/mL [40] and 0.29 ng/mL [24] for SPR sensors, 27 pg/mL [46], 0.2 ng/mL [37], 40 pg/mL [33] and 0.3 pg/mL [38] for fluorescent assay, 0.9 ng/mL [31] for colorimetric, 0.11 pg/mL [41] for SERS, 0.1 ng/mL [42] for electrical immunosensor, 0.6 ng/mL [30] for sandwich-type ELISA impedimetric immunosensor, 0.29 pg/mL [39] for ECL immunosensor and 2.6 pg/mL for PEC immunosensor [47]. A PEC immunosensor [45] has been reported recently and exhibited a wide range of 50 fg/mL – 10 ng/mL by utilizing a nanocomposite of SnS_2@mpg-C_3N_4 with high specific surface area and large pore volume. To further enhance the current response, ascorbic acid (an electron donor) was used which played a significant role to the PEC measurement with LoD 21 fg/mL.

In our work, a wide dynamic range detection (100 fg/mL – 100 ng/mL) with linear regression of 0.963 was obtained which is wider in comparison with previously reported electrochemical (5 pg/mL – 100 ng/mL [44]), ECL (1 – 10 ng/mL [39]), fluorescent (0.1 –100 ng/mL [46], 0.5 – 300 ng/mL [37], 0.1 – 10 ng/mL [38]), electrical immunosensor (1 – 800 ng/mL [42]), colorimetric (0.9 – 60 ng/mL

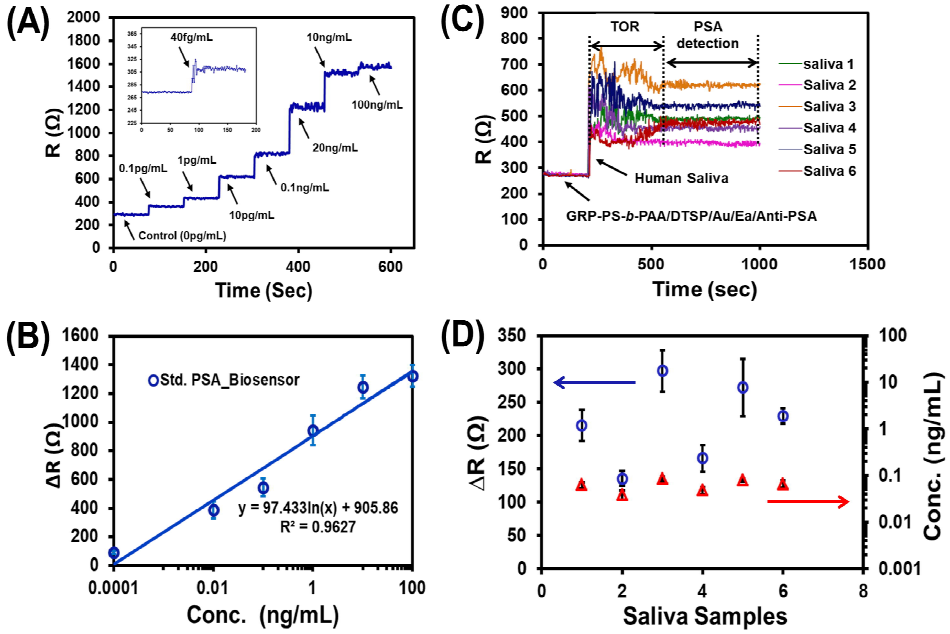

Fig. 2. Standard PSA aqueous solution and human saliva samples testing with electrical biosensor chip. (A) The change in electrical resistance increased with increasing PSA concentration (40 fg/mL – 100 ng/mL). (B) Calibrated range for PSA solution with RoD of 0.1 pg/mL – 100 ng/mL (R^2 = 0.963) and LoD of 40 fg/mL. (C) Real time analysis of human saliva samples (# 1, 2, 3, 4, 5 and 6). TOR indicates time of response to achieve the stable signal for PSA presence in saliva samples. (D) Change in resistance (ΔR) corresponds to PSA concentration 0.063, 0.039, 0.086, 0.048, 0.079 and 0.067 ng/mL in respective samples. ΔR indicates the difference in resistance of the biosensor chip before and after testing saliva samples. Error bars represent the relative SD for n=4.

