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# Detection, Quantification, and Profiling of PSA: Current Microarray Technologies and Future Directions

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Prostate cancer (PCa) is a cancer of prostate gland. The death rate of 13% among the men diagnosed with PCa makes it a second leading cause of cancer death. It has been reported that the monitoring of progression of PCa and response to therapy is done by the measuring the level of blood PSA. Even though, PSA was used for the diagnosis of PCa, the current scenario dictates the necessity of simultaneous detection of more than one biomarker. This critical review evaluates the DNA microarray and Protein microarrays based methods reported in last five years for the detection, quantification, and profiling of PSA.

## 1. Introduction

Prostate cancer (PCa) is a cancer of prostate gland.[1, 2] A person with a family history of PCa has double chances of getting it than that of a person without family history of a PCa.[3] The genetic mutations in the CHEK2 gene and genetic polymorphism in interleukin-1A are associated with the increased risk of PCa.[4,5] According to CDC report, PCa is a most common cancer in United States after skin cancer in men. It is the second leading cause of male death due to cancer in United States.[6]

It was reported in 2013 that approximately 0.2 million living American men have PCa.[6] The death rate among the men diagnosed with PCa is about 13%.[7] PCa is by far the most commonly diagnosed cancer and a second leading cause of cancer death in men. It is reported that the 5 year survival rate of PCa patients in T (tumour), N (spread of PCa to the lymph nodes), and M (metastasis) stages are 100%, 100%, and 28%, respectively.[8] It is important to notice that the early detection and treatment of PCa provides a greatest chance of cure. However, testing for the early detection of PCa remains a source of uncertainty and controversy.[9] It is important to notice that chances of curing the early stage (localized) cancer are much higher than the chances for curing the advanced (metastatic) cancer. Therefore, developments of technologies, which can enable the accurate detection of the PCa at an early stage with simple experimental protocols are highly inevitable.

Many techniques used for the diagnosis of PCa are based on either of two principles. The first being study of morphology of prostate gland and the second is based on the detection of biomarker proteins.[10,11] PCa detection methods based on morphological approach have several disadvantages. The major disadvantage of these methods is that, it cannot be used as a confirmatory test for PCa.[12] The other disadvantage of this method is that it cannot differentiate between PCa and benign prostate hyperplasia (BPH).[13]

Several biomarkers including prostate specific antigen (PSA), are reported for the diagnosis of PCa.[14] However, detection of PSA is a widely accepted approach for the diagnosis of PCa. PSA, a member of the tissue kallikrein family is an androgen-regulated serine protease generated by prostate epithelial cells and PCa.[15] The main function of PSA in the seminal fluids is to cleave the semenogelins. An inactive proPSA is activated to PSA by cleavage of seven N-terminal amino acids after its secretion into prostatic ducts. Protease inhibitors such as *alpha 1*-antichymotrypsin (α1AC) take up the PSA that enters in the circulation and inactivates it to free PSA (fPSA) by proteolysis. When PCa strikes, the proteolytic inactivation and the cleavage of proPSA to PSA is less efficient which results in the increase in the serum total PSA (tPSA) levels.[16,17] It has been reported that the monitoring of progression of PCa and response to therapy is done by the measuring the level of blood PSA, which is a most sensitive maker studied for several years.[18]

This critical review evaluates the current microarray methods and technologies including DNA microarrays and protein microarrays used for the detection of PSA.[19] The aim of this article is to provide a comprehensive review covering the advantages and disadvantages of these PSA detection methods. Emphasis is given for the latest developments microarray technologies for achieving high selectivity and sensitivity for the detection of PSA, thus the early detection of PCa.

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## 2. Label free detection of PSA

There are several reported methods for the label free detection of PSA based on DNA and protein microarrays. It is well known that the formation of a biomolecular complex of the antigen with the antibodies can be affected by the changes in their shapes. Interestingly, one of the advantages of label free detection is that the three dimensional structures of the capture antibodies (cAb) and detection antibodies (dAb) are preserved during the PSA detection process. Label free detection allow the real time monitoring of PSA, because the detection is based on physical, electrical and optical properties of the biomolecular complexes.

### 2.1 DNA Microarray

DNA microarrays have long been used for the detection of genomic DNAs in pathogens. However, DNA microarrays have also been used for the detection of protein biomarkers. The stability of the immobilized DNAs and their three dimensional structures have been exploited for their ability in the detection of biomarkers such as PSA.

#### 2.1.1 Aptamer based PSA detection

The electrochemical detection of biomarkers has been of a great interest to many researchers for the reason that it is one of the label-free detection methods. Recently, aptamer microarray based detection of PSA has been reported.[20]

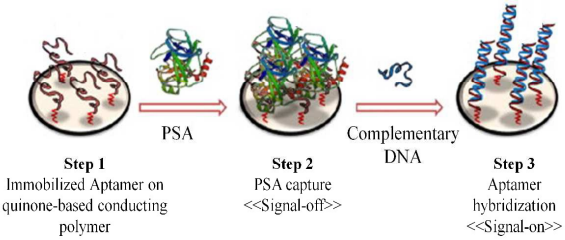



**Figure 1** Working principle for the "off–on" detection of PSA using an aptamer capture probe, followed by hybridization with a complementary sequence. (Adapted from *Biosens. Bioelectron*. 2015, 15, 68, 49-54).

**Figure 1** depicts the Working principle for the detection of PSA. In this technique a quinone-based polymer platform is used to immobilize the PSA selective aptamers. A current flowing through this platform is measured by Square Wave Voltammetry. A decrease in current (signal-off) is observed upon binding of PSA with the immobilized aptamer. Upon addition of a complementary DNA on the platform it displaces aptamer bound PSA molecules resulting in the increase in current (signal on). This technique allows the detection of PSA in the dynamic detection range 1ng/mL to 1µg/mL. This label-free electrochemical biosensor based on a DNA aptamer demonstrate high specificity for PSA. However, the sensitivity of assay is poor as indicated by the detection limit of 1ng/mL. The major drawback of this method is its detection limit. Even though this biosensor can be used for the diagnosis of PCa, it is not suitable for the prognosis and follow up of a treatment. For the application in treatment follow up, a biosensor must detect the PSA with a concentration as low as 1pg/mL.

