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

Pawan Jolly [a,1], Pavel Damborsky [b,1], Narayanan Madaboosi [c], Ruben R.G. Soares [c,d], Virginia Chu [c], João P. Conde [c,d], Jaroslav Katrlik [b], Pedro Estrela [a,*]

[a] Department of Electronic & Electrical Engineering, University of Bath, Bath BA2 7AY, United Kingdom
[b] Department of Glycobiotechnology, Center for Glycomics, Institute of Chemistry, Slovak Academy of Sciences, Dubravska cesta 9, Bratislava 84105, Slovakia
[c] INESC-MN – Microsystems and Nanotechnologies, R. Alves Redol 9, 1000-029 Lisboa, Portugal
[d] Department of Bioengineering, Instituto Superior Técnico, University of Lisboa, Av. Rovisco Pais, 1049-001 Lisboa, Portugal

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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.

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, 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

* Corresponding author.
E-mail addresses: P.Jolly@bath.ac.uk (P. Jolly), Pavel.Damborsky@savba.sk (P. Damborsky), Nsrinivasan@inesc-mn.pt (N. Madaboosi), Ruben.Soares@tecnico.ulisboa.pt (R.R.G. Soares), vchu@inesc-mn.pt (V. Chu), joao.conde@tecnico.ulisboa.pt (J.P. Conde), Jaroslav.Katrlik@savba.sk (J. Katrlik), P.Estrela@bath.ac.uk (P. Estrela).
[1] These authors contributed equally to the manuscript.

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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. (2015) 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. Liu et al. (2011) reported an aptamer-based ELISA approach for detection of rare cells with chemiluminescence (CL) analysis. Yang et al. (2009) reported the development of aptamer based ELISA assay for C reactive protein using magnetic beads in a microfluidic system.

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_3N-$(CH_2)_6$-TTT TTA ATT AAA GCT CGC CAT CAA ATA GCT TT-3′), amine terminated PSA specific DNA aptamer labeled with Cy5 (5′-H_3N-$(CH_2)_6$-TTT TTA ATT AAA GCT CGC CAT CAA ATA GCT TT-Cy5-3′) and a random DNA sequence non-specific to PSA (5′-H_3N-$(CH_2)_6$-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), andwere 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, $w=200$ μm, height, $h=20$ μm and length, $l=1$ cm were sealed to a cleaned glass substrate via an UV–ozone treatment for 6 min at 28–32 mW/cm^2 (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-Glycidyloxypropyl) trimethoxysilane at a flow rate of $Q=0.3$ μL/min for 15 min,