Electrochimica Acta 246 (2017) 399–405

Contents lists available at ScienceDirect

Electrochimica Acta

journal homepage: www.elsevier.com/locate/electacta

Full-length antibodies *versus* single-chain antibody fragments for a selective impedimetric lectin-based glycoprofiling of prostate specific antigen

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ARTICLE INFO

Article history:
Received 20 April 2017
Received in revised form 7 June 2017
Accepted 11 June 2017
Available online 12 June 2017

Keywords:
impedimetric biosensors
full-length antibodies
single-chain antibody fragments (scAb)
lectins
glycan analysis
prostate cancer

ABSTRACT

The main aim of the research was to design a functional impedimetric biosensor able to glycoprofile prostate specific antigen (PSA), a biomarker for prostate cancer (PCa), with high specificity using lectins as glycan recognising proteins. Traditionally, full-length antibody is immobilised on the biosensor interface for specific capture of PSA with subsequent glycoprofiling of PSA by addition of lectins. Since full-length antibodies contain glycans in the Fc domain, particular attention has to be paid to suppress direct binding of lectins to immobilised full-length antibodies, which would compromise accurate glycoprofiling. This issue is addressed here using a recombinant single-chain antibody fragments (scAb), which do not contain any carbohydrate moiety. Surface plasmon resonance was applied to prove negligible interaction of lectins with immobilised scAb fragments, while substantial binding of lectins to full length antibodies was observed. Eight different biosensor designs were tested for their ability to detect PSA. The biosensor device based on scAb fragments covalently immobilised on the gold electrode surface, patterned by a mixed SAM using standard amine coupling chemistry, proved to be the most sensitive. The scAb fragment-based biosensor exhibited sensitivity of $15.9 \pm 0.8\%$ decade^{-1} ($R^2 = 0.991$ with an average RSD of 4.9%), while the full antibody-based biosensor offered sensitivity towards PSA of $4.2 \pm 0.1\%$ decade^{-1} ($R^2 = 0.999$ with an average RSD of 4.8%). Moreover, the selectivity of the scAb-based biosensor was tested using a kallikrein 2 protein, a protein structurally similar to PSA, and the results indicated high selectivity for PSA detection.

1. Introduction

Glycans, which consist of chains of carbohydrates, are present on many biological molecules, such as proteins or lipids, but are also found on the surface of cells [1]. More than 160 different biological roles for glycans have been identified, e.g. cell signalling, immune responses and cell adhesion [2]. Glycans also play a major role in development and progress of many diseases including autoimmune disorders and cancer. Aberrant glycosylation is a fundamental characteristic of malignant transformation and tumour development [3]. For this reason, glycomics is receiving great attention in relation to the diagnosis of various diseases including prostate cancer (PCa). Current diagnostic tests for PCa involve digital rectal examination and determination of the concentration of PSA in blood. PSA is a glycoprotein with a protease activity responsible for liquefying seminal fluid. The majority of healthy men have a PSA concentration of about 0.2–5 mg/mL in seminal fluid while it is below 4 ng/mL in blood [4]. Elevation of PSA levels in PCa is a consequence of disruption of prostate tissue and release of PSA into the blood stream [5].

The most frequent clinical method for detection of PSA is ELISA (Enzyme-linked immunosorbent assays) based on optical reading in a microplate format [6], but there are other bioanalytical methods available for detection of PSA using a diverse range of transducers [7–9] and nanoparticles [1,10]. However, PSA testing has significant drawbacks. More than 20% of men with a PSA below 4 ng/ml were diagnosed with prostate cancer after a biopsy [11].

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http://dx.doi.org/10.1016/j.electacta.2017.06.065
0013-4686/

Moreover, elevated levels of PSA are not PCa-specific, but may be due to benign prostate hyperplasia (enlargement), prostatitis, urinary tract infection or even prostate massage or exercise [12,13]. To circumvent this issue, more specific biomarkers are required. These include prostate stem cell antigen, prostate specific membrane antigen, haptoglobin [14], circulating tumour cells [15], hormones (insulin-like growth factor) [16], gene alterations and gene fusions [17]. In addition, various statistic methods combining results of known analytical approaches (Prostate Health Index, free PSA to total PSA ratio) can provide more accurate diagnosis [6,18]. The study of glycosylation changes has significant potential for PCa diagnostics. The most abundant glycosylation alterations observed were increased branching and fucosylation, especially α-1,2-fucose linked to galactose [5], a significant decrease in α-2,6 sialylation and an increase in terminal α-2,3 sialylated glycans [19]. Precise and specific recognition approaches are required to detect these minute alterations. One of the most reliable methods is mass spectroscopy analysis [20], however, for personalized medical strategies and for point-of-care devices, biosensors and various biochip-based assays are emerging, where antibody-based recognition plays a crucial role.

It is feasible to glycoprofile PSA using biochips/biosensors with immobilised antibodies for a selective capture of PSA from a complex matrix such as blood serum, followed by a final incubation with a lectin in a sandwich configuration. Unfortunately, immunosensors, combined with lectins as a glycoprofiling tool, may provide false positive responses, as a result of interaction of lectins directly with glycans present on the Fc domain of an immobilised full-length antibody [21,22]. For example, Chen et al. addressed this issue by a chemical derivatisation of glycans, which were firstly oxidised by $NaIO_4$ and then blocked by dipeptide [23]. This method, however, required three additional steps in array preparation, was time and labour-intensive and may affect antibody binding.

