



*Citation for published version:*

Jolly, P, Damborsky, P, Madaboosi, N, Soares, RRG, Chu, V, Conde, JP, Katrlík, J & Estrela, P 2016, 'DNA aptamer-based sandwich microfluidic assays for dual quantification and multi-glycan profiling of cancer biomarkers', *Biosensors and Bioelectronics*, vol. 79, pp. 313-319. <https://doi.org/10.1016/j.bios.2015.12.058>

*DOI:*

[10.1016/j.bios.2015.12.058](https://doi.org/10.1016/j.bios.2015.12.058)

*Publication date:*

2016

*Document Version*

Peer reviewed version

[Link to publication](#)

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# **DNA aptamer-based sandwich microfluidic assays for dual quantification and multi-glycan profiling of cancer biomarkers**

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## **Abstract**

Two novel sandwich-based immunoassays for prostate cancer (PCa) diagnosis are reported, in which the primary antibody for capture is replaced by a DNA aptamer. The assays, which can be performed in parallel, were developed in a microfluidic device and tested for the detection of free Prostate Specific Antigen (fPSA). A secondary antibody (Aptamer-Antibody Assay) or a lectin (Aptamer-Lectin Assay) is used to quantify, by chemiluminescence, both the amount of fPSA and its glycosylation levels. The use of aptamers enables a more reliable, selective and controlled sensing of the analyte. The dual approach provides sensitive detection of fPSA along with selective fPSA glycoprofiling, which is of significant importance in the diagnosis and prognosis of PCa, as tumor progression is associated with changes in fPSA glycosylation. With these approaches, we can potentially detect 0.5 ng/mL of fPSA and 3 ng/mL of glycosylated fPSA using *Sambucus nigra* (SNA) lectin, both within the relevant clinical range. The approach can be applied to a wide range of biomarkers, thus providing a good alternative to standard antibody-based immunoassays with significant impact in medical diagnosis and prognosis.

**Keywords:** Microfluidics, DNA aptamer, Glycoprofiling, Sandwich assay, ELISA, Prostate cancer.

## 1. Introduction

Cancer is one of the leading causes of mortality worldwide. When diagnosed accurately and at an early stage, better monitoring and successful treatment of the disease can be achieved (Zieglschmid et al. 2005). Therefore, there is a high demand for improved diagnosis and prognosis tools. The quest for early diagnosis of cancer with high sensitivity and accuracy has led to increasing work on the detection of multiple biomarkers (Ploussard et al. 2011; Velonas et al. 2013). At the same time, one of the fields that have been gaining tremendous attention is that of glycomics for clinical diagnosis. The phenomenon of glycosylation, the covalent attachment of carbohydrate moieties to proteins and lipids, is one of the most abundant and complex post-translational modifications of proteins in human body. These modifications are involved in numerous physiological regulatory processes (Rudd et al. 2001; Rudd et al. 1999; Shental-Bechor and Levy 2009) including cancer (Gilgunn et al. 2013; Christiansen et al. 2014). Since glycosylation is a hallmark of disease states, altered glyco-forms may serve as viable candidate biomarkers for early-stage cancer diagnosis (Adamczyk et al. 2012; Pihíková et al. 2015). It is believed that the simultaneous detection of protein biomarkers and their glycosylation levels is a powerful tool for cancer diagnosis and prognosis.

Protein glycoprofiling can be performed either directly or indirectly after glycan release from intact glycoproteins. The released glycans can be further analyzed using a variety of commonly used analytical techniques such as HPLC, capillary electrophoresis and mass spectroscopy, which are laborious and expensive (Adamczyk et al. 2014; Domann et al. 2007; Thaysen-Andersen and Packer 2014). On the other hand, direct glycoprofiling methods are based on biorecognition elements such as lectins (Katrlik et al. 2010), anti-carbohydrate antibodies (Smith and Cummings 2010) or other glycan binding proteins capable of recognizing specific glycan structures. However, approaches based on the use of proteins (antibodies, lectins) have limitations such as cross-reactivity of the recognition element with some components present in the sample resulting in false positives. Additionally, using antibodies for diagnostic applications have drawbacks such as high costs, poor stability over time and difficulty in engineering them to suit the sensing platforms (Haupt and Mosbach 2000). One alternate promising class of receptor that can address many of these issues is aptamers, which are short

oligonucleotide sequences that can strongly bind to their target with high affinity and specificity by undergoing conformational changes. Selection of aptamers is an *in vitro* process (SELEX) and once selected, they can be synthesized in a controlled fashion with high purity and reproducibility. Additionally, aptamers are chemically more stable than antibodies, retaining most of their functionality even after multiple regeneration steps (Bunka and Stockley 2006; Jolly et al. 2014; O'Sullivan 2002; Song et al. 2008). As oligonucleotides can be easily modified with different reactive chemical groups, their immobilization on surfaces can be easily controlled, unlike antibodies. Because of the numerous advantages over antibodies, aptamers have been studied as emerging bioreceptors for the design and optimization of novel aptamer-based enzyme-linked immunosorbent assay (ELISA) for development of biosensors (Toh et al. 2015).