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[31]) and PEC immunosensor (5 pg/mL – 50 ng/mL [47]). Thus, the proposed immunosensor (Paper/GR-PS-*b*-PAA/Au/DTSP/EA) exhibits the superiority over some earlier reported methods for the detection limit and linear range. This implies that the sensor chip could be utilized clinically for the detection of PSA concentration in human saliva.

In order to investigate the applicability and reliability of the developed sensor chip for clinical applications, human saliva samples collected from six healthy volunteers (# 1, 2, 3, 4, 5 and 6) were tested in real-time. The results indicate that the sensor is highly responsive towards PSA detection as shown in Fig. 2. Without utilizing the standard techniques such as SPR, EIS, CV and fluorescent assay, a simple handheld PCB interfaced with paper-based biosensor was used to monitor the change in electrical resistance (ΔR) before and after testing the human saliva sample. The ratio of input voltage and output current reveals the effective impedance. For each reading, time of response (TOR) was recorded in a real-time manner and the same was repeated atleast four times (n=4) to confirm the actual change in resistance for each sample. Data points were collected and monitored continuously until the sensor exhibited the steady response as shown in Fig. 2c. TOR for the detection of PSA in saliva was varied from 1 – 3 min which is less as compared with previously developed biosensor chips. The low response time was achieved due to the presence of uniform electrical field of square rings and the presence of graphene-polymer composite in between Au electrodes. The change in resistance for all samples (Fig. 2c) was then interpolated with standard PSA calibration curve (Fig. 2b) to determine the level of PSA in the tested saliva samples as shown in Fig. 4d. Herein, without utilizing any redox electrolyte, saliva was directly added to the biosensor chip and the change of resistance was recorded after PSA interacting with anti-PSA antibody attached to modified Au electrode. It can be seen from Fig. 2d that the log concentration of PSA (ng/mL) is labelled correspondingly with the change in resistance (ΔΩ). Matlab algorithm was interfaced with BT module of PCB for data collection in real-time. PSA level in saliva collected from six healthy subjects # 1, 2, 3, 4, 5 and 6 are 0.063, 0.039, 0.086, 0.048, 0.079 and 0.067 ng/mL respectively. These values are in the range of normal saliva-PSA (0.01 – 1.74 ng/mL) as previously recorded using micro-particle enzyme immunoassay technology [51]. The same group also studied the saliva-PSA in low-serum PSA concentration group and results were recorded to be 0.010 ± 0.011 ng/mL (range 0.000 – 0.039 ng/mL) using RT-PCR [51]. Thus, results obtained in our study utilizing a paper-based biosensor chip exhibits acceptable level of PSA in human saliva samples and are in the similar range of the previously published data for PSA in saliva [52] using standard a non point-of-care method.

Results Validity

To further demonstrate the relevance of the prepared biosensor chip and the obtained results for saliva-PSA, the measurements were compared with the ELISA results. ELISA experiments were performed in triplicate with same saliva samples and known PSA concentrations (0.1 pg/mL, 10 pg/mL, 0.1 ng/mL and 1 ng/mL) as shown in Fig. 3. Calibration curve was initially generated using known PSA values (Fig. 3a) and PSA-saliva was then estimated (Fig. 3b) based on the measured intensity. The results obtained using

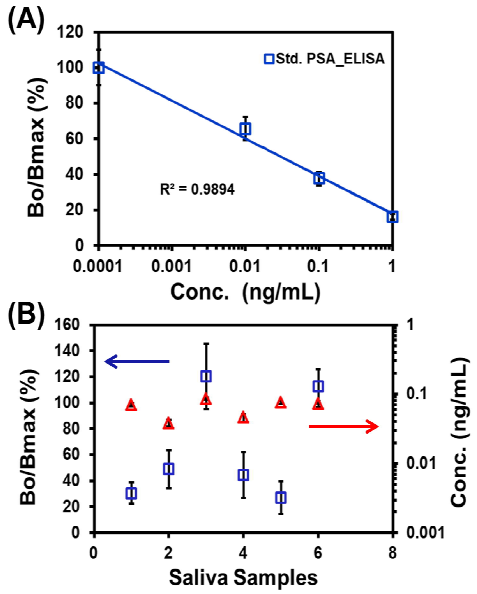

Fig 3. ELISA results for standard PSA solutions and Saliva samples. (A) Calibration curve of standard PSA solution with known concentrations (0.0001, 0.01, 0.1 and 1 ng/mL). (B) PSA concentration of 0.071, 0.038, 0.086, 0.046, 0.076 and 0.073 ng/mL is obtained in saliva samples # 1, 2, 3, 4, 5 and 6 respectively. Error bars represent the relative SD for n=4.