### 2. 2 Protein Microarray

Protein microarrays have been used mainly for two primary objectives, i) to study the qualitative properties of proteins, enzymes etc. and ii) quantitative detection of biomarkers, which has been applied in a range of cancer studies.[21] Label-free detection of PSA on protein microarray avoid the interference due to the labelling of detection antibodies and thus determine the reaction kinetics of biomolecular interactions in real-time.

#### 2.2.1 BioCD protein array

BioCD protein arrays for protein detection are based on the optical interferometry measurement of the protein surface density, which provides quantitative and consistent signals. Thus this technique do not require a fluorophore. For the detection of PSA, cAbs previously immobilized on the BioCD platform are allowed to incubate with the solution containing dAB and target antigen to allow the formation of a sandwich type biomolecular complex cAb-PSA-dAb.

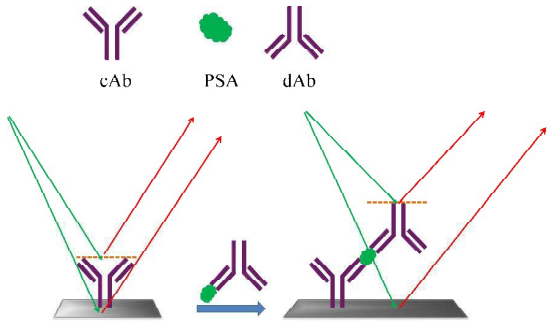



**Figure 2** Interferometric techniques measure the phase differences of the wave fronts and convert it into observable visible intensity fluctuation known as interference fringes.

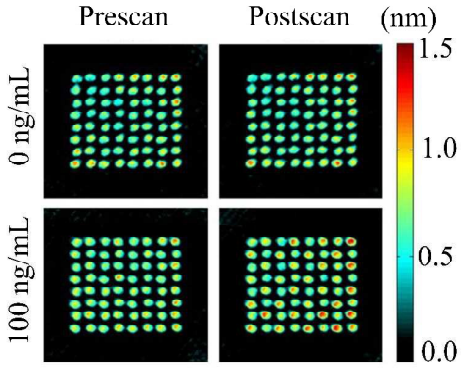



**Figure 3** Protein spot mass profile (protein layer height in units of nm) of each well derived from the reflectance map. (Adapted from *Biosens Bioelectron.*, 2011, 26, 1871–1875).

As shown in the **Figure 2**, the difference between the refractive index before and after incubation is used for the quantification of the captured antigen. The dAB is essentially used to enhance the assay response through the added mass of the dAB.

Scanning of a BioCD platform allow to produce an image as shown in the **Figure 3**.[22] One of the major advantage of the BioCD is that it allows the accurate measurement of PSA in high-level protein backgrounds.[23] BioCD also eliminate the restriction of fluorescence-free biosensors from high background

applications. Thus, BioCD platform appears to have a potential for clinical applications.

The BioCD protein microarray achieved the detection limit of 4ng/mL in clinical samples. Similar to that of the previously discussed aptamer biosensor the BioCD protein array cannot be used for the treatment follow up after the radical prostatectomy. The use of blocking proteins like BSA to reduce the background noise results in the increase in mass of final product and may lead to false results.[24]

### 2.2.2 Nanowire sensor

Nanowire based sensors are reported for the analysis of DNA, proteins, viruses etc. Among the several electrical detection methods, nanowire sensors are highly specific, sensitive, and cost effective.[25,26,27] Recent research on new nanomaterials for biomolecule analysis resulted in the development of silicon nanowires (SiNWs), which are configured as field-effect transistors (FETs). The SiNW as shown in the **Figure 4**, are one of the powerful platforms which allow the label-free, real-time electrical detection of proteins with very high sensitivity.[28,29,30]

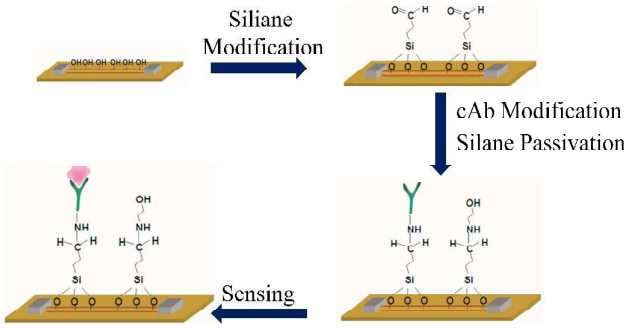



**Figure 4** Antibody modification at SiNW surface, including: silane modification, antibody modification, silane passivation and sensing steps.

The SiNWs based FET biosensors have the potential of label-free electrical detection with the excellent specificity and sensitivity. In SiNWs based FET biosensors, the cAbs immobilized on the surface of the SiNWs are used to capture the respective biomarker proteins. The direction of change in conductance indicates the presence of charge carriers in the semi-conductive channel. The magnitude of the variation in the conductance depends on the binding events of the biomarker with immobilized cAb, which intern used for the measurement of the concentration of a biomarker. For example, binding of the positively charged PSA molecules with the immobilized cAbs on the SiNWs, results in a decrease in the current of an FET device.

Thus, as shown in the **Figure 5**, the decrease in the current is directly correlated with the concentration of the PSA in the samples. It is reported that, in the desalted human serum samples the limit of detection of this device for PSA is 5fg/mL, which is remarkable.[30] Therefore, this biosensor appears to be a very promising candidate for assessing and monitoring the medication therapy, as well as predicting the recurrence of cancer. The SiNWs based FET biosensors are also able to detect multiple biomarkers at a time.

It is very important to note that the sensitivity of SiNWs based FET biosensors is highly depend on the pH and ionic concentration of solution. The change in concentrations of ionic species other than the biomarker protein will severely affect the sensitivity of this device. Therefore, the pretreatment of samples such as filtration, desalting, and buffer exchange is essential.