Replacing one or both of the antibodies in a classical ELISA has enabled development of refined assays which are more robust, reproducible and economical (Tennico et al. 2010; Toh et al. 2015). Such modified ELISA methods have also been demonstrated in microfluidics, which holds promise in biomedical research especially as a potential point-of-care (PoC) device. Su et al. recently reported an electrochemical lab-on-paper cyto-device to demonstrate specific cancer cell detection as well as monitoring of the multi-glycans on living cancer cells (Su et al. 2015). Liu et al. reported an aptamer-based ELISA approach for detection of rare cells with chemiluminescence (CL) analysis. (Liu et al. 2011). Yang et al. reported the development of aptamer based ELISA assay for C reactive protein using magnetic beads in a microfluidic system (Yang et al. 2009).

In this study, we report for the first time the development of aptamer-based ELISA for quantification of free prostate specific antigen (fPSA) in a microfluidic device. PSA is a 33 kDa serine protease (kallikrein-3) secreted by the prostate gland and high concentrations above the cut-off value of 4 ng/ml in blood are often associated with prostate cancer (PCa) thus leading to the consideration of further biopsy procedures (Catalona et al. 1991). However, the levels of PSA in blood in ageing men can also be raised due to other factors such as benign prostatic hyperplasia (BPH) and prostatitis. These factors lead to over-diagnosis (Carter et al. 1992) and as a result of false diagnosis, patients need to undergo unnecessary biopsy surgery, which is painful and also makes the patients vulnerable

to infections, making PSA testing a controversial diagnostic procedure. Nevertheless, PSA is still the most commonly used biomarker by clinicians for the detection of PCa and has thus motivated and led to the refinement of the PSA tests (Filella and Gimenez 2013; Kuriyama et al. 1998; Shariat et al. 2011; Wians et al. 2002).

Along with quantification of fPSA, glycoprofiling could effectively serve as a complementary procedure for enhanced diagnosis and monitoring of PCa and potentially reduce the levels of false positives through blood tests. Thus, we also report for the first time, multi-glycan profiling of fPSA using an aptamer-based sandwich assay in a microfluidic chemiluminescence sensor. Since PSA is a glycoprotein with various glycoforms (Isono et al. 2002; Végvári et al. 2012; Vermassen et al. 2012) the determination of cancer-associated glycoforms of PSA might help to improve early-stage clinical diagnosis of PCa (Meany and Chan 2011). There are studies in the literature showing that altered glycosylation patterns allow to distinguish healthy men from those with PCa or BPH (Peracaula et al. 2003; Tabares et al. 2006; Tajiri et al. 2008). The key aspect of this work is not only the development of an aptamer-based ELISA for dual quantification and glycoprofiling of fPSA but also the simplicity of the microfluidic assay developed and its potential integration in a PoC device. Because of the aptamers' advantages in terms of controlled orientation and smaller size, the fabricated biosensor could potentially detect 0.5 ng/mL of PSA along with distinct differentiation of different glycans involved in PCa.

## **2. Materials and Methods**

### ***2.1 Instruments and reagents***

Amine terminated PSA specific DNA aptamer (5'-H<sub>3</sub>N-(CH<sub>2</sub>)<sub>6</sub>-TTT TTA ATT AAA GCT CGC CAT CAA ATA GCT TT-3'), amine terminated PSA specific DNA aptamer labelled with Cy5 (5'-H<sub>3</sub>N-(CH<sub>2</sub>)<sub>6</sub>-TTT TTA ATT AAA GCT CGC CAT CAA ATA GCT TT-Cy5-3') and a random DNA sequence non-specific to PSA (5'-H<sub>3</sub>N-(CH<sub>2</sub>)<sub>6</sub>-AAA AAT TAA TTT CGA GCG GTA GTT TAT CGA AA-3') used as control DNA were obtained from Sigma Aldrich, UK. Prostate specific antigen

(PSA) from human semen was obtained from Fitzgerald (MA, USA). Human glandular kallikrein 2 (hK2) was obtained from RnD systems, UK. Phosphate buffered saline (PBS) tablets, (3-Glycidyloxypropyl)trimethoxysilane, Ethanolamine, human serum albumin (HSA), and were all purchased from Sigma-Aldrich, Portugal. Streptavidin-HRP was purchased from Invitrogen Life Technologies (MA, USA). Luminol, Pierce (Supersignal® West Femto Substrate Trial Kit – 34094 and Supersignal West Pico – 35065) was used as purchased from Thermo Scientific, Portugal. In addition, anti-equimolar total PSA-HRP antibodies (Ab178776) were purchased from Abcam. Both lectins SNA (*Sambucus nigra* agglutinin) and MAA II (*Maackia amurensis* lectin II) were obtained from VectorLab. All reagents were of analytical grade. All aqueous solutions were prepared using 18.2 MΩ cm ultra-pure water from a Milli Q system (Millipore, MA, USA). All the antibodies were diluted in 10 mM PBS (pH 7.4) filtered through a 0.4 μm syringe filter. Metallic plug adapters for the microchannel and capillary tubing (BTPE-90) were purchased from Instech Solomon (PA, USA). 1 mL syringes were from CODAN, Germany. The liquid flow in the microchannel was controlled using a NE-300 syringe pump from New Era (NY, USA).