ELISA were compared with results of electrical biosensor chip. As seen from Fig. 4, linearity between two methods was found to be 94.02%. The high degree of correlation between two methods shows that the developed biosensor chip with graphene-polymer coated on filter paper with evaporated Au electrodes provides satisfactory sensitivity and reliability for saliva-PSA detection.

Reproducibility, Recovery, Shelf Life and Selectivity

An ultra-sensitivity of the developed biosensor chip to read PSA signals in human salivary samples has been confirmed by demonstrating reproducibility, recovery, shelf life and selectivity experiments. Reproducibility of the fabricated sensors was investigated before immobilizing the anti-PSA antibody and testing human saliva samples. In this regard, 24 sensor chips with similar configuration and size were fabricated and total three batches (72 sensor chips) were investigated to record the electrical resistance for bare Au electrodes deposited on nanocomposite of graphene-polymer. Reproducibility was confirmed with an accuracy of about 88.89%. Among 72 sensor chip, 64 successfully exhibited the net resistance of 416.52 Ω with SD of ±17.82. Description of an individual batch along with success of biosensor chips is shown in Fig. S3. A recovery experiment was then demonstrated using human saliva samples spiked with PSA. All measurements were taken at RT after 15 minutes of PSA addition in samples. A recovery was obtained in the range of 95 – 114 %, 94 – 110 % and 95 – 106% for the developed biosensor chip with PSA concentration of 0.1, 1 and 10 ng/mL respectively as described in Table 1.

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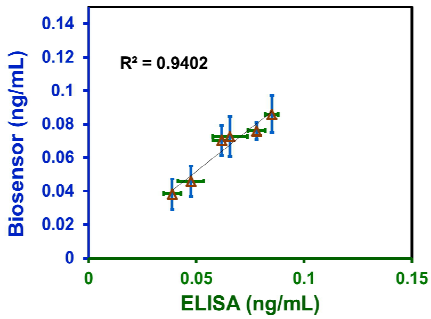

Fig 4. EL A comparison of two methods (Electrical Biosensor Chip and ELISA) to demonstrate the accuracy (% error) based on PSA level determined in saliva samples. Two methods co-relate with regression value (R^2 0.9402) and show strong validity of the proposed biosensor chip for detection of PSA concentration in human saliva. Each error bar represents the individual range of PSA to be detected in one saliva sample.

Shelf life of the biosensor was monitored in terms of change in resistance over the period of eight weeks as shown in Fig. 5a. The results of the study confirm that Au electrodes deposited on graphene-polymer composite with anti-PSA/DTSP were stable for almost 7 weeks. Measurements were taken at intervals of one week at RT as shown in Fig 5b. High resolution SEM image of nanocomposite of GRP/polymer coated on filter paper at week 1 and week 9 is shown in Fig. S4. In order to test the selectivity of the developed biosensor, standard solutions for PSA and other possible competitive proteins including, B2M, LF and AA were prepared in running buffer and tested under similar conditions. A change in resistance was not significant when B2M, LF and AA solutions were pipette onto the sensor chip. The occurrence of this small change could be due interaction of saliva with GRP-polymer. However, a significant change was observed when PSA was tested. As shown in Fig. 5c, these results confirm high selectivity of the sensors for the specific type (PSA) of antigen detection with negligible response towards B2M, LF and AA. We anticipate that developed system could be considered as a promising tool for detection of PSA in clinical samples.

Table 1. Recovery tests for PSA (0.1, 1 and 10 ng/mL) spiked in human saliva samples (# 1, 2, 3 and 4). For all measurements, 10 µL sample volume was utilized. A recovery was obtained in the range of 95 – 114 %, 94 – 110 % and 95 – 106% for the developed biosensor chip with PSA concentration of 0.1, 1 and 10 ng/mL respectively spiked in saliva samples.

