Published in final edited form as:

Bioelectrochemistry. 2017 October ; 117: 89–94. doi:10.1016/j.bioelechem.2017.06.005.

Label-free chronopotentiometric glycoprofiling of prostate specific antigen using sialic acid recognizing lectins

Stefan Belický^a, Hana Černocká^b, Tomas Bertok^a, Alena Holazova^a, Kamila Réblová^c, Emil Paleček^b, Jan Tkáč^a, and Veronika Ostatná^{b,*}

^aInstitute of Chemistry, Slovak Academy of Sciences, Dubravská cesta 9, 845 38 Bratislava, Slovak Republic

^bInstitute of Biophysics, Czech Academy of Sciences, Kralovopolská 135, 612 65 Brno, Czech Republic

^cCEITEC Central European Institute of Technology, Masaryk University, Kamenice 5, Brno 62500, Czech Republic

Abstract

In recent decades, it has become clear that most of human proteins are glycosylated and that protein glycosylation plays an important role in health and diseases. At present, simple, fast and inexpensive methods are sought for clinical applications and particularly for improved diagnostics of various diseases, including cancer. We propose a label- and reagent-free electrochemical method based on chronopotentiometric stripping (CPS) analysis and a hanging mercury drop electrode for the detection of interaction of sialylated protein biomarker a prostate specific antigen (PSA) with two important lectins: Sambucus nigra agglutinin (SNA) and Maackia amurensis agglutinin (MAA). Incubation of PSA-modified electrode with specific SNA lectin resulted in an increase of CPS peak H of the complex as compared to this peak of individual PSA. By adjusting polarization current and temperature, PSA-MAA interaction can be either eliminated or distinguished from the more abundant PSA-SNA complex. CPS data were in a good agreement with the data obtained by complementary methods, namely surface plasmon resonance and fluorescent lectin microarray. It can be anticipated that CPS will find application in glycomics and proteomics.

Keywords

A prostate specific antigen; Lectin-glycoprotein interaction; Sialylated glycan isomers; Chronopotentiometric analysis; Mercury electrode

1 Introduction

Prostate cancer (PCa) is one of the leading issues concerning men worldwide. According to the statistics, PCa is the third most common cause of death of cancerous diseases among

*Corresponding author at: Institute of Biophysics CAS, v.v.i., Kralovopolská 135, 612 65 Brno, Czech Republic. ostatna@ibp.cz (V. Ostatná).

males in European Union [1]. The main problem behind PCa diagnostics is that at an early stage of the disease only mild symptoms are present, or its symptoms may resemble those of benign prostate hyperplasia (BPH). There were also cases reported, when metastatic PCa caused no symptoms at all [2]. Therefore it is often diagnosed only when it reaches a more developed stage and is more difficult to cure. The main diagnostic tool currently used for PCa diagnostics is determination of a prostate specific antigen (PSA) level in a blood serum. Elevated PSA level in patients with PCa is a result of a leakage from disrupted prostate cells, rather than its increased expression [3]. A concentration cut-off point to distinguish between healthy individuals and people with a PCa risk was considered to be $4 \text{ ng}\cdot\text{mL}^{-1}$, nonetheless this limit has lately been reconsidered by many authors to $2 \text{ ng}\cdot\text{mL}^{-1}$ due to the fact that almost 15% of patients diagnosed with PCa had PSA level lower than $4 \text{ ng}\cdot\text{mL}^{-1}$ [4]. Such cases even prompted The US Preventive Services Task Force to issue a recommendation not to use PSA for routine screening of PCa due to its low sensitivity, specificity and prognostic value [5]. This has led to a quest for finding new biomarkers, more specific to PCa, rather than for disease of prostate in general (i.e. BPH).

Since aberrant glycosylation occurs in many intracellular signalling pathways and may eventually lead to the development of cancer, various glycoforms of proteins are often proposed as novel biomarkers [6]. Altered glycosylation patterns were also recognized in tumours that expressed rapid growth or were metastasizing into other parts of organism. This indicates that analysis of various glycoforms of biomarkers may not only be used for disease diagnostics, but also for prognostic purposes to detect aggressive forms of cancer [7]. Until now, there are only 9 protein biomarkers approved by the US Food and Drug Administration (FDA) for clinical use as cancer biomarkers and all of them are glycosylated [8].