## **2.2 Fabrication of PDMS Microchannel structures**

The fabrication of the polydimethylsiloxane (PDMS) microchannels was performed using soft lithography. Fabrication of hard mask, SU-8 mold and PDMS devices were adapted from literature as described by Soares et al. (2014). Briefly, SU-8 (Microchem, Newton, USA) was used to transfer the patterns of an aluminum mask to a silicon mold with a negative photoresist along with UV exposure and development in propylene glycol monomethyl ether acetate (Sigma-Aldrich, Portugal). Later, the mold patterns were transferred to PDMS to obtain the microfluidic devices for the experiments. The PDMS devices consisting of microchannels with width,  $w = 200 \mu\text{m}$ , height,  $h = 20 \mu\text{m}$  and length,  $l = 1 \text{ cm}$  were sealed to a cleaned glass substrate via an UV-ozone treatment for 6 min at 28-32 mW/cm<sup>2</sup> (UVO cleaner 144AX, Jelight Company Inc., CA, USA) for surface oxidation.

### ***2.3 Microfluidic Aptamer Assays***

Surface functionalization of aptamers for both fPSA quantification and glycoprofiling studies were performed according to the schematics shown in Fig. 1. Briefly, the microfluidic channel was first functionalized with pure solution of (3-Glycidyoxypropyl) trimethoxysilane at a flow rate of  $Q = 0.3 \mu\text{L}/\text{min}$  for 15 min, followed by 5 min washing with milliQ water at  $Q = 5 \mu\text{L}/\text{min}$  to remove all non-specifically bound silane molecules. Then, a  $10 \mu\text{M}$  solution of DNA aptamer prepared in PBS was flowed at  $0.4 \mu\text{L}/\text{min}$  for different time spans ranging from 5 to 30 min in order to optimize the aptamers surface density towards maximum PSA binding. The channel was then washed with PBS at a flow rate of  $Q = 5 \mu\text{L}/\text{min}$  for 2 min to remove any non-specifically bound aptamers, followed by ethanolamine blocking (10 mM in PBS, pH 8.5) at  $Q = 0.5 \mu\text{L}/\text{min}$  for 15 min. Finally, the channel was washed with PBS at  $Q = 5 \mu\text{L}/\text{min}$  for 2 min prior the assays. The subsequent analysis was always performed immediately after the immobilization procedure. For every individual experiment, new structures with fresh functionalization steps were performed.

For the fPSA quantification assay, HRP labelled anti-PSA antibodies were used at a concentration of  $100 \mu\text{g}/\text{mL}$ . For the glycoprofiling assay, biotinylated lectins and streptavidin-HRP were used at a concentration of  $100 \mu\text{g}/\text{mL}$ .