Sample #	Measured PSA (ng/mL)	Spiked PSA (ng/mL)	Recovery (%)
1	0.114	0.1	114
	0.940	1	94
	10.611	10	106
2	0.098	0.1	98
	1.050	1	105
	10.223	10	102
4	0.097	0.1	95
	1.073	1	107
	9.771	10	98
6	0.110	0.1	110
	1.013	1	101
	9.501	10	95

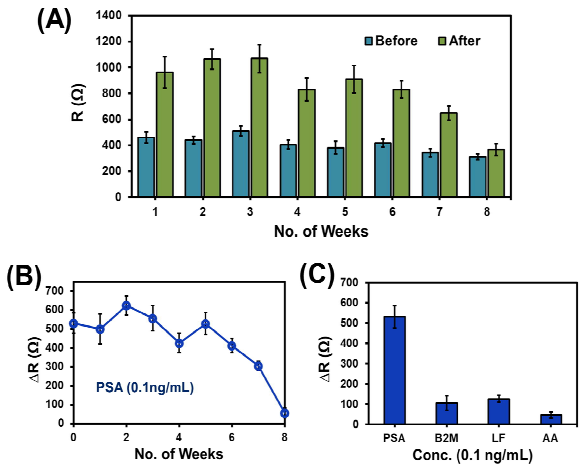

Fig 5. Shelf life and specificity of the electrical biosensor chip. (A) Actual resistance of the sensor chip with Anti-PSA antibody conjugated Au-GRP-Polymer and after testing PSA sample (0.1 ng/mL) over the period of 8 weeks. (B) Change in resistance exhibits the similar characteristic of the sensor chip for 7 weeks. This testing confirms the integrity of the developed biosensor chip. A minimal change of 37 Ω was observed for 8th week. (C) Selectivity of the sensor was determined by testing other proteins such as B2M, LF and AA with Anti-PSA antibody conjugated Au-GRP-Polymer.

Experimental

Materials and Reagents:

HRP-Streptavidin, substrate solution, Na_2CO_3, $NaHCO_3$, $NaBH_4$, washing buffer, antibody Mix-n-Stain Kit, polymer (PS_{67}-*b*-PAA_{27}), acetone, ethanolamine, Whatman filter paper, Lactoferrin, ascorbic acid and dithiobis (succinimidyl propionate) DTSP were purchased from Sigma Aldrich (Saint Louis, MO-USA). Blocking Buffer (BSA), anti-PSA antibody, beta 2-microglobuin (B2M) antigen was purchased from Thermo-Fisher Scientific (Waltham, MA-USA). Graphene nano-platelets and PSA antigen used in experiments were purchased from Strem Chemicals (Newburyport, MA-USA) and Lee Biosolutions (Maryland Heights, NO-USA) respectively. Polyvinylidenefluoride (PVDF) membrane filter (0.22 µm) and 10 mL syringe were ordered from Millipore Sigma (Temecula, CA-USA) and centrifuge tubes from Eppendorf (Hauppauge, NY-USA). Phosphate buffer solution with pH 7.4 was used for all electrochemical measurements.

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Fabrication of Biosensor Chip

The fabrication procedure and detection principle of the developed biosensor for PSA are illustrated in Scheme 1. The base layer of the sensor was developed by spin-coating the composite of graphene nanoplatelets with average thickness of 5 – 10 nm and amphiphilic polymer (PS_{67}-*b*-PAA_{27}) on a Whatman filter paper (diameter 9 mm) [17]. Initially, a mixture of graphene-polymer composite (1:1 w/w; 20 mL) was probe sonicated (Qsonic Sonicators, Newtown, USA) at amplitude 4, with a pulse rate of 5 sec (on) and 2 sec (off) for 4 h, with intermittent cycles of 30 min at 37 °C. The spin-coating technique was preferred over conventional drop-cast method to produce a thin and uniform layer of graphene-polymer composite with minimum surface defects. This was achieved in three steps: i) spinning the solution at 150 rpm for 45 sec, 300 rpm for 600 sec and 600 rpm for 120 sec. Samples were dried using a nitrogen spray gun for 10 min and were further dried overnight under a biosafety hood at room temperature (RT). Electron beam evaporator was finally employed using a pre-pattern shadow mask to evaporate the Au electrodes design (thickness of 50 nm, width of 50 nm and interspacing between two electrodes of 50 nm) on thin graphene-polymer layer spin-coated on Whatman filter paper. Samples were finally stored in wafer carrier at RT until further used.