Composition of PSA glycan is well known, however study of glycan changes as a result of PCa is more challenging. PSA protein from healthy donor is reported to contain fucosylated complex biantennary N-glycan with one or two terminal sialic acids attached [9]. Several research groups study glycan changes of PSA from cancerous origin [7,9–12]. An overexpression of $\alpha(1,6)$ -fucosyltransferase resulting in an increased $\alpha(1,6)$ -fucosylation of PSA was reported to be a sign of an aggressive form of PCa and thus being a perspective prognostic marker [7,13]. Monitoring of an increased branching (i.e. presence of tri- and tetraantennary glycans) is also a valuable predictive marker with a potential to predict a castration resistant PCa [14]. Another important cancer-related modification of PSA glycan is aberrant sialylation. N-acetylneuraminic acid (Neu5Ac) is a predominant sialic acid found in humans. Terminal Neu5Ac present on the PSA glycan is in healthy individuals linked to galactose predominantly via $\alpha(2,6)$ -glycosidic linkage. Contrary to this, presence of $\alpha(2,3)$ -glycosidic linkage of Neu5Ac to Gal is significantly increased in prostate cancer [15]. Since PSA samples from patients with BPH do not show this increase, monitoring of altered sialylation pattern is a promising tool to distinguish between PSA of cancerous and non-cancerous origin [16].

The main approaches for glycan analyses are robust and reliable methods such as mass spectrometry in conjunction with liquid chromatography or capillary electrophoresis [17]. Nevertheless, these methods require an experienced operator and are highly time-consuming. An alternative way for glycan analyses are different lectin-based methods, such

as enzyme-linked lectin binding assay (ELLBA), fluorescent microarray or lectin-based biosensors and biochips [18]. Number of studies are focused on preparation of a sensitive biosensor for analysis of biological molecules using various approaches [19]. Alterations in glycan composition occur not only during cancer development, but also in various autoimmune diseases (systemic sclerosis, rheumatoid arthritis etc.) or even as a result of an aging process [20,21]. Electrochemical platforms of detection provide several advantages, such as a minute sample consumption, low detection limits, low price and a possibility to miniaturize such devices. Jolly et al. [22] combined an aptamer detection with a molecular imprinted polymer to develop a biosensor resistant to non-specific interactions able to detect PSA down to $1 \text{ pg}\cdot\text{mL}^{-1}$. Tzouvadaki et al. reported a memristive aptasensor capable of detecting $0.7 \text{ fg}\cdot\text{mL}^{-1}$ of PSA – among the most sensitive electrochemical biosensor for PSA detection published [23]. However, as previously stated, determining only the concentration of PSA marker is not sufficient for a definite PCa confirmation, further investigation of aberrant glycosylation changes is necessary to determine the origin of a biomarker. Pihikova et al. described an impedimetric biosensor for quantification of PSA and its subsequent glycoprofiling, both on the same interface [24]. When detecting various interactions, such as protein-protein or glycan-protein interactions, it is often necessary to modify electrode surfaces, i.e. using self-assembled monolayers (SAMs).

Palecek's group in Brno invented (i) new methods of chemical modification of glycans and glycoproteins by complexes of six-valent osmium (binding to 1,2 diglycols), followed by electrochemical [25] and/or immunochemical assays [26] and (ii) new approaches in electrochemical analysis of non-conjugated proteins [6,27–31] based on the ability of proteins to catalyse hydrogen evolution at mercury electrodes. In constant current chronopotentiometric stripping (CPS) proteins produce the so-called peak H [6]. Using this peak, the structure-sensitive analysis has been developed, capable to detect small changes in protein structures resulting e.g. from a single amino acid exchange in a mutated protein [6,29]. Moreover, it has been shown that the same principles can be applied to study interactions of proteins with DNA, such as the specific binding of tumour suppressor protein p53 to DNA [28].

