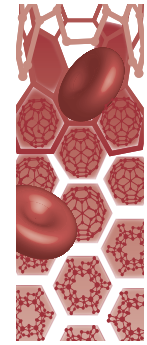
Research Article

For reprint orders, please contact: reprints@futuremedicine.com

Nanomedicine



Electrical nanowell diagnostics sensors for rapid and ultrasensitive detection of prostate-specific antigen

Aim: Alumina nanowell based disposable diagnostic biosensor for detecting and quantifying levels of prostate-specific antigen (PSA) from human serum has been designed, fabricated and tested. **Materials & methods:** The biosensors were designed by integrating nanoporous alumina membranes onto printed circuit board platforms, resulting in the generation of high-density nanowell arrays with gold base electrodes. The size and density of the nanowells were leveraged toward achieving sieving action for size-based exclusion of nonspecific molecules and size-based confinement of the target PSA molecules. **Results & conclusion:** We demonstrated PSA detection between 0.01 and 1000 ng/ml and detection and quantification of PSA from a 17 patient cohort validated using the Beckman Access system with >95% correlation.

Keywords: alumina nanowells • charge screening • electrochemical impedance spectroscopy • nanowell biosensor diagnostics • prostate-specific antigen

Anjan Panneer Selvam[1], Shalini Prasad*[,1], Thomas W Barrett[2] & Steven C Kazmierczak[3]

[1]Department of Bioengineering, University of Texas at Dallas, Richardson, TX 75080, USA

[2]Department of Veterans Affairs Portland, Oregon Health Sciences University, OR, USA

[3]Department of Pathology, Oregon Health & Science University, Portland, OR, USA

*Author for correspondence:
Tel.: +1 972 883 4247
Fax: +1 927 883 4657
Shalini.prasad@utdallas.edu

Long-standing goals in cancer biology have been to accurately detect cancer at the earliest time possible, predict prognosis and optimize therapeutic selection [1]. The use of biomarker tests could greatly accelerate the development of targeted cancer therapies by identifying the patients that are most likely to benefit from a given therapy. Recent technological advances, especially in the fields of genomics and proteomics, have made it easier to identify many biomarkers simultaneously using high-throughput screening. New genomic and proteomic molecular tools have been designed to identify the molecular signature of a targeted disease [2–5]. These include genetic and epigenetic signatures, changes in gene expression, protein profiles and post-translational modification of proteins. Such advanced diagnostic tools are not always readily adapted to clinical cancer screening due to their complexity, costs and the requirement for highly qualified operators [6]. Widespread adoption of effective new biomarker tests for cancer detection, diagnosis, therapy selection and monitoring, may lead to a paradigm shift in the way clinical medicine is practiced, with potential economic consequences.

Existing cancer screening methods include: the Papanicolau test for women to detect cervical cancer, mammography to detect breast cancer, prostate-specific antigen (PSA) level detection for men to detect prostate cancer, occult blood detection for colon cancer and endoscopy for colon cancer [7–11]. These traditional diagnostic methods are not as reliable when it comes to cancer detection at early stages. In addition, some of the screening methods are cost prohibitive for worldwide implementation. Therefore, the development of technology that is ultrasensitive and reliable for detecting cancer at an early stage as a point-of-care (POC) diagnostic tool has significant value. In this paper, we present a nanowell biosensor for ultra-sensitive detection of PSA from human serum.

At nanoscale, a target binding event can have a significant effect on nanomaterial properties, thereby providing a mode of signal transduction not available with a bulk structure made of the same material. Addi-

Future Medicine part of fsg

tionally, along with synthetic advances for varying the size, shape and composition of nanostructured materials, techniques for surface modification and patterning have significantly advanced for conjugating biomolecules to the surface to functionalize the biosensors for biomarker detection.

Generally, nanoscale biosensors are based on nanomaterials, nanoporous materials and nanoelectrodes [12–14]; that are conjugated to a targeting ligand where the ligand finds the specific marker of interest, giving the biosensor specificity, and the nanostructured surface acts as the generator or detector of a signal, assigning sensitivity. The specific binding interactions are usually provided by nature, such as antibody–antigen, nucleic acid hybridization, biotin–streptavidin and enzyme–substrate interactions.

Important clinical applications for nanotechnology enabled biosensors include early disease detection, healthcare system cost reduction, productivity augmentation and ensuring the technology improves quality of care. The key benefit of POC diagnostic devices is that they have the potential to accelerate the entire process from sample collection to obtaining a result that can be used for diagnosis or to implement treatment [15–17].

Successful detection of a wide variety of molecules has been demonstrated on POC biosensor platforms. The binding of charged molecules on such a sensor surface alters the carrier density through charge transfer resulting in changes to the measured current. The charge-detection based sensing mechanism has many advantages, including label-free detection, femtomolar sensitivity [18–21] and electronic read out capability. However, ultrasensitivity in an electronic biosensor is dependent on its ability to detect charges in high ionic strength solutions. The ability to achieve this capability is fundamentally impeded by ionic screening.

A charged surface in an ionic solution attracts counterions from the solution, forming an electrical double layer (EDL) and effectively screening off the charges. The columbic potential due to the surface falls off exponentially as we move away from it. This ionic screening effect is characterized by the Debye screening length λ_D,

$$\lambda_D = \sqrt{\frac{\varepsilon k_B T}{q^2 c}}$$

where ε is the dielectric permittivity of the media, k_B is Boltzmann's constant, T is the temperature, q is the electron charge and c is the ionic strength of the electrolyte solution.