Anti-PSA Antibody Conjugation

Biosensor chip was initially incubated for 1 h for formation of a self-assembled monolayer (SAM) layer onto Au electrodes. This was achieved by incubating a sensor chip with a mixture of DTSP (25 mg/mL in acetone) and sodium borohydride ($NaBH_4$, 10 mg/mL in DI water) at RT. The sensor was then rinsed with acetone to remove any unbound DTSP, followed by another rinse with PBS buffer (three times) to remove the residual acetone. Anti-PSA antibody (20 µg/mL) was prepared in PBS (10 mM, pH 7.4) and immobilized on preformed SAM layer covalently attached with Au electrodes for another 1 h at RT. This allows for a covalent bonding of the amine group of the PSA-antibody with the succinimidyl group of DTSP. The sensor was gently washed with PBS. Finally, a mixture of 20 µL of ethanolamine (1%), glycine (1%) solution in PBS (v/v 9:1) was prepared and used for 15 min to block the unreacted succinimidyl group of DTSP with anti-PSA antibody. Finally, the sensor was dried using a nitrogen spray gun for 2 min and stored in an airtight container at 4 °C until further used.

Collection and Storage of Human Saliva Samples

The protocols used to collect and storage human salivary samples have been described previously [15] with some modifications. Seven saliva samples were collected from healthy volunteer participants in the present study. A procedure approved by the University of Illinois at Urbana-Champaign (UIUC) Institutional Review Boards (IRB) was followed for non-invasive collection of human bodily fluid. Each subject was asked to thoroughly rinse the mouth with clean water for 30 sec followed by holding their saliva in mouth for 20 sec. They were given a 10 mL sterilized vial to collect saliva directly into it. Saliva samples were filtered using PVDF membrane filter (0.22 µm) attached to a 10 mL syringe. Samples were squeezed into centrifuge tubes (1.5 mL), stored separately at -20 °C and sealed with an aluminium foil to avoid any chemical loss or contamination. Samples were thawed to RT before their use and centrifuged at 2000 rpm for 10 min.

Surface Characterization

Raman Spectroscopy, X-Ray photoelectron Spectroscopy (XPS), and Scanning Electron Microscopy (SEM) were employed to characterize the layer-by-layer development of the biosensor chip before and after testing with anti-PSA and Saliva. All Raman measurements were taken on a Nanophoton Raman instrument with a 532 nm wavelength laser. For each spectrum, a grating (600 l mm^{-1}) scan was taken over the range of 120–2700 cm^{-1}. For Raman micrographs, samples were imaged using line scans at 50% laser power and a 5X objective for 20 seconds per scan. XPS was obtained on the fabricated chip before and after incorporation of Au electrodes, followed by deposition of PSA samples. A Physical Electronics PHI 5400 spectrometer was used equipped with Al Kα 8620 (1486.6 eV) radiation lamp. The spectrum was referenced to the adventitious C 1s feature at 284.8 eV. SEM was utilized to image the developed sensor chip before and after using saliva sample on sensing area. Biosensor chips were mounted on SEM sample holder using a piece of carbon tape. The images were taken 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 10 kV, extracting current of 10 µA, and working distance of 25 mm for scanning.

Miniaturized Printed Circuit Board

The impedance monitoring system was designed to record time-impedance scanning. All electrical measurements were demonstrated using programmable impedance based analyzer assembled with other electronic components built in our lab. Microcontroller (ATmega328P) was reconfigured to control the input supply, Bluetooth (BT) module and LCD. Matlab was used to record the data in real-time monitoring using BT module of PCB. The BT device used in this study was a commonly used Bluetooth module (HC-05) that has a standard baud rate of 9600 and operates at 2.4 GHz for its transmission with input voltage of 3.3V. LCD was used to display the concentration of PSA-Saliva. 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 in Electrical and Computer Engineering facility and integrated with battery and biosensor chip at Medical Research Centre of Carle Foundation Hospital Urbana.