Recently, Ostatna et al. [31] extended these efforts for studies of lectin-glycoprotein interactions. They used CPS and a bare hanging mercury drop electrode (HMDE) to investigate interactions of Concanavalin A lectin (Con A, from *Canavalia ensiformis*) with mannosylated glycoproteins [31] and showed that, based on changes in peak H, free lectins and free glycoproteins can be distinguished from the lectin-glycoprotein complexes and the time course of the complex formation can be followed.

In this study, we attempted to apply this methodology for analysis of sialylated protein biomarker PSA and its interactions with two important lectins: (i) *Sambucus nigra* agglutinin-I (SNA, specific for Neu5Ac(α 2–6)Gal) and (ii) Maackia amurensis agglutinin (MAA, specific for Neu5Ac(α 2–3)Gal). We show that CPS analysis is able to recognize specific binding of PSA to SNA from its less abundant interaction with MAA.

2 Material and methods

2.1 Chemicals

KH_2PO_4 , K_2HPO_4 , NaH_2PO_4 and Na_2HPO_4 , hydrochloric acid, N-hydroxysuccinimide (NHS), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), *Maackia amurensis* agglutinin (MAA, Neu5Ac(α 2–3)Gal-specific, 130 kDa, 2 subunits, pI 4.7, a glycoprotein without Cys residues) were purchased from Sigma Aldrich (USA). Prostate specific antigen (PSA, a serine protease, human kallikrein 3, pI 7.26, a glycoprotein containing single complex N-type glycan and 10 Cys residues) (98%) from a human seminal fluid was purchased from Fitzgerald, USA. *Sambucus nigra* agglutinin-I (SNA, Neu5Ac(α 2–6)Gal-specific, 140 kDa, 4 subunits, pI 5.4–5.8, a glycoprotein containing 8 Cys residues) was purchased from EY Labs, USA. Biotinylated lectins (*Maackia amurensis* and *Sambucus nigra* agglutinin) and carbo-free blocking solution for lectin microarray experiments were purchased from Vector Laboratories (USA). A CF647-streptavidin fluorescent label was purchased from Biotium (USA). All solutions were prepared in 0.055 μS ultrapure deionized water and were subsequently filtered prior to use using 0.2 μm sterile filters.

2.2 Apparatus

2.2.1 Lectin microarrays (LMA)—LMA experiments were run using SpotBot3 Microarray Protein edition (Arrayit, USA) on epoxide coated slides Nexterion E (Schott, Germany) using a previously optimized protocol and scanned using InnoScan710 scanner (Innopsys, France) at the wavelength of 630 nm [21]. The slide image was evaluated using the Mapix 5.5.0 software.

Fluorescent protein microarray experiment was performed using 10 mM K-phosphate pH 7.0 as a printing and washing buffer and containing a 10 $\times$ diluted carbo-free blocking solution (VectorLabs, USA) as a blocking buffer. Shortly, six different concentrations of diluted PSA (including a 1 $\text{mg}\cdot\text{mL}^{-1}$ stock solution) were spotted using SpotBot3 Microarray Protein edition (Arrayit, USA) on epoxide coated slides Nexterion E (Schott, Germany) using a previously optimized protocol [21]. Spotting temperature was set to 10 $^{\circ}\text{C}$ and humidity to 60%. Subsequently, the slide was blocked using a blocking buffer at room temperature for 1 h, rinsed under a gentle stream of a printing buffer and drained. Then, 100 μL of 25 $\mu\text{g}\cdot\text{mL}^{-1}$ biotinylated lectin (SNA and MAA respectively) in a binding buffer was applied to the slide surface and incubated for 1 h. After lectin incubation, the slide was incubated with the Biotium CF647-streptavidin solution (1 $\mu\text{g}\cdot\text{mL}^{-1}$ in a printing buffer) for 15 min. After a washing procedure, the slide was scanned using an InnoScan710 scanner (Innopsys, France) at a wavelength of 635 nm. The slide image was evaluated using the Mapix 5.5.0 by evaluation of the intensity of fluorescence and intensity of all independent array spots on the array (normalized to the background).