For a typical 100 mM buffer solution, λ_D is approximately 1 nm and the surface potential will be completely screened at a distance of a few nanometers. As the result, most of existing nanoelectronic sensors operate in their most optimal conditions in low ionic strength solutions (~1–10 mM); otherwise the sample will need to undergo a desalting process. Since physiologically relevant ionic strength is ~100 mM, mitigating ionic screening effect is critical for POC nanoelectronic biosensors where detection needs to be carried out at the patient site with limited sample processing capability.

The primary objective of this paper is the demonstration of a nanoelectronic sensor device that can detect protein biomarkers associated with cancer diagnostics at ultralow concentrations from patient blood in a rapid and label-free manner. We have developed an electrical immunoassay technology that works on the principle of creation of the EDL at the liquid/metal interface. The device demonstrates the ability to perform this detection by specifically measuring charge perturbations in the capacitive EDL as a function of measuring the capacitance dominated impedance at a specific frequency. The major roadblock in using nonpurified samples in electrical biosensors is the charge screening effects due to presence of nonspecific components in the diffuse layer. This device overcomes the charge screening effects through the architecture of the device that is comprised of a high density array of nanowells integrated with a metal electrode. These size selective nanowells help minimize charge screening effects through size exclusion [22–24] and increasing the EDL region by providing a scaffold for charge distribution [25] (walls of the nanowell). We have leveraged naturally occurring miniaturization in nanopores of manufactured nanoporous alumina membranes to miniaturize microtitter well-plate technology to a nanowell plate, which has picoliter volume, and integrate that with an electrical detection and measurement system to improve sensitivity [26]. To achieve this, we integrated a 'nano' fabricated nanoporous alumina membrane onto a microfabricated electrical platform. In order to establish the performance efficacy of the device we have selected PSA, a well-established biomarker. PSA at a concentration of 2.5 ng/ml and higher has been identified to have predictive value toward diagnosis, assessment of treatment responses and follow-up among patients with prostate cancer [27]. While specificity of PSA as a biomarker as well as its concentration levels for early diagnosis of prostate cancer is heavily debated, the goal of this paper is to use PSA as a study marker for demonstrating the performance capabilities of the nano-electronic sensor device.

Materials & methods

Fabrication of nanowells biosensor

Figure 1 shows the fabricated nanowells biosensor device by integrating features in both the micro and nano scales. The nanowells biosensor device consists of three components: microelectrode printed circuit board, nanofabricated polymer membrane and polymer microfluidic encapsulant. Concentric gold microelectrodes fabricated on a FR-4 printed circuit board were used. The spacing between the microelectrodes was maintained at 1 mm. Nanoporous alumina membrane with a pore size of 200 nm was overlaid on the microelectrode platform to create nanoscale spaces (nanowells). The membrane was aligned with the microelectrodes followed by treatment in a vacuum suction chamber to uniformly adhere to the microelectrodes. AFM measurements were performed (n = 10 replicates) on the electrode surface post and prior membrane placement. The change in thickness prior to membrane placement was from 152.8 to 206.8 microns. The protein molecules are captured in the nanowells to ensure effective bonding and minimize noise associated with nonspecific binding. The microfluidic sample chamber is fabricated with polydimethyl siloxane (PDMS). The microfluidic chamber was designed to situate and hold the test sample over the circular electrode pattern. The dimension of the sample chamber is 13 mm in diameter and 2 mm in height. The total volume of the chamber is 75 µl, and inlet/outlet ports transport the sample. The three components are integrated by a manual and simple assembly line process. The assembled nanowell biosensors are shown in Figure 1A. The overall dimension of the device is 3 cm by 2 cm. The two terminals of the electrodes are connected to a potentiostat which is used to perform impedance measurements on the sample of interest.

Immobilization of capture probe

An affinity-based electrical immunoassay was implemented on the surface of nanoelectronic sensor device. A highly specific monoclonal antibody to PSA was cho-