ELISA

A self-designed primary ELISA protocol was developed to compare the accuracy of PSA detection by electrochemical sensor. A biotinylated antibody was used to form a covalent binding with anti-PSA antibody. Anti-PSA antibody (10 µg/mL) was diluted in a blocking solution (1% BSA in PBS) to reduce non-specific binding. Mix-n-Stain reaction buffer and storage buffer vials were centrifuged to collect the solutions at the bottom of the vials. 1 µL of the centrifuged reaction buffer solution was pipetted into a micro-centrifuge tube with 9 µL of anti-PSA antibody solution. The solution was mixed by pipetting up and down for a few times and then transferred to the vial containing the biotin followed by

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vortexing for a few seconds. Reaction mixture was allowed to incubate in the dark for 30 min. The labelled antibody solution was diluted with the provided storage buffer by transferring the entire labelled antibody solution into the storage buffer and stored at 4 °C. For binding method, the antigen (standard PSA) was diluted to concentrations of 0.1 pg/mL, 10 pg/mL, 0.1 ng/mL and 1 ng/mL in a coating buffer (15 mg Na_2CO_3, 30 mg $NaHCO_3$ and 10 mL distilled water) and was used to coat the microliter plate wells with sample volume of 50 µL. They were left for incubation at 4 °C overnight by covering the plate with an adhesive plastic. The coated solution was then washed three times by filling the wells with 150 µL of washing solution. The remaining solution was removed by flicking the plate over a sink followed by patting the plate on a paper towel. Blocking solution (100 µL of 1% BSA) was added to each well followed with covering the plate with an adhesive plastic and incubated for 3 h at RT. Washing step was again carried out for 3 times using a wash buffer as described previously. The prepared biotinylated-antibody solution (50 µL) was added to each well and incubated for 1 h at RT followed by washing 3 times in wash buffer. Then 50 µL of HRP-Streptavidin was added to each well and incubated for 1 h RT followed with washing 3 times in wash buffer. The appropriate substrate solution (100 µL) was added to each well and incubated at RT for 20 min. Finally, stop solution (100 µL) was used followed by tapping the plate to ensure thorough mixing. Absorbance was measured at 450 nm within 30 min of the final solution. The analysis of measured data was done by preparing a standard curve from the data produced using standard PSA concentration from 0.1 pg/mL to 1 ng/mL.

Conclusion

To the best of our knowledge, this is the first paper-based electrical biosensor chip to detect the PSA in human saliva. A novel analytical sensor was prepared using a hybrid nanocomposite of graphene-polymer and Au electrodes (GRP-PS_{67}-*b*-PAA_{27}-Au) followed by immobilization of anti-PSA antibody using DTSP/SAM chemistry. Six human saliva samples collected from healthy volunteers were tested and PSA level was estimated. The biosensor exhibited promising performance in terms of sensitivity (0.875 $\Omega fg^{-1}mL$), LoD (40 fg/mL), RoD (0.1 pg/mL – 100 ng/mL) with regression 0.9627 and TOR (3 – 5 min). To study the relevance of the obtained results using biosensor chip, ELISA was performed for the same saliva samples and the results were in good agreement with accuracy 94.02%. A recovery was obtained in the range of 94 – 114% by spiking the PSA (1, 10 and 100 ng/mL) in selected saliva samples. The specificity of the sensor chip for PSA detection was demonstrated by utilizing other antigens/proteins such as B2M, LF and AA and a significant change in resistance was observed only in case of PSA. Finally, the biosensor showed excellent shelf life of up to 7 weeks before and after testing with same concentration of PSA sample (0.1 ng/mL). The area of simple miniaturized PCB integrated with paper-based sensor has great significance and prospects of commercialization as a handheld device at low-cost offering great potential for clinical use in a POC setting.

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Supporting information

This section details the comparison of previously developed biosensors to detect PSA in serum/whole blood/PSA aqueous solution. SEM images of the fabricated electrical biosensor chip before and after testing saliva sample (Fig. S1) and XPS narrow scan regions for C1s and O1s present in GRP-polymer layer coated on filter paper (Fig. S2). Reproducibility of the biosensor chip with bare Au electrodes and high resolution SEM images for the coated graphene-polymer on filter paper are shown in Fig S3 and Fig. S3 respectively.

Note: †MSK and KD have contributed equally.

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