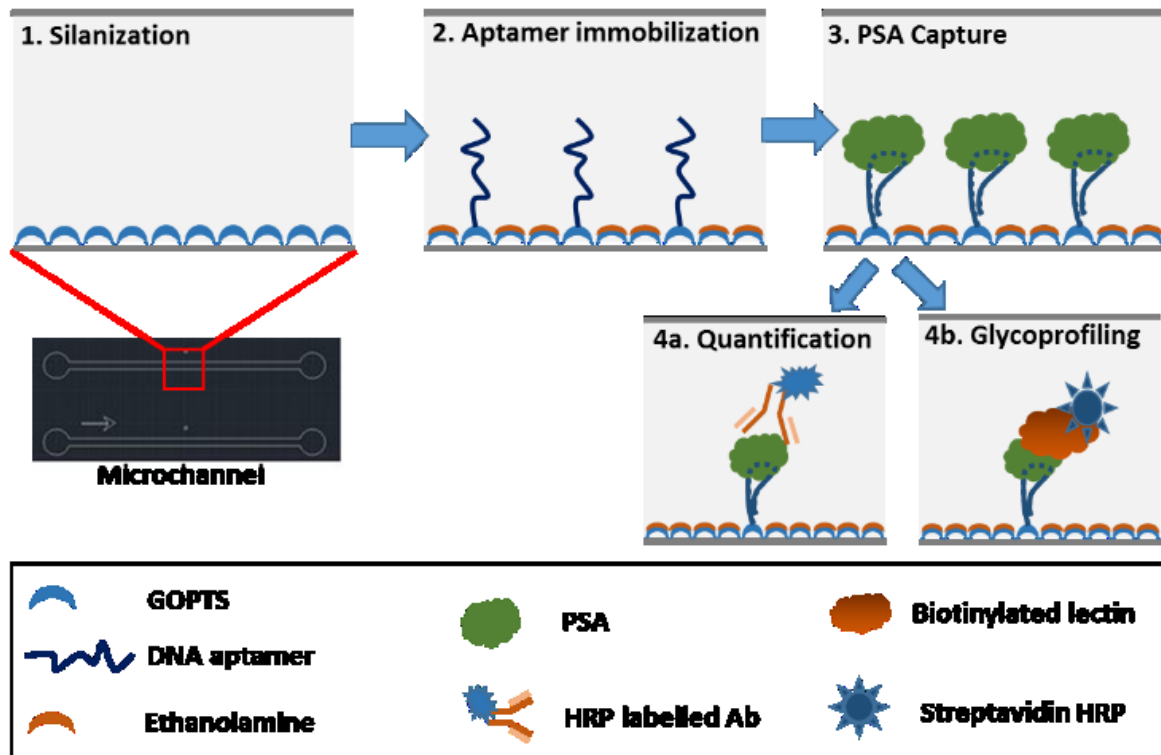


Fig. 1. Schematic of microfluidic channel fabrication scheme.

#### 2.4 Nanospotting of DNA aptamers on PDMS film

The DNA aptamers labelled with Cyanine3 on the 3' end were spotted on a thin PDMS film surface as detailed by Novo et al. A non-contact spotter (Nanoplotter NP2.1, GeSiM, Germany) was used to spot different concentration of DNA aptamer solutions with a piezoelectric pipette (Pico tip with a capacity to dispense single droplets with volumes down to ~56 pL) mounted on a computer controlled stage (Novo et al. 2011). The total number of times a droplet solution was dispensed and the degree of hydrophilicity of the ozone treated PDMS film at a particular location determines the diameter and the amount of DNA aptamers at that spot. The temperature was fixed at 16 °C and humidity was adjusted to near the dew point to increase the evaporation time of the droplets, ensuring homogeneous drying.

#### 2.5 Image acquisition and analysis

A fluorescence microscope (Leica DMLM) connected to a digital camera (Leica DFC300FX) was used for imaging the microchannels and recording the signal. The chemiluminescent signal was acquired using a microscope after flowing luminol  $Q = 5 \mu\text{L}/\text{min}$  for 1min, with an exposure time of 10 s and 10x optical gain, all in a dark background. The acquired images were analyzed using ImageJ software (National Institutes of Health, USA). The values correspond to the background normalized average measurements from three independent experiments, each with three regions of interest along the microchannel.

### **3. Results and Discussion**

#### ***3.1 Optimization of DNA aptamer surface coverage***

Aiming at miniaturizing an assay for the quantification and glycoprofiling of f-PSA, it is essential to perform first an optimization of both the physical and chemical aspects of the biosensors, namely the microchannel dimensions, the flow-rates used, residence time and molecular immobilization strategies. The first three parameters were previously optimized and validated with a system using antibodies to capture PSA (Madaboosi et al. 2015) and used herein without further modification.

Surface density of probes in particular plays a vital role in fabricating an efficient biosensor as reported with different techniques (Jolly et al. 2015; Keighley et al. 2008a; Keighley et al. 2008b; Madaboosi et al. 2015). An optimum spacing between the aptamers on the surface is required so that upon target capture, the aptamer can undergo conformational changes with minimal steric hindrance effects. In order to have efficient binding of PSA in the microfluidic channel via DNA aptamers, an optimization of the immobilization time of DNA aptamers was made. The optimization was performed using an antibody as a detector molecule, where fPSA was captured using DNA aptamers immobilized on the microfluidic channel, followed by detection with an anti-fPSA antibody labeled with the reporter enzyme horseradish peroxidase (anti-fPSA Ab-HRP). A fixed concentration of DNA aptamer equal to  $10 \mu\text{M}$  in  $10 \text{ mM}$  PBS was flowed for different time intervals and a fixed fPSA concentration of  $10 \text{ ng/mL}$  was chosen for the experiment. From Fig. 2a, it can be seen that as we

increase the time of immobilization of DNA aptamers from 5 min to 15 min, an increase in the chemiluminescence signal was observed.