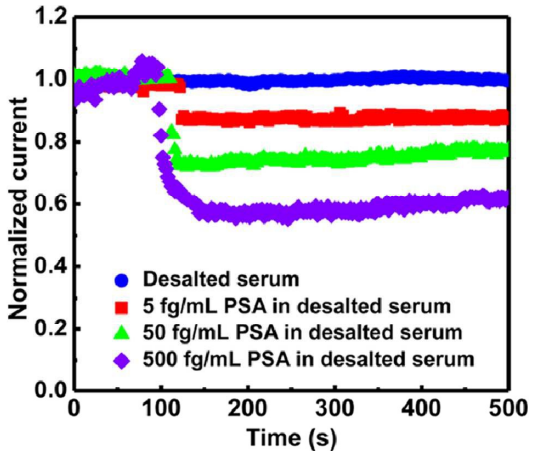
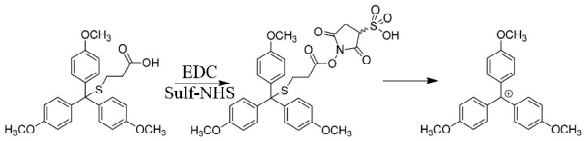



**Figure 5** Normalized current with respect to various PSA concentrations in desalted human serum. Desalted serum without PSA exhibited no electrical response. (Adapted from *Anal. Chem.,* 2013, 85, 7912−7918).

In another example of real time protein detection, a biosensor is based on an electrostatically formed nanowire is reported to detect the PSA at a concentration of 100fg/mL. The electrostatically formed nanowire biosensor is different from a conventional silicon nanowire with respect to the confinement potential, charge carrier distribution, surface states, dopant distribution, moveable channel, and geometrical structure.[31] Though the electrostatically formed nanosensor are label free, sensitive, and can detect PSA in fg/mL concentration, they cannot be used to detect multiple biomarkers at a time. Furthermore, the sensitivity of the instrument is highly depend on the pH of the solution.

### 2.2.3 Mass-tag mass spectroscopy

In recent years, photo-cleavable mass-tags as probes in matrix-assisted laser desorption/ionization-mass spectrometry (LDI-MS) are used for detection of biomarkers such as PSA. The trityl(3-[tris(4methoxyphenyl)methylsulfanyl]propanoic acid) tags as shown in the **Scheme 1** are photo labile trityl thioesters which are connected to the probe or carrier via its free amino groups.[32,33,34,35] The tags connected to the probe or carrier are ionized by radiation. The ionized tags which are very stable cations are then detected by the LDI-MS.




**Scheme 1** Synthesis of the mass-tag, its activation, and the resulting carbocation.

The major advantage of mass-tags is that any size of a targeted biomolecule can be tagged thus there is no need of target protein digestion before use. Moreover, the target protein can be sensitively detected irrespective of its abundance as compared to other proteins in the sample.

Recently, the LDI-MS is used for the detection of PSA in the clinical samples.[36]

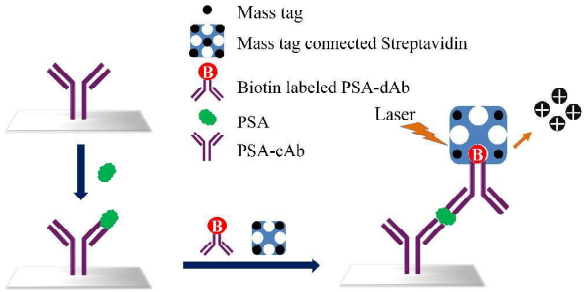



**Figure 6** Normalized current with respect to various PSA concentrations in desalted human serum. Desalted serum without PSA exhibited no electrical response. (Adapted from *Anal. Chem.*, 2013, 85, 7912−7918).

In a first stage of sandwich assay as shown in the **Figure 6**, the PSA in a sample is allowed to form a complex with cAbs immobilized on the silicon surface. Whereas in second step, the biotinylated dAb is incubated with mass-tagged avidin to form an immuno-complex and then incubated on the PSA-cAb complex formed in the first step. When subjected to laser irradiation, the mass-tags are ionized, released, and recorded by the mass detector.

In a non-sandwich assay, PSA can be directly detected if it is allowed to connect to the mass tags and captured by the immobilized cAbs, the release of ionized mass-tags upon laser irradiation can be used to detection the concentration of PSA in the sample. In such a non-sandwich assay, PSA was detected with the concentration of 5μg/mL. However, in a sandwich assay in which the biotin labelled dAb and mass-tab connected streptavidin are used for the signal amplification allowed to detect the PSA with a concentration of 186pg/mL. A double amplification effect which involves multiple avidin-biotin interactions endows antibody-mass-tag system with a very low LOD. Several tag molecules can be bound to avidin, every antibody has several biotin residues which can bind to an avidin complexed with the tagged molecules, thus formation of multiple complexes of biotin labelled antibody with mass-tagged avidin results in a signal amplification.

It is important to note that the process of tag conjugation need an overnight incubation step. Furthermore the variations in reaction conditions can lead to the variation in results. The variation in the tags per molecule have also been reported which could directly affect the specificity of this approach.

### 2.2.4 Dip-pen Lithography

Techniques used for the fabrication of protein microarray includes inkjet printing,[37] electron beam lithography,[38] and photolithography.[39] The Dip-pen nanolithography (DPN) is also exploited for the fabrication of miniaturized protein microarrays.[40] The biomarker proteins can be detected by various spectrophotometric methods including Raman spectroscopy, which is known for its various applications in many fields. According to the recent report, Raman spectroscopy has been successfully used for the detection of PSA in serum samples on the miniaturized microscale arrays fabricated by dip-pen nanolithography.[41] The advantages of DPN for the fabrication of microarray are its accuracy, use of easily available material, higher resolution, cost effectiveness, and possibility of high scale fabrication.[42]