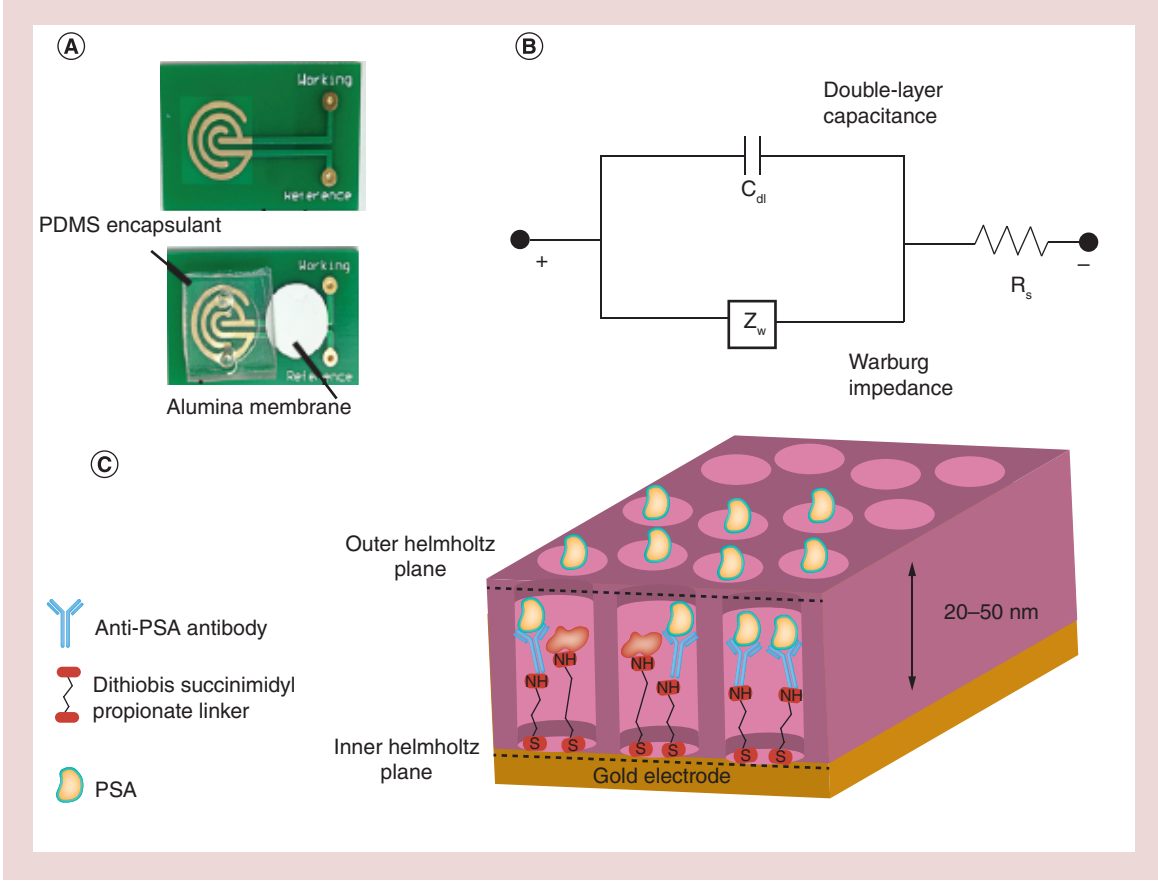


Figure 1. Electrical nanowell biosensor design, assembly and operation. Sensor device and operation **(A)** optical micrograph showing the gold microelectrodes, PDMS encapsulant and nanoporous alumina membrane **(B)** modified Randles equivalent circuit for label-free nonfaradaic impedance spectroscopy and **(C)** schematic representation of binding events at the electrical double layer.
PDMS: Polydimethyl siloxane; PSA: Prostate-specific antigen.

sen as the capture probe (Santa Cruz Biotechnologies, catalog number sc-7638, TX, USA). The primary role of the capture antibody was to detect PSA with high specificity in the test samples. The capture antibody was immobilized on the sensing regions using dithiobis succinimidyl propionate linker (DSP, Thermo Fisher Scientific Inc., IL, USA). The microelectrodes were treated with 10 mM DSP dissolved in DMSO (Thermo Fisher Scientific Inc.) for complete functionalization of the sensing regions. Then the electrodes were incubated with anti-PSA antibody at a saturating concentration of 1 µg/ml diluted in PBS for 15 min. The electronic sensor device was then treated with 1X blocking buffer solution (SuperBlock, Thermo Fisher Scientific Inc.) to block nonspecific sites and unbound DSP linker sites.

Nonfaradaic electrochemical impedance spectroscopy for detection & quantification of PSA

The presence of specific analytes of interest in a sample is detected through impedance measurements. A schematic representation of this electrical immunoassay is shown in Figure 1C. This immunoassay formation is enabled on top of the electrodes and within the array of nanowells. The changes occurring to the EDL formed at the electrode-solution interface are studied before and after the inoculation of the test target sample on the nanoelectronic sensor [28]. Electrochemical impedance spectroscopy (EIS) measurements were performed using a Gamry Reference 600 potentiostat (Gamry instruments, PA, USA). All measurements were performed at a 10mV AC voltage over a frequency spectrum of 20 Hz–10,000 KHz. All measurements were performed in human serum. The experimental results were fitted with an equivalent circuit model as shown in Figure 1B to identify the dominant parameter responsible for sensitive and selective quantification of PSA.

Debye length screening

In order to measure specific changes occurring at the electrode-solution interface, we performed debye length screening by varying the frequency of applied voltage and measured current response. The binding of biomolecules on the electrode surface resulted in the creation of a columbic potential. The formation of an electrical immunoassay on the microelectrode surface resulted in the formation of a range of columbic potentials associated with multiple EDLs. The screening of the EDLs for specific impedance change was then used toward the classification of binding effects (specific vs nonspecific) on the microelectrode surface. The impedance changes (ΔZ) for assay steps were calculated by subtracting the modulus of impedance between assay steps. The dominant parameter in the modulus of impedance was identified as the double layer capacitance over the frequency range of 20 Hz to 10,000 KHz for an applied AC voltage of 10mV.

Detection of PSA from human serum

The DSP linker-anti-PSA antibody conjugate is used as the capture probe for detection of PSA in human serum. For the initial calibration response study, PSA was serially diluted in human serum in the range of 0.001–10,000 ng/ml and tested on the nanoelectronic sensor device. Detection was carried out by performing EIS measurements. The presence of PSA in the test sample was confirmed by the impedance changes observed due to charge modulation occurring at the EDL where specific binding between PSA and anti-PSA antibody occurs. The calibration response study was performed for a total of n = 5 replicates and error was calculated as the SD from mean. A quadratic fit was performed for the calibration response data to establish a correlation between concentration of PSA and impedance measured. As a negative control, blank human serum samples from two female patients were tested for a total of n = 5 replicates.