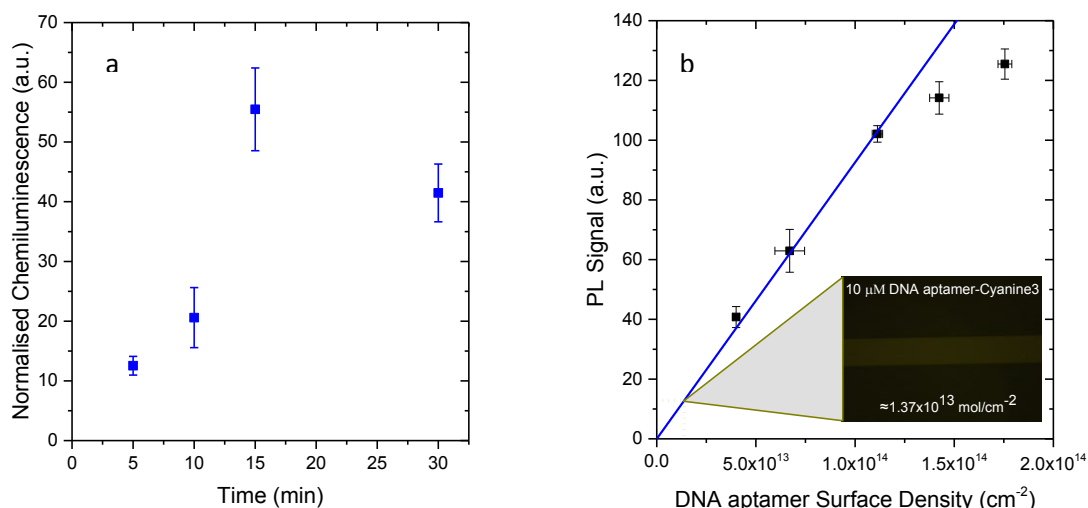


Fig. 2. Chemiluminescence signal with different time periods of DNA aptamer immobilization upon PSA binding, detected by anti-fPSA Ab-HRP (a). Nanospotting of DNA aptamer labelled with Cy3 to estimate the surface density. The inset image shows the optical micrograph of DNA aptamers labelled with Cy3 covalently immobilized in the microchannel (b).

However, as the time was further increased to 30 min, the chemiluminescence signal was reduced. This may be explained by considering that the density of the DNA aptamer layer on the surface becomes too densely packed to allow further effective PSA binding due to steric hindrance effects (Formisano et al. 2015). Thus, to achieve an effective PSA detection, 15 min was chosen as the immobilization time for subsequent experiments.

The relative coverage of the immobilized DNA aptamers on the surface of a microchannel was estimated from a nanospotting experiment. This method allows a robust and precise deposition of a controlled volume of DNA aptamers onto a surface using a computer-controlled microspotter (Novo et al. 2011). In the experiment, the aptamer solution is filled in a Pico tip that enables spots on the surface by dispensing the DNA aptamer solution in a non-contact mode (50 drops per spot). A calibration graph of the DNA aptamers labeled with Cy3 fluorophore (DNA aptamer-Cy3) was

obtained from fluorescence microscopy. Known volumes of varied concentrations of DNA aptamer-Cy3 was spotted on a thin PDMS film. The fluorescence resulting from the adsorbed DNA aptamer-Cy3 was measured in the spotted areas and the total number of DNA aptamers adsorbed per unit area was calculated. Each DNA aptamer occupies an area of  $6.16 \times 10^{-6} \mu\text{m}^2$  assuming that all the DNA aptamers are in their single stranded state and lying perpendicular to the surface, where a single stranded DNA has a hydro-dynamic radius of 1.4 nm.

Data obtained can be used to make a correlation between the total number of DNA aptamer-Cy3 in the spot and the fluorescence intensity from the spot. The curve obtained was then used for determining the surface density of the immobilized DNA aptamers in the microfluidic channel at a known concentration. Fig. 2b shows a correlation between the average fluorescent signal obtained from the spotting experiments and the DNA aptamer-Cy3 relative surface coverage, which can be used to estimate the surface density of the DNA aptamer molecules in a microfluidic channel used for PSA-specific experiments. The inset in Fig. 2b shows the fluorescence signal of a typical relative surface coverage when DNA aptamers are immobilized in a microchannel using GOPTS surface chemistry. The microchannel was prepared according to the protocol described in the methods section and the inset image represents one of the channel with immobilized DNA aptamer-Cy3 via GOPTS. A surface coverage of  $1.37 \times 10^{13}$  molecules/cm<sup>2</sup> was estimated from the obtained fluorescence signal in the microchannel (PL signal:  $12.80 \pm 1.68$  a.u.) with the immobilized DNA aptamer-Cy3.

### ***3.2 Quantification of PSA***

The DNA aptamer functionalized microfluidic channel, as described in the fabrication section, was used for the quantification of fPSA. The assay was based on a sandwich ELISA format, where anti-fPSA antibodies labeled with HRP were used to detect the fPSA captured by the immobilized DNA aptamers (Aptamer-Antibody Assay). A wide working range of fPSA concentrations ranging from 0.01 ng/mL to 50 ng/mL were used in the current study. It is important to highlight that each assay was performed in a new microchannel immediately after immobilization, since PDMS structures with such small footprint can be easily produced in large numbers and disposability avoids the use of

complex generation procedures (such as those described in *e.g.* Damborsky et al. 2015) and potential accumulation of non-specific binding. As seen from Fig. 3, a significant increase in the chemiluminescence signals is observed between 1 ng/mL and 25 ng/mL of fPSA concentrations, which lies within the cutoff range (grey area) of PSA in blood. It can be immediately observed that the background signal is not limiting the detection sensitivity at the clinically relevant concentrations, while still being detectable using the CCD camera of the microscope. This confirms that the ethanolamine blocking works effectively in providing a fit-for-purpose level of non-specificity.