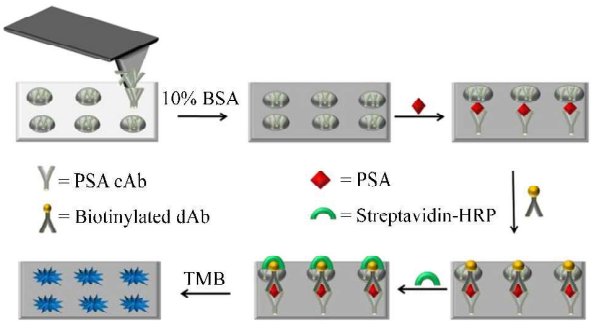



**Figure 7** Schematic representation of the PSA assay development. (Adapted from *Anal. Chem. 2013, 85, 5617−5621*).

As depicted in the **Figure 7**, the cAb is printed using DPN on the nitrocellulose surface. Remaining nitrocellulose surface is blocked with BSA to obtain the miniaturized microscale array. For the detection of PSA, a sample containing PSA is added on the microarray followed by the addition of biotinylated dAb, streptavidin-HRP, and TMB to complete the assay. The HRP converts TMB to a charge transfer complex indicated by formation of a blue precipitate around the spots. The microarrays are then analysed by Raman spectroscopy. This technique allows the ultrasensitive detection of PSA as intensity of the Raman signal has linear relationship with the concentration of PSA. The reported detection limit of this assay is 25 pg/mL. Because the detection limit is well below the normal levels of PSA (4−10 ng/mL) found in blood, this assay can be applicable for diagnosis of PCa in the early stages of post-surgery recurrence. It is important to note that during laser irradiation a care must be taken to prevent degradation of nitrocellulose surface.

### 2.2.5 Electrochemical detection

Electrochemical biosensor has always attracted wide scientific community for their sensitivity, low cost, and simple experimental protocols. In a recent report,[43] a simple and novel electrochemical multiplexed immunosensor on a flexible polydimethylsiloxane (PDMS) slice deposited with 8 × 8 nano-Au film electrodes was used for the simultaneous detection of PSA, prostate specific membrane antigen (PSMA), and interleukin-6 (IL-6).[44,45,46] The construction of flexible microchip array and the further sensing approach is depicted in the **Figure 8**.

In this biosensor, cAb linked magnetic beads are attached to the nano-Au film electrodes via magnetic force. When the solution containing respective antigen, horse radish peroxidase

and dAb–conjugated gold nanorods (HRP-Ab2-gold NRs) is loaded on the biosensor a sandwich complex cAb-antigen-HRP-Ab2-gold NRs is formed, which then reduces the $H_2O_2$ resulting in the electrochemical signals.

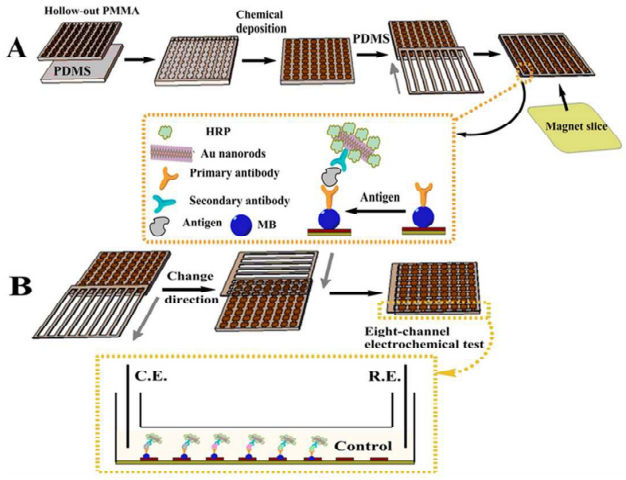



**Figure 8** (A) Preparation of the flexible microchip and the construction of multiplexed immunoassay interface; (B) Sketch map of eight spots in one microchannel in detection of three antigens for prostate cancer. (Adapted from *ACS Appl. Mater. Interfaces* 2014, 6, 20137−20143).

The immunosensor provide good performance with respect to the sensitivity and specificity during the multiplex detection of above mentioned biomarkers. The chemical deposition method used for this biosensor is reproducible and needs very less instrumentation. The linear relation observed between the PSA concentration and current allows the quantitative measurement of the PSA concentration in the serum samples. The reported detection limit of this method for PSA detection is 100pg/mL. In contrast to the nanowire biosensor this detection limit is very high. This indicates that the signal amplification strategy used in this biosensor do not allow as sensitive detection as that of the SiNWs based FETs. The detection process is highly sensitive to the change in pH, which is a common drawback of the electrochemical biosensors.

## 3. Label based detection of PSA

Labelling of dAb with fluorescent molecules, enzymes, etc. is a common method adopted in many label based PSA detection methods.

### 3.1 DNA microarray

Almost in all cases of protein detection, decrease in the specificity of the immobilized cAb over a period shadows the sensitivity and specificity of a biosensor. It is well accepted that the approach of a direct immobilization of the cAb on the surface results in the loss of specificity of cAb over a period. Thus the non-specific interactions of cAb with non-target biomarkers results in the in the low sensitivity of the respective biosensor device. [47] Therefore, to solve these problems several approaches such as DNA nanostructures, DNA directed immobilization (DDI), [48] DNA-Guided Detection (DAGON) method [49] which provide lateral and vertical spacing have been exploited.

#### 3.1.1 DNA Nanostructure-Based Universal Microarray

The fabrication of high-performance biological microarrays greatly depends on the approach used for the immobilization of biomolecules on a solid support. According to a recently reported, a self-assembly of four oligonucleotides allowed formation of DNA tetrahedra carrying three amino groups on three vertices and one single-stranded DNA extension on the fourth vertex. [50]

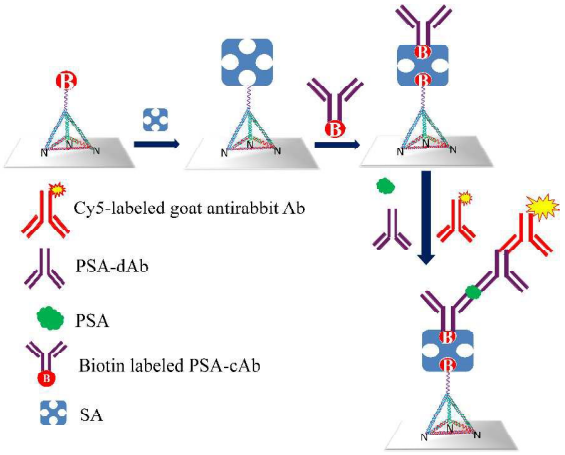



**Figure 9** DNA Nanostructure-Based Universal Microarray for PSA detection.

As shown in **Figure 9**, the DNA tetrahedron-based microarrays were fabricated by covalent coupling of DNA tetrahedron on the glass substrate. The DNA tetrahedron microarrays were utilized for the analysis of cancer biomarkers such as PSA. The array demonstrated good capability to anchor capture biomolecules for improving bio-recognition. The reported limit of detection for PSA by this method is 40pg/mL. It is important to note that the DNA nanostructured based microarray platform can detect the PSA in clinical serum samples with good relativity to the results of conventional chemi-luminescent immunoassay.