Patient cohort study samples

For the evaluation of ultra-sensitive protein biomarker detection, we measured PSA in serum samples from 17 males. Heparinized plasma was collected from discard blood from patients at the Oregon Health & Science University in October 2013. Specimens were deidentified and maintained at -80°C until analysis. A total of 100 µl sample volume was used for testing with the nanoelectronic sensor device. The Oregon Health & Science University Institutional Review Board approved this study. The samples were first measured with the Beckman Access (Beckman-Coulter, CA, USA) which utilizes a two-site immunoenzymatic 'sandwich' assay. The linear range of operation for the Beckman Access system was 0.01–20 ng/ml. For this study, PSA concentration measured with the Beckman Access ranged between 0.01 and 1000 ng/ml. In addition, we also obtained serum from two female patients to use as a PSA-negative sample. Figure 2 shows a comparison of PSA measurements performed using the Beckman Access versus measurements performed with the nanoelectronic sensor device. The quadratic equation derived from the calibration response was used to estimate concentration of PSA from patient samples. Regression analysis was performed for the comparison of performance for the two technologies.

Results

Electrical characterization of functionalized sensor surface

The integration of the nanowells on the sensor surface results in the formation of nanoconfined spaces for protein biomarker binding and detection. DSP linker at the concentration of 10 mM was used to treat the sensor surface for effective functionalization. To validate the success of treatment, we performed impedance measurements before and after DSP treatment. Figure 3A shows the trend of impedance changes measured for a blank solution- DMSO only (14 kΩ) followed by the increase in measured impedance (32 kΩ) observed when treated with 10 mM DSP for 30 min. The increase in impedance was attributed to the highly electrical resistive nature of DSP. ΔZ between measurements for assay steps were highest at an applied signal frequency of 130 Hz. Hence, the impedance changes were calculated at this frequency for the consecutive assay steps.

Following treatment with DSP, a stable impedance change which was statistically negligible (ΔZ <5%) was observed for consecutive DMSO washes that demonstrated the successful linker conjugation on the sensor surface. For immobilization of capture antibodies, the sensor surface was first washed with 0.15 M 1X PBS to remove unbound linkers.

The sensor was next incubated with anti-PSA antibody for 15 minutes at room temperature. Impedance measurements were carried out before and after antibody treatment and were quantified as 3.9 and 1.5 kΩ respectively. Figure 3B shows the impedance changes observed at a frequency of 130 Hz. The decrease in impedance from the PBS assay step to the addition of anti-PSA antibody, demonstrated the immobilization of the capture antibodies. Anti-PSA antibody is electrically conductive; hence the immobilization resulted in the decrease in measured impedance. To further validate the immobilization, the sensor surface was washed three-times with PBS and impedance measurements were repeated. There were no statistically significant differences between the three washes, $p < 0.05$. The decrease in impedance due to antibody addition is as a result increase in capacitive charges in the EDL (inversely proportional to impedance).

Noise signal estimation

To estimate the signal due to background buffer and nonspecific binding, we performed a noise-signal classification study using human serum matrix aliquots from two female patients. The nanoelectronic sensor device was immobilized with anti-PSA at saturating concentration and then incubated with the control human serum matrix for 30 min. Impedance measurements were carried out for a total of n = 5 replicates. The change in impedance for noise signal was calculated as the difference (ΔZ_{noise}) obtained by subtracting the impedance obtained at PBS wash after SuperBlock (control) from the impedance measured with control human serum matrix. The impedance measured at the control assay step was 1.346 kΩ and for the female serum was 1.306 kΩ. Figure 4 shows the observed impedance change ΔZ_{noise} as 40 Ω. For a measured signal to be classified as specific signal, the specific signal threshold (SST) was defined as three-times ΔZ_{noise} (S/N = 3) and hence estimated as 120 Ω.

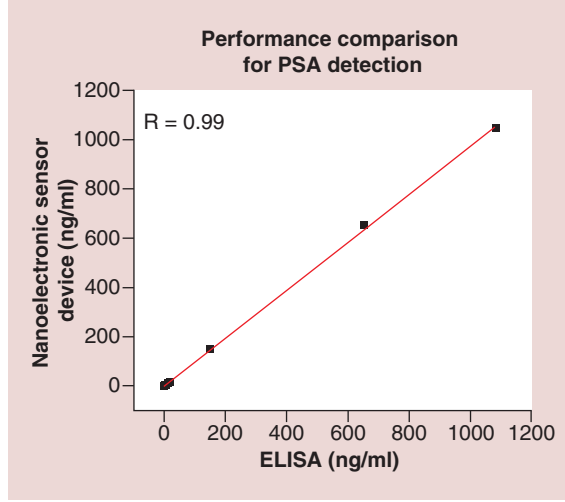


Figure 2. Testing and validation of patient sample cohort with the nanoelectronic sensor device.
Quantification of PSA in a total of 17 male patients was performed and the performance was compared with concentration estimated using Beckman Access system. Linear range of the Beckman Access system is 150 ng/ml and hence samples with concentration >150 ng/ml were diluted from the original specimen for detection with the Access system.
PSA: Prostate-specific antigen.

Identifying the impedance-concentration relation for different PSA concentrations

A serial dilution of PSA from 10,000 to 0.00001 ng/ml in phosphate buffer solution (PBS) was used for the calibration response study and to identify the analytical parameters of the nanoelectronic sensor device. The anti-PSA antibody saturated sensor surface was treated with SuperBlock buffer to seal-off any unbound linker sites. A PBS wash was performed following this, and the impedance measurements were recorded. The impedance measurement at this step was defined as the baseline/control value (1.346 kΩ).