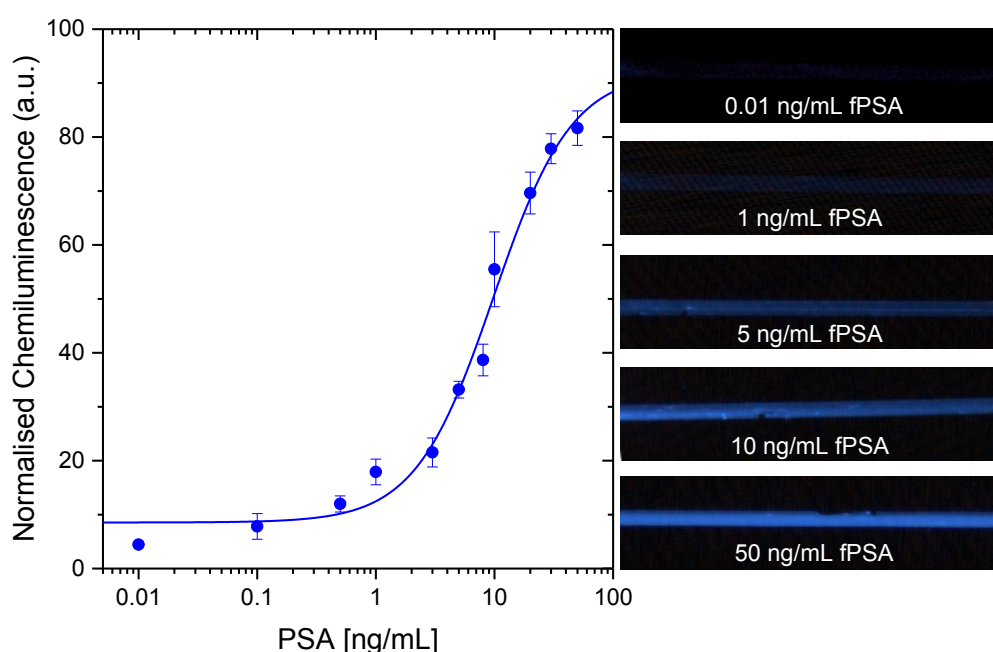


Fig. 3. Quantification of fPSA using anti-fPSA Ab-HRP. Images represent the chemiluminescence signals from microscopy.

The response obtained for fPSA quantification follows a Hill dose-response equation of the type  $y = y_0 + (y_{max} - y_0) \frac{c^n}{(K_d^n + c^n)}$  where  $c$  is the concentration. The dissociation constant ( $K_d$ ) for the dose response was calculated as 9.82 ng/mL with a root mean square (RMS) value of 0.98. The value of  $y_{max}$  which is the end value calculated from the fitting was 92.17 a.u. with  $n = 1.32$ . The fabricated biosensor could potentially differentiate 0.5 ng/mL of fPSA with a chemiluminescence value of  $11.90 \pm 1.49$  a.u., which is higher than the  $y_0$  value (8.5 a.u.) calculated from the fitting.

### ***3.3 Selectivity study***

The development of a reliable biosensor and its potential applications depends on many factors including selectivity. Various control experiments were investigated in order to confirm that the signals obtained with different PSA concentrations in the earlier section were due to specific capture of fPSA by the DNA aptamers immobilized on the microchannel surface. As the current study is a demonstration of the potential of an aptamer-based ELISA, it is important to test the role of aptamers as a probe to capture fPSA in the channel. This was performed by using two control experiments (see Fig. 4). In the first experiment, the silanized channel was blocked with ethanolamine, so that the aptamers were not eventually immobilized (Fig. 4, #3). In the second study, a random DNA of same length as of the PSA-specific DNA aptamer was immobilized on the microfluidic channel (Fig. 4, #4). The DNA aptamer specific to PSA used in this study was reported by Savory et al. (2010) where different DNA sequences were tested for the most efficient PSA binding sequence. Hence, for the current study, a random sequence was used as a control in order to demonstrate specific capture of fPSA and that there are no non-specific fPSA-DNA electrostatic interactions. It was done in order to confirm the role of DNA aptamer as a capture probe and to ensure that the signal is obtained only when fPSA has been captured by the aptamers. When the channel was observed under the microscope, a negligible change of less than 2 a.u. when compared with blank measurement was noticed.