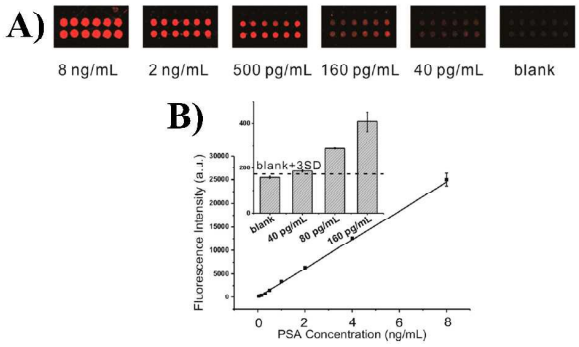



**Figure 10** A) Microscopic fluorescence images of DNA tetrahedron-based microarrays for PSA assay, B) Linear calibration curve of PSA assay (Inset: Histogram showing the LOD of PSA assay).

It is important to notice that this bioassay demonstrates the linear correlation between the signal and PSA concentration. As shown in the **Figure 10**, the dynamic detection range of this assay is claimed to be from 40pg/mL – 8ng/mL. The signal intensity difference between the blank and 40pg/mL is statistically reasonable. However, variations in the assay conditions may lead to the false positive results. The other drawback of this method is the decrease in the hybridization efficiency as a result of distortion in the tetrahedral geometry of immobilized DNA tetrahedron.

### 3.1.2 DDI and DAGON Technology

As mentioned earlier the surface immobilization of cAb is key for high sensitivity and specificity of the detection platform.[58] Conventional immobilization techniques are either based on the physical adsorption, covalent bonding, or non-covalent interaction between streptavidin and biotin.[51] Each of these methods have their pros and cons. The steric crowding of immobilized cAbs results in two problems, firstly sterically crowded cAbs demonstrate non-specific interaction and secondly, immobilized cAbs degrade over a period resulting in the decrease in the sensitivity of the platform.[52]

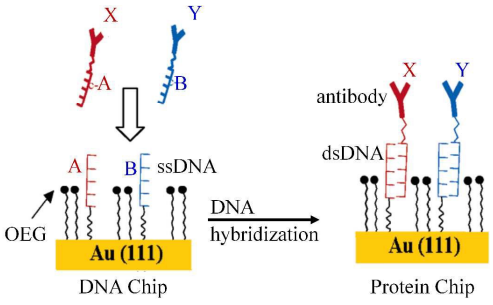



**Figure 11** DNA directed immobilization of cAb.

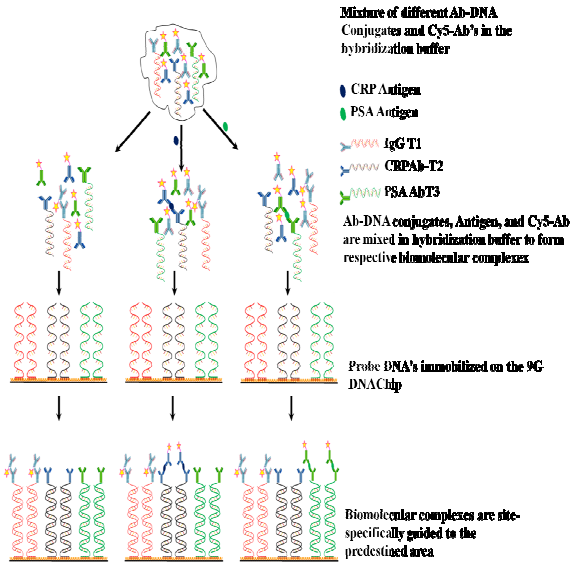



**Figure 12** Multiplex detection of various biomarkers including PSA by DAGON method on a 9G DNAChip.

To avoid the non-specific binding due to steric crowding, DNA directed immobilization (DDI) which provides lateral spacing in immobilized cAbs, as shown in the **Figure 11** was introduced. The immobilization of proteins through DNA-DNA hybridization has advantages of fast immobilization which can be done at room temperature, less steric interaction between nearby proteins.[53] The detection limit of this method is 100pg/mL.[54,55] The disadvantage of this method is that the cAbs should be immobilized on the surface.

The DNA guided detection (DAGON) method based on the 9GDNA technology was reported recently. In this method a 9G DNAChip was used as a platform for detection of PSA. The 9G DNAChips are obtained by the immobilization of the probe DNAs appended with the nine consecutive guanines (9G) on the aminocalix[4]arene modified glass surface.[56,57,58,59,60]

The immobilized DNA maintains the vertical as well as longitudinal spacing. As shown in **Figure12**, the protein detection do not need cAbs to be previously immobilized. In the DAGON technology, the cAbs are conjugated to oligonucleotide which are complimentary to probes on chip. The conjugation of amine group of cAb and amine modified oligonucleotide ($NH_2$-DNA) is done by using 2-iminothiolane and sulpho-SMCC as linker to produce PSAcAb-DNA conjugate. For detection PSA, the PSAcAb-DNA, PSAAg and Cy5-labeled PSAdAb are mixed in the hybridization buffer to allow the formation of a biomolecular complex (Cy5-PSAdAb-PSAAg-PSAcAb-DNA). Then the solution is loaded on the 9G DNAChips for final detection. As shown in the **Figure 13**, DAGON method allows the multiplex detection of various biomarkers at a time in a dynamic detection range of 1ng/mL to 1pg/mL.