2.2.2 Surface plasmon resonance (SPR)—For the SPR measurements, a carboxymethyl dextran hydrogel (CMD) modified gold chip (12 $\times$ 12 $\times$ 0.3 mm, 50 nm Au thickness, medium density, Xantec Bioanalytics, Germany) was used. The chip was activated using EDC/NHS (1+1 ratio of 0.2 M EDC and 0.05 M NHS, respectively) and subsequently

PSA was covalently immobilized on the chip surface from a stock solution with a concentration of $0.33 \text{ mg}\cdot\text{mL}^{-1}$ ($11.6 \text{ }\mu\text{M}$) for 10 min at a flow rate of $5 \text{ }\mu\text{L}\cdot\text{min}^{-1}$. After washing step, MAA and SNA lectins as binding analytes were injected on a chip in five different concentrations (prepared by dilution from their $0.33 \text{ mg}\cdot\text{mL}^{-1}$ stock solutions). After each binding step, the chip surface was regenerated by 20 mM HCl. The sensorgram was recorded and evaluated using SPR Autolink software 1.1.7 (Reichert, USA). Surface coverage of bound PSA, as well as the ratio of SNA/MAA lectin binding was obtained using a SPR machine (SR7000DC, Reichert, USA) operated with an autosampler. All proteins were dissolved in 10 mM K-phosphate buffer pH 7.0 prepared from ultra-pure deionized water ($0.0055 \text{ }\mu\text{S}$).

2.2.3 Electrochemical measurements—Electrochemical measurements were performed on an Autolab analyser (PGSTAT30, EcoChemie the Netherlands) connected with VA-Stand 663 (Metrohm Switzerland) with a three-electrode system. HMDE(0.4 mm^2) as a working electrode, Ag|AgCl|3MKCl as a reference one and Pt wire as an auxiliary electrode were used in a standard thermostated cell open to air. $1 \text{ }\mu\text{M}$ PSA (if not stated otherwise) was adsorbed at the working electrode from $5 \text{ }\mu\text{L}$ of 50 mM Na-phosphate, pH 7.0 at open current circuit for 60 s without stirring (Schematic 1A) to reach full electrode coverage. The HMDE modified by PSA was incubated in $1 \text{ }\mu\text{M}$ lectin in 50 mM Na-phosphate, pH 7.0 at open current circuit for 120 s under stirring at 1500 rpm (Schematic 1B). The protein-modified electrode was washed and transferred into a blank background electrolyte followed by recording of chronopotentiogram (Schematic 1C) or C-E curve by alternating current voltammetry (a.c. voltammetry) in phase-out mode at $20 \text{ }^\circ\text{C}$ (if not stated otherwise). A.c. voltammetry: scan rate 7.5 mV/s , frequency 223 Hz, amplitude 50 mV. CPS measurements: stripping current $I_{\text{str}} - 45 \text{ }\mu\text{A}$ (if not stated otherwise). Experiments were replicated at least 3 times for each measurement.

Denaturation of $12.6 \text{ }\mu\text{M}$ PSA in sodium-phosphate buffer, pH 7 solution with 7 M urea was performed overnight at $4 \text{ }^\circ\text{C}$. The protein solution was then diluted by the background electrolyte to the final protein $1 \text{ }\mu\text{M}$ concentration and immediately measured; the final non-denaturing concentration of urea was 560 mM.

3 Results and discussion

3.1 Chronopotentiometric analysis

We have shown that CPS in combination with a high electron-yield electro-catalytic process is convenient for protein analysis [6]. Such analysis is based on the ability of some amino acid residues to catalyse hydrogen evolution on mercury-containing electrodes [6,32]. Our approach to study PSA-lectin interactions was based on our previous paper dealing with lectin-ovalbumin interactions [31]. In difference to that paper, in which the Concanavalin A-ovalbumin was prepared in a test-tube and then measured at the electrode, here we interacted the surface-attached PSA with a lectin in solution. This arrangement is more convenient for sensing of differences in PSA glycosylation, but it strongly depends on orientation of the PSA molecules on the electrode surface. Earlier we showed that HMDE could be easily modified with thiols and particularly with dithiols, such as DTT [27]. The thiol SAM

protects the surface-attached protein from the denaturing effect of the electric field in the vicinity of the negatively charged electrode surface. PSA molecule contains 10 Cys residues, all of them involved in disulfide binding. Among these residues eight are located close to the surface of the PSA molecule in the side opposite to the glycan moiety (Fig. 1). Considering the PSA structure and high affinity of sulphur to mercury, we decided to use bare HMDE because we expected that the four cystine dithiols will strongly bind to mercury electrode surface, while the glycan will be available for its interactions with the environment, capable to form specific complexes with lectins.