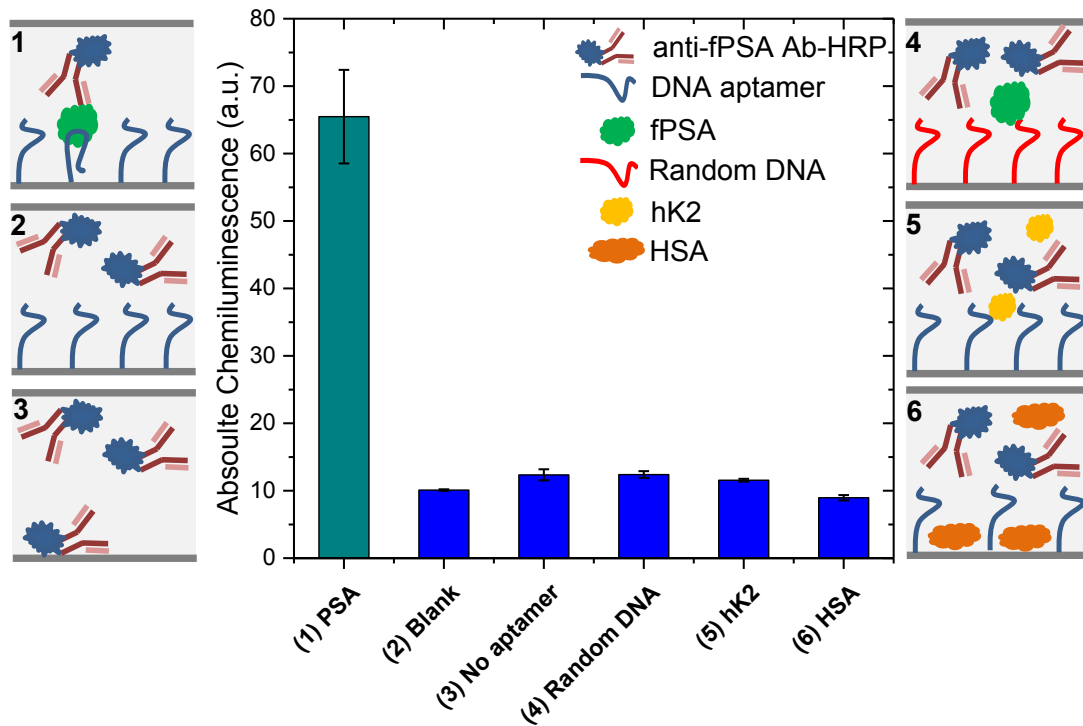


Fig. 4. Selectivity studies for fPSA measurements. (1) Specific capture of fPSA (10 ng/mL) by DNA aptamers and thereafter, detected by anti-fPSA HRP. (2) Signal change when no fPSA was flowed. (3) Signal change when no aptamer was immobilized in the microchannel and 10 ng/mL fPSA was flowed. (4) When a random DNA sequence which is equivalent to the size of PSA specific DNA aptamer was used and 10 ng/mL fPSA was flowed (5) Signal change when 10 ng/mL hK2 was flowed. (6) Control with 4% HSA.

We also performed two selectivity studies using the control proteins human Kallikrein 2 (hK2) (10 ng/mL) (fig. 4, #5) and human serum albumin (HSA) (4%) (fig. 4, #6). hK2 is another serine protease from the same kallikrein family as PSA. It was chosen as a control protein candidate of interest since it shares 80% homology with the PSA. The levels of hK2 are 100 fold lower than that of PSA in clinical samples; still this protein was stringently chosen since it served as an appropriate control to evaluate the aptamer-based approach over an antibody-based approach using ELISA (Hong 2014; Vaisanen et al. 2006). With an aptamer-based ELISA system, an appreciable differentiation between hK2 and PSA can be noticed; the former when compared with blank showed a difference of

less than 2 a.u. only (fig. 4, #5). This can be distinctively differentiated from 10 ng/mL PSA (fig. 4, #1) with a difference of 55 a.u.

As a comparison, classical ELISA was performed by adsorption of capture antibodies in the microfluidic channel and followed by the flow of 10 ng/mL hK2 protein, and finally a detector antibody labeled with HRP. The channel was then analyzed under a microscope. The results show a high level of cross reactivity of up to 20 a.u. and the inability of the antibodies to differentiate hK2 from PSA. The high cross reactivity could be due to the fact that most of the antibodies raised against PSA exhibit certain cross-reactivity with the hK2 due to similar epitopic regions. Also, 4% HSA was used to test matrix interference effects and a slight decrease in chemiluminescence ( $\sim 1$  a.u.) was observed. This could be attributed to the blocking effect of HSA, leading to decreased non-specific binding of detector antibodies.

### **3.4 Glycoprofiling of PSA**

The change in carbohydrate linkage of particular carbohydrate moieties such as sialic acid play a major role in glycoprofiling of PSA. It can indicate not only the presence of a pathological process but, more importantly, in some cases also its stage. Thus, a similar aptamer based assay design was also used for fPSA glycoprofiling, utilizing lectins as glyco-recognition elements rather than an anti-PSA antibody (Aptamer-Lectin Assay). This is demonstrated by profiling the fPSA carbohydrate content using two biotinylated lectins specific for sialic acid: (i) *Sambucus nigra* lectin (SNA) binding preferentially to sialic acid attached to terminal galactose in  $\alpha$ -2,6 linked sialic acid and (ii) *Maackia amurensis* lectin II (MAA II), a lectin recognizing terminal  $\alpha$ -2,3 sialylation of PSA. The increase of  $\alpha$ -2,3 linked sialic acid is highly presented in PCa patients (Ohyama et al. 2004).