All impedance changes for the subsequent testing with PSA dilutions were calculated with reference to the baseline/control impedance. Figure 2 shows the calibration response for the PSA concentration range

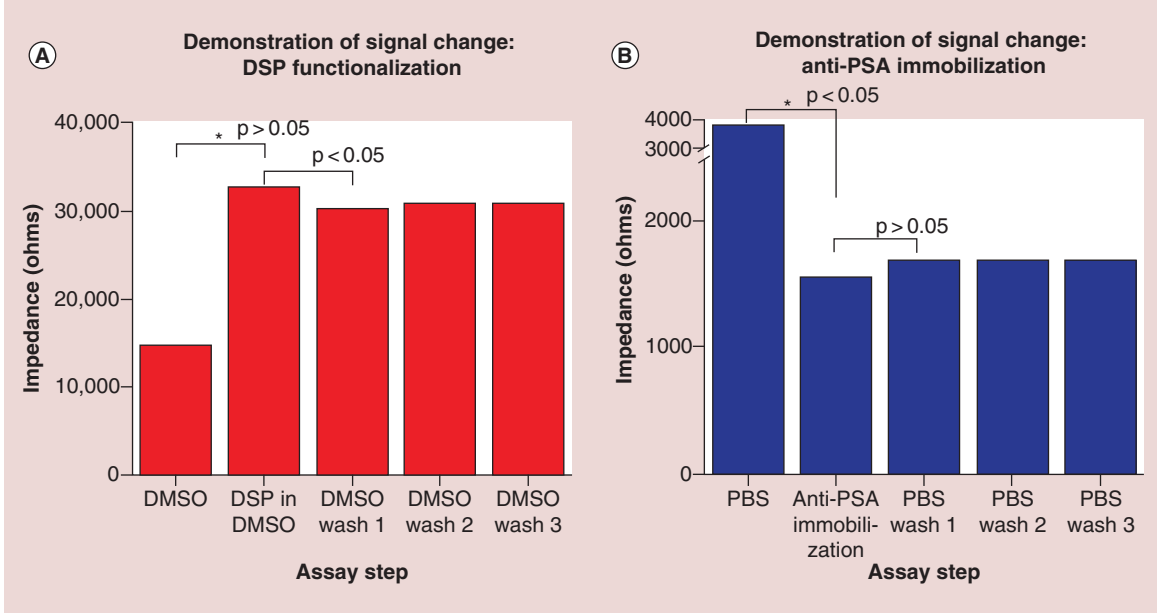


Figure 3. Estimation of noise using linker and antibody. **(A)** Validation of DSP linker conjugation through debye length screening and impedance spectroscopy measurements. Resistive nature of DSP conforms with the increase in impedance upon conjugation to gold electrode surface. **(B)** PSA immobilization to DSP-conjugated surface validated through impedance spectroscopy. Conductive nature of anti-PSA conforms to decrease in impedance observed after immobilization of anti-PSA antibody.
DSP: Dithiobis succinimidyl propionate; PSA: Prostate-specific antigen.

of 0.00001 to 10,000 ng/ml. The error bars represent SD over mean for a total of n = 5 replicates. The impedance changes observed for concentrations < 0.0001 ng/ml were 98 Ω – less than the 120 Ω specific signal threshold.

At 0.0001 ng/ml, the impedance change was 125 Ω, so was greater than the specific signal threshold. Following this, 0.0001 ng/ml was defined as the lowest detectable concentration of PSA by the nanoelectronic sensor device. For PSA concentrations in the range of 0.0001–10,000 ng/ml, the sensor demonstrated an impedance change greater than the noise signal of the system. The overall dynamic range of impedance change was between 125 and 868 Ω. The impedance-concentration correlation was defined through a calibration equation which was used for the estimation of PSA in human serum test samples.

Detection of PSA in human serum samples

A cohort consisting of 17 male patients was used to clinically validate the detection capability of the nanoelectronic sensor device. The predicate device for the nanoelectronic sensor was the Beckman Access system. The range of PSA concentrations in the patient samples were (approximately) 0.01–1,000 ng/ml. The nanoelectronic sensor performed direct detection of PSA in the human serum samples and demonstrated detection over the range of 0.001– 1000 ng/ml for the test samples. Two samples in the study demonstrated concentrations of < 0.01 ng/ml as estimated with the Beckman Access system. Both of these samples were quantified as 0.01 ng/ml by the nanoelectronic sensor. The coefficient of variation for both the Beckman Access and the nanoelectronic sensor were <5%.

Discussion

The common approach for quantification of protein biomarkers depends on chemi luminescence assays. In addition, current laboratory-based methods are not suitable for ultrasensitive PSA detection, especially when measured using a POC device. Reporter labels are limited by nonspecific and interfering biomolecules in complex sample media such as human serum and whole blood. EIS is a rapid and robust technique for screening protein biomarkers based on affinity binding. Traditional EIS utilizes redox reporters and labels which transduce the electrical signal based on specific biomolecular interactions. In summary, traditional EIS and ELISA use a label which critically limits the sensitivity and selectivity of the sensing system when testing with complex media samples. In addition, it limits the applicability of such technologies for POC situations.

In comparison to the two commonly used approaches (ELISA and redox based EIS), EIS based on debye length screening is a high sensitivity and specificity method which can be used for label-free biomarker detection. The label-free operation is achieved by using a nonfaradaic detection modality that depends on EDL

screening for detecting biomolecule binding events. In this paper, we demonstrate the ability to detect the PSA protein biomarker in PBS dilutions and in patient serum matrix samples with enhanced sensitivity and selectivity. Our method provides a promising approach for screening a range of biomarkers directly in complex media in a rapid and robust manner. This can significantly help in the early detection and diagnosis of cancer or cancer recurrence.