1 μM PSA was adsorbed on HMDE from a 5 μL drop for t_A 60 s. After a gentle rinse, the PSA-modified HMDE was transferred into a solution of 1 μM MAA or 1 μM SNA lectin in 50 mM sodium phosphate, pH 7 and incubated for 2 min. After this incubation the PSA-modified electrode was transferred into a blank electrolyte followed by CPS analysis at $I_{\text{str}} - 45 \mu\text{A}$ at 20 $^{\circ}\text{C}$ (Schematic 1). PSA alone yielded two small peaks H at -1.86 V and -1.89 V vs. $\text{Ag}|\text{AgCl}|3 \text{ M KCl}$ (Fig. 2A), respectively, suggesting that PSA was on the HMDE surface in its native form (denatured PSA yielded more than 4 times higher peak H than that of native PSA, Fig. 2B). Under these conditions MAA lectin (not containing any Cys residues) produced no peak H (Fig. 2A). In contrast SNA lectin yielded a small peak at -1.90 V vs. $\text{Ag}|\text{AgCl}|3 \text{ M KCl}$ (Fig. 2A). MW of these two lectins are similar, but SNA contains 8 Cys residues. These SNA Cys residues are most likely less available for the electrocatalysis and might be more distant from the HMDE surface as PSA molecule, which is about 5 times smaller (Fig. 2A). After the incubation of PSA-modified HMDE with SNA lectin, peak Hof PSA (peak potential of -1.86 V vs. $\text{Ag}|\text{AgCl}|3\text{-M KCl}$) increased more than twice (Fig. 2A). Similarly, specific interaction of ovalbumin with Concanavalin A in solution resulted in an increase of the ovalbumin peak H [31]. This phenomenon may be explained by the change in the PSA orientation on the HMDE surface after the lectin binding to PSA N-glycan, or by a conformational change in PSA after this biorecognition process. Peak H height of PSA after SNA binding was about 25% lower than that of urea-denatured PSA (Fig. 2B), suggesting that not all electroactive residues of PSA were involved in catalytic hydrogen evolution reaction after the biorecognition. In contrast, peak H of PSA alone was almost the same as that obtained after PSA incubation with MAA lectin (Fig. 2A). These results can be due to almost no binding of MAA to the surface-attached PSA as compared to the specific SNA binding to PSA. Electrochemical data (Fig. 2) appeared thus in a good agreement with impedimetric assay [24] and the lectin microarray results (see below).

In CPS the rate of potential changes increases non-linearly with a current density and may reach very high polarization rates [6]. By using high negative intensity I_{str} , the exposure time of molecules attached to the surface may be reduced to milliseconds. Exposure to the electric field can induce denaturation of the surface-attached protein [6,27,33] as well as disintegration of the DNA-protein [28] or lectin-glycoprotein complexes [31]. Susceptibility of proteins and their complexes to the effects of an electric field at negative potentials decreases with decreasing temperature [6,28,33]. We studied effect of the stripping current (I_{str}) intensity on the CPS responses of PSA and PSA after the interaction with MAA and SNA lectins at a constant temperature (Fig. 2C) and effect of temperature at a constant current (Fig. 2D). Fig. 2C and D are showing a structural transition of PSA alone between -40 and $-45 \mu\text{A}$ at 20 $^{\circ}\text{C}$ and at constant current $-45 \mu\text{A}$ between 25 and 30 $^{\circ}\text{C}$. These