In this work we investigated an entirely different approach from the protocol developed by Chen et al. [23] i.e. by replacing full-length antibodies with recombinant antibody fragments such as scAb, which do not carry a carbohydrate moiety. Recombinant antibody fragments (scFv – single-chain fragment variable, Fab – fragment antigen binding, scAb – single-chain antibody fragment *etc.*) may be generated with high reproducibility/reliability, and can be tailored for sensitivity, specificity and stability [24]. In this work SPR was used to demonstrate low background binding of lectins to immobilised scAb fragments, optimisation of the biosensor construction based on scAb fragments and proof-of-concept for glycoprofiling of PSA by the lectin *Sambucus nigra* agglutinin (SNA).

2. Experimental section

2.1. Material and Methods

Purified free prostate specific antigen (PSA) isolated from human seminal fluid was purchased from Fitzgerald Industries International (USA), anti-PSA mouse monoclonal antibody Ab10185 (specific for total PSA, no cross reactivity for human kallikrein 2) was purchased from Abcam (UK), human kallikrein 2 (KLK2) was purchased from R&D Systems, Inc. (USA), *Sambucus nigra* agglutinin (SNA, recognising Neu5Acα6 Gal/GalNAc), *Phaseolus vulgaris* erythroagglutinin (PHA-E, recognising Galβ4GlcNAcβ2Manα6), wheat germ agglutinin (WGA, recognising GlcNAc) and carbo-free blocking solution (CFB) were purchased from Vector laboratories (USA). In designating the glycan preference of the lectins the following abbreviations were applied: Neu5Ac – *N*-acetylneuraminic acid (sialic acid), Gal – galactose, GalNAc – *N*-acetylgalactosamine, GlcNAc – *N*-acetylglucosamine and Man – mannose. A 2-(2-pyridinyldithio) ethaneamine hydrochloride (PDEA) thiol coupling reagent was purchased from GE Healthcare Life Sciences (USA), 2-mercaptoethanol (ME), 6-mercaptohexanol (MH), 11-mercaptoundecanoic acid (MUA), cysteamine, ethanolamine hydrochloride, gelatine from porcine skin (type A), hydrogen peroxide (30% w/w), N-hydroxysuccinimide (NHS), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), phosphate buffered saline tablet (PBS, one tablet dissolved in 200 mL of deionized water yields 0.01 M phosphate buffer, with 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4, at 25 °C.), potassium ferricyanide (III), potassium ferrocyanide(II) trihydrate, sodium hydroxide, sulphuric acid, Tween 20, and all general chemicals were purchased from Sigma-Aldrich (USA). All solutions were prepared in deionized water (DW) and were filtered prior to use through 0.2 μm sterile filters. Protein solutions were prepared in PBS (10 mM, pH 7.4), unless otherwise stated.

2.2. SPR measurements

Interaction between lectins and full length antibodies or scAb fragments was monitored by a surface plasmon resonance (SPR) dual channel Reichert SR7000DC SPR system, thermostated at 23 °C, operated using SPR Autolink System software (AMETEK, Reichert Technologies, USA). The flow cell was equipped with a three-dimensional carboxymethyldextran hydrogel SPR Sensorchip CMD50 m (Xantec bioanalytics, Germany), which was pre-treated by sodium acetate buffer (AcB), pH 5.0, prior to amine coupling, to enhance the immobilisation efficiency. The optimal pH for immobilisation was determined prior to the immobilisation itself (data not shown). The CMD50 m sensorchip was functionalised using the amine coupling protocol recommended by the manufacturer i.e. by covalent attachment of proteins via $—NH_2$ towards activated —COOH groups. Initially, both the working and the reference channel were activated by a 1:1 mixture of 0.2 M EDC and 0.05 M NHS at a flow rate of 20 μL/min for 420 s. After the activation, 20 ug/mL of Ab10185/scAb in 10 mM sodium AcB pH 5.0 was injected into the working channel for 10 min at a flow rate of 20 μL/min. Ethanolamine hydrochloride (1 M, pH 8.5) was then injected into both channels for 10 min to deactivate active carboxylic groups and, finally, 20 mM HCl was injected into the system. SNA, PHA-E and WGA lectins, diluted in a running buffer (PBST) to a concentration of 25 μg/mL, were sequentially injected at a flow rate of 50 μL/min. The injection time was 210 s and the dissociation time was 420 s. Regeneration was achieved with 20 mM NaOH injected for 60 s. The response of the system is represented by arbitrary refractive index units (μRIU, 1 mRIU = 1 pg /mm^{2}according to the manufacturer) and the monitored interaction is assessed by subtracting the reference response from the signal obtained in the analytical channel. Data obtained were processed by the Scrubber software (BioLogic Software Pty, Australia).

2.3. Electrode pre-treatment

For the electrochemical measurements, polycrystalline gold disc electrodes (1.6 mm diameter, BASi, USA) were cleaned according to the protocol developed by Tkac et al. [25]. Briefly, electrodes were cleaned mechanically, chemically and electrochemically. Initially, any remaining thiols were removed from the gold surface by electrochemical reductive desorption, under a N_2 atmosphere, using cyclic voltammetry (CV) in 100 mM NaOH (100 scans, potential range from −500 mV to − 1,500 mV, a scan rate of 100 mV/s) under stirring to dissolve thiol molecules and to prevent subsequent re-adsorption of thiols on the electrode surface. The next step was a mechanical polishing of the electrodes on the