We have demonstrated the ability of the nanoelectronic sensor device to detect PSA over the dynamic range of 0.0001–10,000 ng/ml in human serum through a calibration dose response study. The ultrasensitivity in detection is achieved through: use of nanowells to overcome charge screening effects, and EDL screening for detecting specific binding of PSA to anti-PSA antibody. The integration of the insulating nanowells to the metal microelectrodes resulted in the formation of a charge screening system with reduced noise and interfering electrical signals from the bulk solution media. Hence, this helped in the measurement of electrical impedance changes with a high S/N for ultrasensitive quantification PSA.

In the design of the nanoelectronic sensor, nanoporous alumina was embedded on the base metallic gold electrodes. One of the aspects that this nanoporous structure serves is spatial confinement, to support this there is a compelling argument in the realm of biophysical understanding of the proteins and there structures. There exists a crowded environment inside a cell structure, the cytoplasm which is typically different from the dilute solutions that are generally used in *in vitro* studies of proteins and there exists a school of thought which states that this may significantly affect the behavior of the proteins. The high concentration of proteins, nucleic acids and complex sugars in the cells have various energetic consequences and size constraints on the smaller molecules, the effect of these bigger structures is known as 'macromolecular crowding' [22–24]. The binding affinities and the rates of self-assembly can change by orders of magnitude as a result of this phenomenon. Crowding is therefore a very important factor when we are trying to perform an *in vivo* study *in vitro*. Thus, it is imperative to use this idea in a device that would be used to measure the accurate concentration of proteins as it is measured from physiological fluids.

The charge screening offered by the nanowells helped in the implementation of specific electrical debye length screening for the detection and quantification of protein biomarker binding. Previous studies conducted by the authors have demonstrated the efficacy of using a nanoporous material on biosensing electrodes toward enhanced signal response and detection using EIS [29]. The electrical immunoassay was implemented at the electrode-solution interface. By screening electrical impedance changes at this height from the electrode surface, specific binding events occurring due to PSA interaction with the capture probes (anti-PSA antibody) were detected. The modulus of impedance was

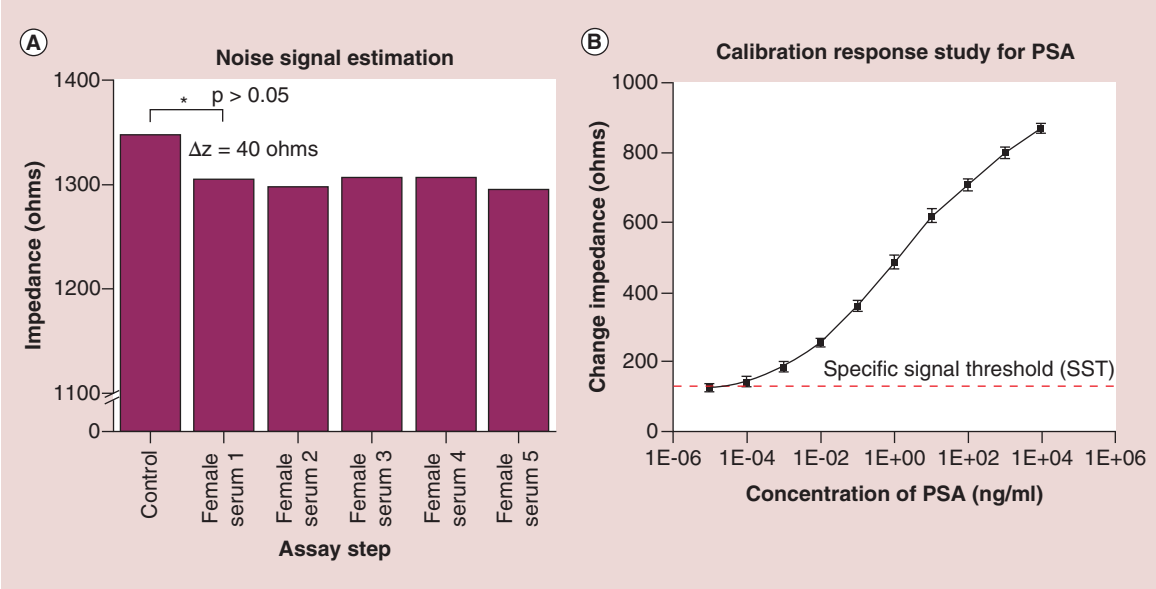


Figure 4. Electrical nanowell biosensor performance. (A) Estimation of noise signal due to buffer and nonspecific binding. Female serum samples were used as a control and noise signal was estimated. **(B)** Calibration dose–response correlating concentration of PSA and change in impedance observed. PSA over the concentration of 0.00001 and 10,000 ng/ml was tested. Specific signal threshold was estimated as three-times the noise signal. PSA: Prostate-specific antigen.

used for subsequent conversion to meaningful protein biomarker concentrations. From the circuit modeling, we identified that the dominant component of impedance was the double layer capacitance (Figure 1B). The changes in C_{dl} correlated to binding events occurring in the electrical immunoassay at the debye length of interest. This confirmed the nonfaradic nature and the specific detection of binding events in the electrical debye length.

The PSA impedance change measured from a female patient serum sample was used as the noise signal of the nanoelectronic sensor device. As the sensor was an electrical system, a highly competitive S/N of 3 (S/N = 3) was defined for the system. Specific signal threshold (SST) was defined as the impedance change that was three-times the noise signal. The lowest dose of PSA that demonstrated an impedance change greater than SST was 0.0001 ng/ml (100 fg/ml). Below this dose, PSA could not be detected with confidence using the nanoelectronic sensor device. The sensor was tested with PSA concentrations up to 10,000 ng/ml. In the range of 0.0001–10,000 ng/ml, the nanoelectronic sensor device demonstrated a linear response for change in impedance.