results are in a good agreement with the results obtained for other proteins [6, 33], where structural changes between native and denatured forms of proteins were caused by an external electric field [33]. PSA with MAA and PSA alone displayed almost the same curves, suggesting very weak or no interaction between PSA and MAA (Fig. 2C). On the other hand, incubation of PSA with SNA resulted in an increase of peak H at $I_{str} = -45 \mu A$ and at more negative I_{str} intensities (Fig. 2C). This increase can be explained by partial protein unfolding after PSA-SNA interaction. At less negative I_{str} intensities, where the surface-attached PSA is denatured (due to the prolonged electric field effects at negative potentials) PSA alone and PSA-SNA complex yielded almost the same peak H areas (Fig. 2C). It cannot be excluded that, in addition to the protein denaturation, disintegration of the PSA-SNA complex took place under these conditions at the electrode surface. The observed dependence of peak H on I_{str} (Fig. 2C) is supported by the results of temperature dependence at constant I_{str} of $-45 \mu A$ (Fig. 2D), which shows a structural transition between 25 and 30 °C in PSA and PSA-MAA but not in PSA-SNA in agreement with our assumption that protein unfolding resulted already from the interaction of SNA with the surface-attached PSA.

3.2 Adsorption/desorption behaviour of PSA

Electrochemical double layer capacitance is a sensitive indicator of adsorption processes [34]. Protein adsorbed on an electrode surface displaces solvent molecules and ions and reduces the capacity because of higher dielectric permittivity of solvent compared to protein solution. We measured a dependence of capacity (C) of PSA-modified HMDE electrochemical double layer on polarization potential E (C-E curves). PSA lowers the C in the potential range of about -0.1 to -1.7 V vs. Ag|AgCl|3 M KCl (Fig. 3A), giving two peaks: first at -0.57 V and second (less developed) at -1.56 V vs. Ag|AgCl|3 M KCl. Presence of the first one is caused by the protein reorientation on the surface after Hg-S(Cys) bond reduction at a potential of about -0.6 V vs. Ag|AgCl|3 M KCl. Signals of proteins at negative potentials, far from the potential of the zero charge (p.z.c.) are markedly influenced by processes linked to denaturation of the surface-attached protein. We therefore considered E-C values obtained close to p.z.c., which are better comparable to the CPS results at high $-I_{str}$. Fig. 3B shows that at -0.3 V vs. Ag|AgCl|3 M KCl, there is an observable increase in the C correlating to an increasing concentration of PSA (from 50 to 750 nM). For higher PSA concentrations, no further increase was observed, suggesting that we already obtained a full surface coverage for this protein.

To obtain more information about arrangement of PSA and lectins layers at the electrode surface, we measured their specific capacity. We did not observe any peak around -0.6 V vs. Ag|AgCl|3 M KCl on C-E curves of free MAA lectin, while SNA yielded the peak much smaller than that of PSA alone (Fig. 3A). C-E curves of both lectins significantly differed from that of PSA at potentials more negative than -1.2 V vs. Ag|AgCl|3 M KCl. We incubated the PSA-modified electrode with lectin in solution (50 mM Na-phosphate, pH 7) and transferred the electrode into the blank electrolyte. In this way we obtained C-E curves, which were at potentials close to p.z.c., similar to that of free PSA (Fig. 3A), suggesting that PSA remained at electrode surface even after the interactions with the lectins.

Furthermore, we studied the effect of the lectin concentration on peak H of surface-attached PSA (Fig. 3C). Additions of SNA lectin up to the 250 nM concentration significantly increased the peak H area until reaching almost 1.5-fold area of this peak as for PSA alone. Further increase in the SNA concentration resulted in only slight increase. Peak area almost twice as much as for PSA alone or PSA-MAA, respectively, was obtained for 3 μ M SNA concentration. At full surface coverage of PSA, we were able to selectively distinguish between specific and nonspecific lectin recognition for lectin concentrations above 250 nM.

CPS as a simple and inexpensive method shown to be a useful tool for the in situ glycoprofiling of widely used PSA biomarker without previous release of the intact glycan (using PNGase F). It is of great importance to switch from the study of only the protein part to the study of their glycan part as well, especially for this biomarker, since its glycosylation is changed during the development of PCa. The main advantage of this method is the reproducibility, which (due to atomically smooth HMDE surface) is much better than that obtained with most of the popular solid electrodes [35,36]. Slightly higher concentrations of PSA are needed for this analysis than contained in human sera (present prostate cancer diagnostic test), this approach can be used for PSA isolated from urine or seminal fluid where the PSA concentration can be relatively high ($\sim 1 \text{ mg}\cdot\text{mL}^{-1}$) [37,38].