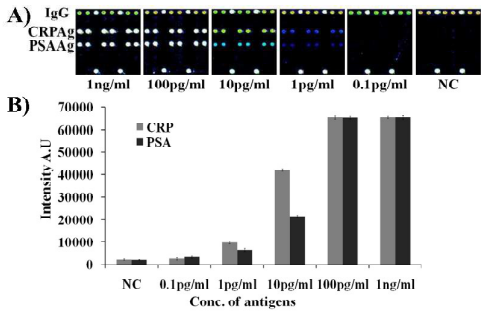



**Figure 13** Multiplex detection of various biomarkers (1ng/mL – 0.1pg/mL) on 9G DNAChip.

It is reported earlier that the 9G DNA technology allows DNA-DNA hybridization with 80% efficiency in less than 30min on 9G DNAChips. This this method is fast, sensitive, and allows specific detection of biomarkers. Reported detection limit of this method is 1pg/mL, which is 1000 time sensitive than other reported methods.[61]

### 3.2 Protein Microarray

The high-throughput technology for labelled detection of biomarkers on protein microarray was relatively easy to develop since it is based on the technology developed for DNA microarrays. Thus, for the fabrication of protein microarrays by immobilizing cAbs on the surface, several covalent attachment chemistries involving various functional groups on the surface

have been exploited. It is reported that the conventional immobilization methods can result in the loss of the activity of the immobilized cAbs.[62,63] Current technologies used in the protein microarrays allow the high-throughput screening and detection of a multiple biomarkers on a single platform.
Several protein microarray based biomarker detection methods suffer from a common drawback of high LOD, which is generally in nanomolar range [111]. Detection of only one biomarker at a time is another drawback of conventional biomarker detection methods. These drawback limits the use of newly developed protein microarray technologies [112].

### 3.2.1 Porous silicon (P-Si) microarray

Recently, a protein microarray based on the porous silicon (P-Si) is reported for the detection of PSA. Due to large surface area and higher capture capacity P-Si is a good choice for the cAb immobilization.[64,65,66] As shown in the **Figure 14**, the cAbs are immobilized on the P-Si surface by physisorption.[67] It is claimed that enriching the surface density of immobilized cAbs enhances the detection signal of antibody sandwich microarrays.

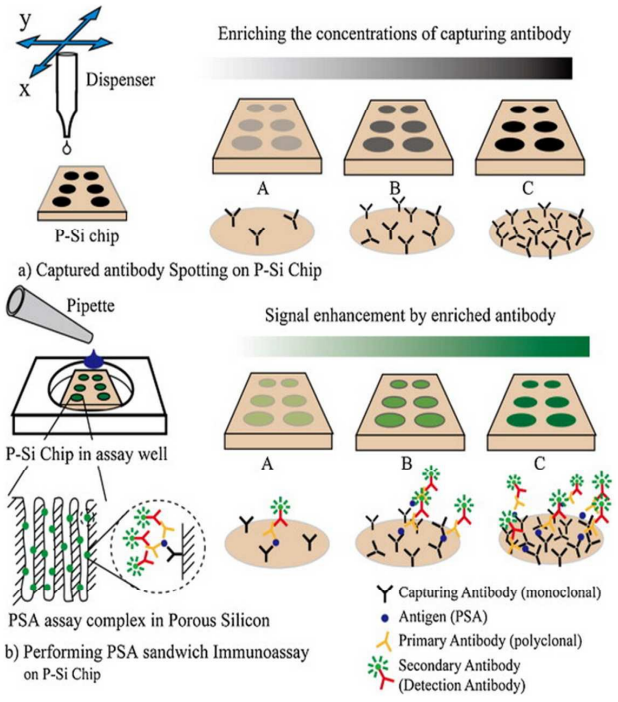



**Figure 14** Fabrication and application of P-Si chip for PSA detection. (a) By increasing the concentration of cAb the surface density of the spotted cAb can be increased. (b) Higher density of cAb microarray allows enhanced signal readouts. (Adapted from *Anal Chim Acta. 2013, 796, 108-14*).

Moreover, it is considered that the P-Si maintains the native structure of protein as method of physical adsorption is used for the immobilization of cAbs. The hydrophobic P-Si surface favours the immobilization of cAbs. The approach of P-Si based microarray has great advantage as it does not require any chemical treatment for antibody immobilization. Furthermore, this approach does not require any laborious procedures such as incubation, humidity control, and temperature control.[77,68]

In simultaneous detection of fPSA, tPSA on P-Si microarray, the detection limit for tPSA and fPSA were reported as 140pg/mL and 760pg/mL, respectively.[69] However, by improving the capacity of immobilized cAb, a pronounced effect on the intensity was observed. As shown in **Figure 15**, increased amount of immobilized cAbs results in the pronounced increases in the signal intensities which further lower the detection limits to 800fg/mL PSA.[70]

Though the freshly prepared microarray demonstrate excellent performance, a shelf-life study is essential for evaluation of this technique for the commercial clinical applications. The physical adsorption coupled with high dose of cAb may make this microarray prone to steric crowding, which in turn leads to non-specific interactions.

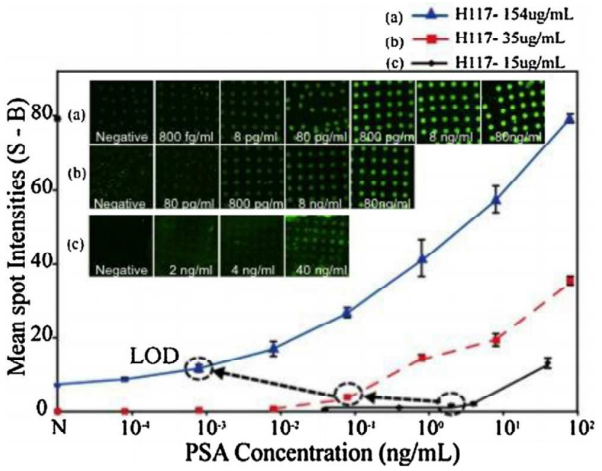



**Figure 15** Effect of increased concentration of immobilized cAb on the PSA detection sensitivity of P-Si protein microarray. (Adapted from *Anal Chim Acta. 2013, 796, 108-14*).