To validate the applicability of the sensor for PSA detection we measured PSA in serum from 17 males. The concentration of PSA in the patient cohort was also measured using the Beckman Access system. To compare metrics with the Beckman Access system, the detection range of interest chosen for and demonstrated using direct patient samples is 0.01–1500 ng/ml. This performance confirmed the ability of the sensor to perform in a robust manner in a clinical setting. The immediate clinical application of this technology for PSA is to detect prostate cancer recurrence after prostatectomy, either in a laboratory or POC setting. The overall cost for manufacturing

Conclusion

An ultrasensitive nanoelectronic sensor for protein detection was designed and fabricated. This sensor has several advantages over existing clinical immunoassay techniques: it is label-free, hence no external chemicals or tags are needed thus reducing the risk of contamination, has been designed to function as a portable device and uses nonsilicon fabrication processes that enables a low cost of approximately US$1 per sensor (fabricated in batches of 1000) for manufacturing versus the traditional Beckman Access system which can cost >US$250 per test. In addition to these advantages, it has a rapid response time of <3 min after sample addition, requires low sample volumes (~100 µl per test) for detection and has demonstrated ultra-sensitivity; with detection sensitivity in the pg/ml regime for PSA. For cancer diagnostics, detection of multiple protein biomarkers from small volumes in a rapid manner is important for cost-effective disease diagnosis. The sensor technology has potential for 'point-of-care' disease diagnostics, as it is highly portable and inexpensive. There is a great promise for this technology in the areas of laboratory clinical diagnostics, healthcare and in pharmaceutical sectors.

Future perspective

The nanowell biosensor designed for PSA sensing has the sensitivity to detect PSA and potentially distinguish the different isoforms of PSA through the implementation of multisized nanoporous structure (for size based biomarker diffusion) or antibodies with specific binding pockets (frequency based detection at specific heights in the EDL). Ultimately, the key to better diagnosis of prostate cancer is finding more effective ways to use PSA as a screening tool – ultimately, making PSA a better prostate cancer detection tool in the modestly-elevated PSA range. PSA is very specific for prostate cancer over 10 ng/ml, detection of PSA between 4 and 10 ng/ml is typically associated with the lack of specificity in diagnosis. Strategically, an improved PSA test capable of detecting multiple biomarkers and different isoforms of markers could contribute in clinical situations, such as detecting aggressive prostate cancer, which would more likely affect a man's quality or length of life, and in identifying indolent forms of prostate cancer, which probably would not affect a man's quality or length of life. The nanowell biosensor demonstrates the potential of designing a rapid POC test that can be administered in a physician's office and potentially be self-administered for longitudinal monitoring.

Financial & competing interests disclosure

TW Barrett and S Prasad have a significant interest in Pacific Nanoscience Inc., a company that may have a commercial interest in the results of this research and technology. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Ethical conduct

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

Nanowell biosensor design & fabrication

- Mimics macromolecular crowding in biological cells to maintain prostate-specific antigen (PSA) efficacy while detection.
- Provides size exclusion for reducing nonspecific molecules in the electrical double layer.

Debye length & charge screening effects

- Debye length is enhanced within the nanowells that mitigates charge screening effects due to highly ionic human serum and bulk solution resistance.

Nonfaradaic electrochemical impedance spectroscopy

- Label-free biosensing has been demonstrated using a nonfaradaic or nonredox based implementation of electrochemical impedance spectroscopy. The technique works uniquely with the achieved charge screening to detect binding events on the electrode surface without the need for a label or external charged molecule.

Detection of PSA in human serum samples

- Detection of PSA was achieved over the range of 0.0001–10,000 ng/ml with 0.95% correlation with the predicate Beckman Access system.

References

Papers of special note have been highlighted as: • of interest

1 Siegel R, Ma J, Zou Z, Jemal A. Cancer statistics, 2014. *CA Cancer J. Clin.* 64(1), 9–29 (2014).

2 Hassanein M, Callison JC, Callaway-Lane C, Aldrich MC, Grogan EL, Massion PP. The state of molecular biomarkers for the early detection of lung cancer. *Cancer Prev. Res.* 5(8), 992–1006 (2012).

3 Varambally S, Yu J, Laxman B *et al.* Integrative genomic and proteomic analysis of prostate cancer reveals signatures of metastatic progression. *Cancer Cell* 8(5), 393–406 (2005).

4 Wulfkuhle J, Espina V, Liotta L, Petricoin E. Genomic and proteomic technologies for individualisation and improvement of cancer treatment. *Eur. J. Cancer* 40(17), 2623–2632 (2004).

5 Wulfkuhle JD, Liotta LA, Petricoin EF. Proteomic applications for the early detection of cancer. *Nat. Rev. Cancer* 3(4), 267–275 (2003).

• **Reviews the value of proteomic analysis for early diagnoses of prostate cancers. The ability of physicians to effectively treat and cure cancer is directly dependent on their ability to detect cancers at their earliest stages. Proteomic analyses of early-stage cancers have provided new insights into the changes that occur in the early phases of tumorigenesis and represent a new resource of candidate biomarkers for early-stage disease.**

6 Smith RA, Manassaram-Baptiste D, Brooks D *et al.* Cancer screening in the united states, 2014: a review of current american cancer society guidelines and current issues in cancer screening. *CA Cancer J. Clin.* 64(1), 30–51 (2014).