Since we employed commercial fPSA obtained from healthy donors, no interaction with MAA II lectin was expected. Thus, MAA II lectin was used as a negative control for a commercial non-malignant fPSA. The fabricated biosensor using lectins was able to distinguish between  $\alpha$ -2,6 linked sialic acid and  $\alpha$ -2,3 linked sialic acid as shown in Fig. 5. This finding is in agreement with published studies (Saldoval et al. 2011). The working range is slightly shifted when compared to quantification



experiments, the concentrations ranging from 3 ng/mL to 50 ng/mL fPSA. As seen from Fig. 5, the fabricated biosensor could potentially differentiate 3 ng/mL fPSA from the blank measurement with a difference of  $5.64 \pm 0.50$  a.u. using chemiluminescence. However, a significant increase in the chemiluminescence signal was observed between 5 ng/mL of PSA to 50 ng/mL of PSA concentration which lies within the cut-off range (grey zone) of PSA in the blood. Nevertheless, here the major focus was on the potential occurrence of sialic acid with different linkages on PSA surface in a multiglycan profiling approach, thus establishing the difference between the two lectin forms using an aptamer-based ELISA.

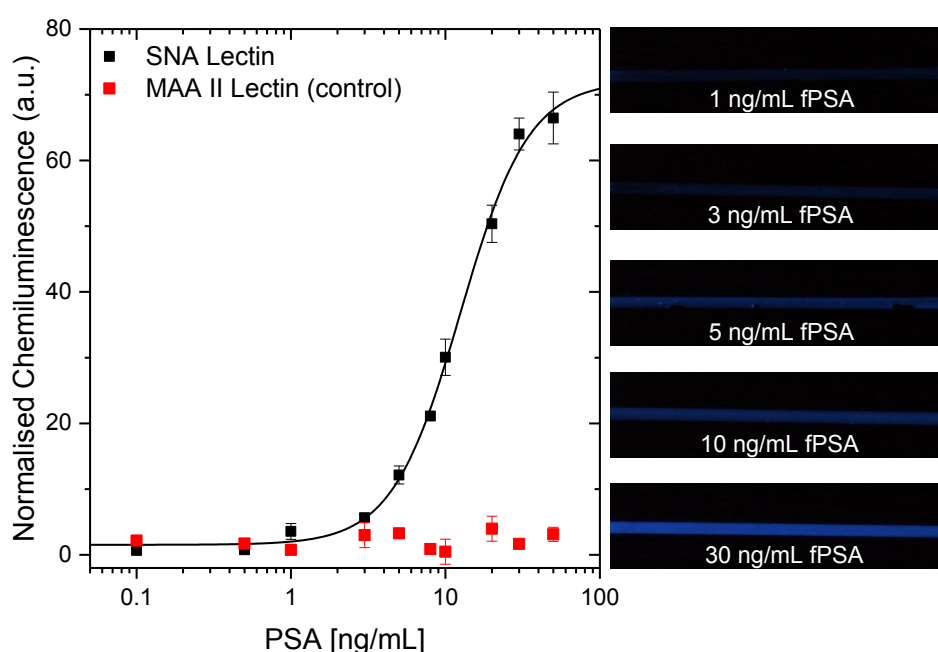


Fig. 5. Multi-glycan profiling for fPSA detection. Blue data points were obtained using a SNA lectin whereas, red data points were obtained using an MAA II (control) lectin. Images represent the chemiluminescence signals from microscopy.

Again, a dose response fitting was performed using the same Hill equation that was used with quantification of PSA in Fig. 5, on the data points obtained with SNA lectin. A dissociation constant ( $K_d$ ) of 12.5 ng/mL of PSA with a root mean square value of 0.99 was obtained. The value of  $y_{max}$  which is the end value calculated from the fitting was 72.08 a.u. with  $n = 1.96$ .

Considering the  $y_0$  value calculated to be 1.53 a.u. from the fitting, the fabricated biosensor could differentiate 3 ng/mL of fPSA with chemiluminescence value of  $12.20 \pm 1.36$  a.u.. In the control experiments, MAA II lectin did not show any binding to PSA in the whole concentration range studied. The differentiation in signal from the two lectins could be observed for fPSA concentrations above 3 ng/mL.

#### **4. Conclusions**

We present a simple novel aptamer based microfluidic ELISA assay for the optical detection of fPSA in the clinical range with high sensitivity. The integration of quantification of PSA with multi-glycan profiling of fPSA could serve as a potential platform for PoC devices in PCa diagnosis. We demonstrated a sensitive quantification of PSA of 0.5 ng/mL and also demonstrated multi-glycan profiling where the specific lectin (SNA) could differentiate response from control lectin (MAA II) at fPSA concentration down to 3 ng/mL.

The study addresses how the use of aptamers could be an effective methodology to handle the current antibody limitations with cross reactivity issues in glycoprofiling. It also shows how aptamers could be used as a potential tool for multi-glycan profiling of biomarkers with high sensitivity and selectivity in a simple microfluidic channel. This approach can be easily extended to a wide range of other biomarkers available for PCa, a step towards multiplexing, which is of great interest for PCa diagnosis. The simultaneous detection of protein cancer biomarkers together with their levels of glycosylation provides enhanced diagnosis and prognosis of the disease.

#### **Acknowledgements**

This work was funded by the European Commission FP7 Programme through the Marie Curie Initial Training Network PROSENSE (grant no. 317420, 2012-2016)

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