### 3.2.2 Acoustic whole blood plasmapheresis chip

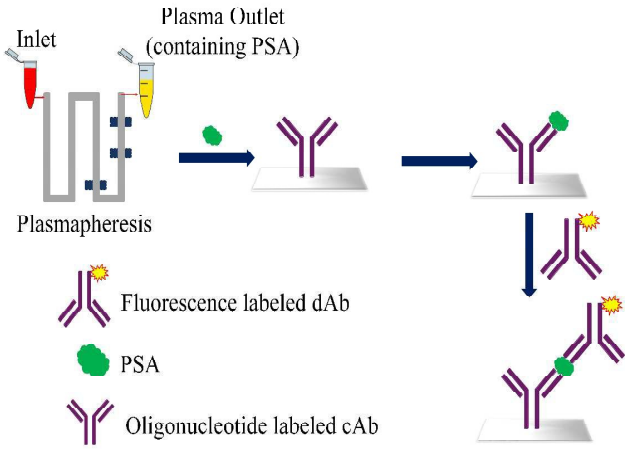



**Figure 16** Blood purification followed by protein detection.

To be a potential point-of-care detection platform, detection of biomarkers in the whole blood or blood serum is very important. Most of the reported techniques do not allow to use the direct

blood sample. Because blood sample contain large amount of blood cells and other cellular contents. The major challenge faced by many reported microfluidic separation devices is to process fluids with high cell content, such as whole blood. Therefore, biomedical analyses and clinical diagnostic methods can be improved by a method which can generate high quality plasma from whole blood. Recently reported acoustophoresis based separation chip prepares the diagnostic plasma by acoustic separator.[71] The acoustic separator allows the removal of the blood cells in multiple steps to yield high quality plasma, which is then directly loaded on a detection platform. As shown in the **Figure 16**, by directly connecting the plasmapheresis microchip to the porous silicon sandwich antibody microarray chip allows the highly sensitive and specific detection of PSA. PSA was detected by good linearity in the generated plasma via fluorescence readout without any signal amplification at levels of 0.19ng/mL to 21.8ng/mL. It is important to note that the lower limit of detection by this method is much higher than the P-Si protein microarrays, which achieves the LOD of 800fg/mL PSA.

### 3.2.3 Lectin based immunoassay

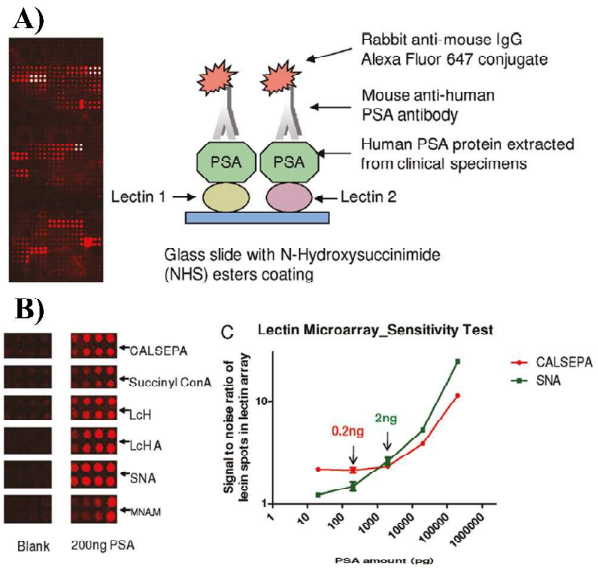



**Figure 17** (A) Lectin microarrays employed in the analysis of target glycoprotein, (B) Binding of lectins to PSA on the lectin microarrays, and (C) sensitivity of a lectin microarray for the detection of lectin PSA interaction. (Adapted from *Anal. Chem. 2011, 83, 8509–8516*).

The lectin microarray is a relatively novel platform for glycan analysis, having emerged only in recent years.[72] Unlike other conventional methods, it enables rapid and highly sensitive detection of glycoproteins. As PSA is a glycoprotein, the analysis of glycan profiling is one of the way for detection and quantification of PSA. Also changes in glycosylation pattern is very much related with the disease state. Thus the detection of glycosylation pattern is also a key to diagnosis. Lectin microarray is well known for glycan profiling. The binding affinity of lectins and glycan are in range of 1-10uM. The study of glycosylation pattern of PSA can be used to differentiate between aggressive cancer (AC) and non-aggressive cancer (NAC).

Recently, a lectin microarray with a two-phase approach is reported for the detection and verification of the glycosylation patterns.[73] As depicted in the **Figure 17**, in a first phase the glycosylation changes are detected by high-density lectin microarrays. In a second phase, a lectin-based immunosorbent assays is used to verify the lectins. This approach was applied to analyse the glycosylation patterns of two glycoproteins PSA and a membrane metallo-endopeptidase (MME), which are highly expressed in PCa from aggressive (AC) and nonaggressive prostate cancer (NAC) tissues.

It is important to note that though this assay demonstrate a linearity in PSA concentration and its increased binding with the immobilized lectins, it is not used for the quantification of PSA. Rather, this assay provides the differences in glycosylation patterns of PSA and MME, which represent a significant clinical importance for diagnosis of aggressive prostate cancer. It is reported that the sensitivity of lectin microarray may be an issue when only a small amount of the target glycoprotein is available. Low sensitivity is attributed to the weak lectin-glycan interaction (dissociation constant, Kd > $10^{-6}$ M).[74]

### 3.2.4 Tyramide Signal Amplification

To overcome the problems associated with the traditional lectin microarray, researchers invented tyramide signal amplification (TSA) technology for the antibody-overlay lectin microarray.[75,76] Reportedly, TSA improved the sensitivity by over 100 times for glycan profiling of PSA. The principle of detection of the target glycoprotein PSA on the lectin microarray using TSA is depicted in the **Figure 18**. After allowing the formation of a complex between the cAB, PSA and biotinylated dAb the microarray is incubated with the streptavidin-labelled HRP (SA-HRP). The HRP catalyses the localized deposition of biotin tyramide, which results in the higher levels of biotin. Multiple biotins attached to the biomolecular complex is then allowed to bind to a Cy3 labelled streptavidin resulting in the signal amplification.