3.3 Evaluation of lectin binding specificity by other methods

Binding of SNA and MAA lectins to PSA was evaluated also by standardly used methods like SPR and LMA, respectively. For calculation of the total amount of bound protein during SPR measurements, the conversion $1 \text{ } \mu\text{RIU} = 1 \text{ pg}\cdot\text{mm}^{-2}$ (according to the manufacturer) was applied. Surface coverage of PSA on a SPR chip was calculated as $\Gamma_{\text{PSA}} = (212 \pm 60) \text{ pg}\cdot\text{mm}^{-2}$ i.e. 30% of a theoretical surface coverage of a full protein monolayer. SPR analysis further revealed that MAA lectin from 25 nM protein solution was bound to immobilized PSA with $\Gamma_{\text{MAA}} = 0.55 \text{ pg}\cdot\text{mm}^{-2}$, while lectin SNA with much higher surface coverage of $\Gamma_{\text{SNA}} = 10.1 \text{ pg}\cdot\text{mm}^{-2}$. SPR assays thus showed that the amount of MAA lectin bound to PSA was only 5.4% of the amount of SNA lectin attached to PSA. Similar results were obtained by fluorescent lectin microarray experiment (Fig. 4) using lectins in a concentration range of PSA from 317 nM to 7.69 μ M with a response ratio observed for MAA bound to PSA compared to SNA attached to PSA of $(7.6 \pm 0.1)\%$. These data well-correlated with electrochemical results (Fig. 2).

4 Conclusions

We studied chronopotentiometric and a.c. voltammetric behaviour of the PSA and its interactions with lectins capable to recognize PSA glycans occurring in healthy people or in patients with prostate cancer. We show for the first time that CPS analysis in combination with HMDE can be used to distinguish specific interaction of SNA lectin with PSA (occurring in healthy men) from the PSA interaction with lectin MAA, specific for prostate cancer (PCa) PSA. Earlier we showed that proteins are not significantly denatured when adsorbed at mercury electrode under solution conditions close to physiological but the surface-attached protein can be denatured due to prolonged exposure to the electric field effects at negative potentials [6,33]. In CPS using high current densities, this exposure can

be limited to milliseconds, preventing thus the denaturation of the surface-attached protein. Here we found conditions under which the surface-attached PSA was not denatured in CPS experiments and showed that as a result of PSA-SNA interaction some protein unfolding was taking place in the PSA-SNA complex under conditions at which the PSA alone remained native.

For several reasons, in this paper no attempt was made to develop a sensor. At this stage we prefer to get more data about lectin-glycan interactions and about chemical modification and electrochemical behaviour of glycoproteins in our further work. Moreover, we are aware that HMDE has some unique properties, but it is not the best electrode for sensor development. On the other hand, it has been shown that the HMDE can be substituted by solid amalgam electrodes in the analysis of nucleic acids [36], proteins [6,39] and glycans [40] and we wish to test this possibility in a near future. Knowing that the problem of improving specificity of biomarkers used in cancer and other diseases is very difficult and urgent we believe that attempts to apply electrochemical methods for this purpose are promising and desire increased attention of electrochemists and their involvement in the interdisciplinary research.

Acknowledgement

This study was supported by project No. 13-00956S to VO, No. 15-15479S to EP from the Czech Science Foundation and by project CEITEC 2020 (LQ1601) to KR from the Ministry of Education, Youth and Sports of the Czech Republic. The financial support received from the Slovak Scientific Grant Agency VEGA 2/0162/14 and the Slovak Research and Development Agency APVV-14-0753 is acknowledged. The research leading to these results received funding from the European Research Council under the European Union's Seventh Framework Program (FP/2007-2013)/ERC grant agreement number 311532. This publication was made possible by NPRP grant number 6-381-1-078 from the Qatar National Research Fund (a member of the Qatar Foundation). The statements made herein are solely the responsibility of the authors. This publication is the result of the project implementation: Centre for materials, layers and systems for applications and chemical processes under extreme conditions – Stage I, ITMS No.: 26240120007, supported by the Research & Development Operational Program funded by the ERDF.