7 Koss LG. The papanicolaou test for cervical cancer detection: a triumph and a tragedy. *JAMA* 261(5), 737–743 (1989).

8 Bleyer A, Welch HG. Effect of three decades of screening mammography on breast-cancer incidence. *N. Engl. J. Med.* 367(21), 1998–2005 (2012).

9 Partin A, Yoo J, Carter HB *et al.* The use of prostate specific antigen, clinical stage and gleason score to predict pathological stage in men with localized prostate cancer. *J. Urol.* 150(1), 110–114 (1993).

10 Mandel JS, Bond JH, Church TR *et al.* Reducing mortality from colorectal cancer by screening for fecal occult blood. *N. Engl. J. Med.* 328(19), 1365–1371 (1993).

11 Karita M, Tada M, Okita K, Kodama T. Endoscopic therapy for early colon cancer: the strip biopsy resection technique. *Gastrointest. Endosc.* 37(2), 128–132 (1991).

12 Merkoçi A, Braga S, Fàbregas E, Alegret S. A potentiometric biosensor for D-amygdalin based on a consolidated biocomposite membrane. *Analyt. Chim. Acta* 391(1), 65–72 (1999).

13 Albareda-Sirvent M, Merkoci A, Alegret S. Configurations used in the design of screen-printed enzymatic biosensors. A review. *Sens. Actuators B Chem.* 69(1), 153–163 (2000).

• **The referenced papers demonstrate previous work with the use of nanomaterials and electrodes toward biosensing.**

14 Stortini A, Moretto L, Mardegan A, Ongaro M, Ugo P. Arrays of copper nanowire electrodes: preparation, characterization and application as nitrate sensor. *Sens. Actuators B Chem.* 207, 186–192 (2015).

15 Daniels JS, Pourmand N. Label-free impedance biosensors: opportunities and challenges. *Electroanalysis* 19(12), 1239–1257 (2007).

• **Review on the use of label-free impedance spectroscopy and its application is presented in this journal article. The basic parameters affecting impedance biosensors, validity of circuit models are presented.**

16 Wang Y, Xu H, Zhang J, Li G. Electrochemical sensors for clinic analysis. *Sensors* 8(4), 2043–2081 (2008).

17 Selvam AP, Wong J, Flanagan K, Prasad S. Cellular level classification of breast cancer through proteomic markers using nanochannel array sensors. *Nanomedicine* 1–13 (2013).

18 Stern E, Vacic A, Li C *et al.* A nanoelectronic enzyme-linked immunosorbent assay for detection of proteins in physiological solutions. *Small* 6(2), 232–238 (2010).

19 Panneer Selvam A, Prasad S. Nanosensor electrical immunoassay for quantitative detection of nt-pro brain natriuretic peptide. *Future Cardiol.* 9(1), 137–147 (2013).

20 Sorgenfrei S, Chiu C-Y, Johnston M, Nuckolls C, Shepard KL. Debye screening in single-molecule carbon nanotube field-effect sensors. *Nano Lett.* 11(9), 3739–3743 (2011).

- **Authors study the effect of in ionic screening from charged buffers on single-molecule electronic sensors. Both charge in proximity to the defect site and buffer concentration are found to affect noise amplitude in a manner that follows from simple Debye length considerations.**

21 Zhang G-J, Zhang G, Chua JH *et al.* DNA sensing by silicon nanowire: charge layer distance dependence. *Nano Lett.* 8(4), 1066–1070 (2008).

22 Hall D, Minton AP. Macromolecular crowding: qualitative and semiquantitative successes, quantitative challenges. *Biochim. Biophys. Acta* 1649(2), 127–139 (2003).

23 Minton AP. Models for excluded volume interaction between an unfolded protein and rigid macromolecular cosolutes: macromolecular crowding and protein stability revisited. *Biophys. J.* 88(2), 971–985 (2005).

24 Minton AP. Macromolecular crowding. *Curr. Biol.* 16(8), R269–R271 (2006).

25 Simon P, Gogotsi Y. Charge storage mechanism in nanoporous carbons and its consequence for electrical double layer capacitors. *Philos. Trans. A Math. Phys. Eng. Sci.* 368(1923), 3457–3467 2010).

- **Presents an explanation on the modification to the electrical double layer in the presence of nanoporous scaffolds. This is critical for implementing the frequency-modulated impedance spectroscopy at the electrical double layer.**

26 Bothara M, Venkatraman V, Reddy RK, Barrett T, Carruthers J, Prasad S. Nanomonitors: electrical immunoassays for protein biomarker profiling. *Nanomedicine (Lond.)* 3(4), 423–436 (2008).

27 Thompson IM, Pauler DK, Goodman PJ *et al.* Prevalence of prostate cancer among men with a prostate-specific antigen level ≤4.0 ng per milliliter. *N. Engl. J. Med.* 350(22), 2239–2246 (2004).

28 Panneer Selvam A, Vattipalli KM, Prasad S. Design of a high sensitive non-faradaic impedimetric sensor. Presented at: *Engineering in Medicine and Biology Society (EMBC), 2012 Annual International Conference of the IEEE.* CA, USA, 28 August–1 September 2012.

- **Paper that explores the technique of label-free impedance spectroscopy with a major focus toward electrical characterization of the immunoassay on nonfaradaic biosensors. A circuit model for nonfaradaic biosensing and relevance to the detection process is presented.**

29 Vattipalli K, Feikert P, Brandigampala S, Prasad S. Study of nanoporous membranes with applications in the enhanced detection of cadiovascular biomarker proteins. *Nano Life* 1(03n04), 175–183 (2010).