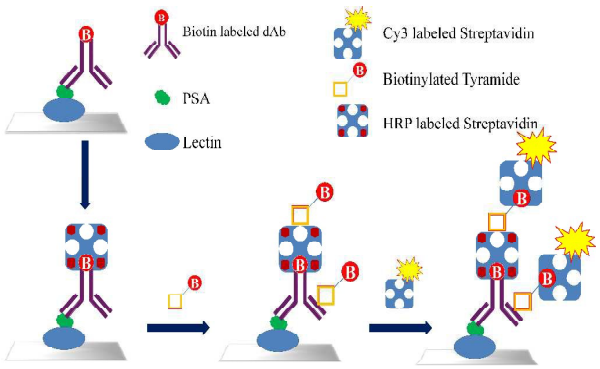



**Figure 18** Scheme depicting the principle of tyramide signal amplification technology for the detection of a glycoprotein on the antibody overlay lectin microarray. (Adapted from *J. Proteome Res. 2011, 10, 1425–1431*).

It is important to note that the TSA, which is a horseradish peroxidase (HRP)-mediated signal amplification method do not require specialized instruments and can be easily integrated into the PSA profiling process of the antibody-overlay lectin microarray. Though this method achieved high sensitivity the specificity remains an issue. The specificity of this assay depends on

overall binding of PSA with the immobilized lectins. Oxidation of immobilized lectins over a period of time may lead to decreased sensitivity and specificity of this assay. The other disadvantage of this method is that it is laborious and time consuming.

**Table 1:** comparison of the methods used for biomarker detection

| Detection Type | Type | Assay Technology | Sensitivity (ng/ml) | Advantages | Drawbacks |
|---|---|---|---|---|---|
| Label free | DNA Microarray | Aptamer based PSA detection | 1 ng/mL | Label free detection<br>High specificity | Lower detection limit<br>Unsuitable for prognosis and follow up of a treatment |
| | Protein Microarray | BioCD protein array | 4 ng/mL | Provides quantitative and consistent signal<br>Decrease in background signal | Cannot be used for treatment follow-up |
| | | Nanowire sensor | 1 fg/mL | Treatment follow-up and recurrence can be monitored | Highly depend on the pH and ionic concentration of solution<br>Requires Sample pre-treatment<br>Multiplex detection is not possible |
| | | Mass-tag mass spectroscopy | 186 pg/mL | No size limitation of the targeted biomolecule,<br>Digestion of the targeted protein is not needed | Variation in the mass tags per molecule affects results<br>Tag conjugation is time consuming process |
| | | Dip-pen Lithography | 25 pg/mL | Accuracy,<br>Possibility of large scale fabrication | Laser irradiation may degradation of nitrocellulose surface |
| | | Electrochemical detection | 100 pg/mL | High sensitivity and specificity<br>Allows quantitative measurement | Highly sensitive to the pH change and buffer condition |
| Label based | DNA microarray | DNA Nanostructure-Based Universal Microarray | 40 pg/mL | Improves bio recognition by increasing anchoring capability | Variation in result may lead to false positive result |
| | | DDI and DAGON Technology | 1 pg/mL | Less steric interaction between nearby proteins<br>Multiplex detection is possible<br>Fast, sensitive and specific | Long term storage of immobilized proteins on DDI results in non-specific interactions |
| | Protein Microarray | Porous silicon (P-Si) microarray | 800 fg/mL | Native structure of protein is maintained<br>No chemical treatment required<br>It do not require any laborious procedure | Physical adsorption of cAbs can result in steric crowding and thus non-specific interaction |
| | | Acoustic whole blood plasmapheresis chip | 190 pg/mL | Allows direct detection of blood sample by purification of cellular debris | Non-specific interactions of immobilized antibodies associated with immobilization method (physical adsorption) |
| | | Lectin based immunoassay | 2 ng/mL | Allows differentiation in glycosylation pattern in proteins | Unsuitable for samples containing less amount of target glycoprotein<br>Low sensitivity |
| | | Tyramide Signal Amplification | 2 ng/mL | TSA 100 times higher signal intensity compared to the detection without TSA | Specificity depends on the overall binding of PSA with the immobilized lectins |

## 4. Future Directions

A comparative summary of the methods discussed in this article is presented in the **Table 1**. The **Table 1** clearly indicate that each method has their own advantages and disadvantages. There have been several reports on the methods which can be applied to reduce or eliminate the various disadvantages related to immobilization of cAbs. According to a recent report, the problem associated with non-specific adsorption can be eliminated by increasing the immobilization efficiency and improving orientation of immobilized proteins.[77,78]

Though there is enormous technological improvement in the field of PSA detection, a fast and low-cost analytical method which can be employed at the bedside of patients is still of the highest interest for clinicians.

Therefore, development of technologies which can detect the PSA at very low concentrations (fg/mL ~ pg/mL) in a very short time are crucial for the monitoring of PCa treatment. Current reports suggest that the emerging biomarkers may be useful for the screening and prognosis of diseases. Therefore, a novel platform should be highly sensitive allowing the fg/mL ~ pg/mL detection of biomarkers as well as it should allow the multiplex detection of various biomarkers at a time. The identification and development of the novel biomarkers will depend on the development of the novel techniques which can detect the biomarker proteins with ultra-low concentrations. The commercial availability of the diagnostic kits based on the technologies explained in this article would have been of great importance. However, most of the products in the market are based on the ELISA based immunoassays. Therefore, considering the available biomarker detection technologies and their advantages and disadvantages there is a huge scope for the development of these and other novel technologies for commercial purposes.

Trends in the clinical diagnostics indicate the necessity of a diagnostic test to be done near the patient sites. The biomarker detection platforms must therefore be adapted for a point-of-care testing, which requires the ability to design affordable, portable, and user-friendly immunoassay systems. The point of care testing immunoassay systems should be capable of rapid and sensitive detection of biomarkers.

## 5. Conclusion

In this review we have illustrated different methods which have been reported in last five years for the detection, quantification, and profiling of PSA. Significant progress has been made in technologies for the detection and quantification of PSA in recent years. These techniques can be relevant for their use in point-of-care cancer diagnostics. It is very important to consider the concentration range of cancer biomarkers and cancer cells in the early stage of the cancer and metastases in the development of novel diagnostic technology. The ideal diagnostic platform should allow the detection of biomarkers at ultra-low concentrations can be used for the dual purposes, i) the development and evaluation of novel biomarkers, ii) early detection of cancer and treatment follow-up.

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‡ SBN and MDS contributed equally for this work, hence both of them should be considered as a first author of this manuscript.

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