References

- [1]. Ferlay J, Steliarova-Foucher E, Lortet-Tieulent J, Rosso S, Coebergh JWW, Comber H, Forman D, Bray F. Cancer incidence and mortality patterns in Europe: estimates for 40 countries in 2012. *Eur J Cancer*. 2013; 49:1374–1403. [PubMed: 23485231]
- [2]. Ng SJK, Sinha AK, Loi HY, Khor LK. Asymptomatic metastasis to cricoid from prostate carcinoma: an incidental finding detected on 18F-choline PET/CT. *Jpn J Radiol*. 2015; 33:298–301. [PubMed: 25820451]
- [3]. Wong LIL, Labrecque MP, Ibuki N, Cox ME, Elliott JE, Beischlag TV. p,p'-Dichlorodiphenyltrichloroethane (p,p'-DDT) and p,p'-dichlorodiphenyldichloroethylene (p,p'-DDE) repress prostate specific antigen levels in human prostate cancer cell lines. *Chem Biol Interact*. 2015; 230:40–49. [PubMed: 25682698]
- [4]. Llop E, Ferrer-Batallé M, Barrabés S, Guerrero PE, Ramírez M, Saldova R, Rudd PM, Aleixandre RN, Comet J, de Llorens R, Peracaula R. Improvement of prostate cancer diagnosis by detecting PSA glycosylation-specific changes. *Theranostics*. 2016; 6:1190–1204. [PubMed: 27279911]
- [5]. Moyer VA. Screening for prostate cancer: U.S. preventive services task force recommendation statement. *Ann Intern Med*. 2012; 157:120–134. [PubMed: 22801674]
- [6]. Pale ek E, Tká J, Bartošík M, Bertók TS, Ostatná V, Pale ek J. electrochemistry of nonconjugated proteins and glycoproteins. toward sensors for biomedicine and glycomics. *Chem Rev*. 2015; 115:2045–2108. [PubMed: 25659975]
- [7]. Li QK, Chen L, Ao M-H, Chiu JH, Zhang Z, Zhang H, Chan DW. Serum-fucosylated prostate-specific antigen (PSA) improves the differentiation of aggressive from nonaggressive prostate cancers. *Theranostics*. 2015; 5:267–276. [PubMed: 25553114]

- [8]. Badr HA, Alsadek DM, Darwish AA, Elsayed AI, Bekmanov BO, Khussainova EM, Zhang X, Cho WC, Djansugurova LB, Li CZ. Lectin approaches for glycoproteomics in FDA-approved cancer biomarkers. *Expert Rev Proteomics*. 2014; 11:227–236. [PubMed: 24611567]
- [9]. Pihikova D, Pakanova Z, Nemcovic M, Barath P, Belicky S, Bertok T, Kasak P, Mucha J, Tkac J. Sweet Characterisation of Prostate Specific Antigen Using Electrochemical Lectin-Based Immunosensor Assay and MALDI TOF/TOF Analysis: Focus on Sialic Acid. *Proteomics*. 2016; 16:3085–3095. [PubMed: 26920336]
- [10]. Gilgunn S, Conroy PJ, Saldova R, Rudd PM, O'Kennedy RJ. Aberrant PSA glycosylation – a sweet predictor of prostate cancer. *Nat Rev Urol*. 2013; 10:99–107. [PubMed: 23318363]
- [11]. Vermassen T, Speeckaert MM, Lumen N, Rottey S, Delanghe JR. Glycosylation of prostate specific antigen and its potential diagnostic applications. *Clin Chim Acta*. 2012; 413:1500–1505. [PubMed: 22722018]
- [12]. Murthy V, Rishi A, Gupta S, Kannan S, Mahantshetty U, Tongaonkar H, Bakshi G, Prabhash K, Bhanushali P, Shinde B. Clinical impact of prostate specific antigen (PSA) inter-assay variability on management of prostate cancer. *Clin Biochem*. 2016; 49:79–84. [PubMed: 26506115]
- [13]. Wang X, Chen J, Li QK, Peskoe SB, Zhang B, Choi C, Platz EA, Zhang H. Overexpression of α (1,6) fucosyltransferase associated with aggressive prostate cancer. *Glycobiology*. 2014; 24:935–944. [PubMed: 24906821]
- [14]. Hatakeyama S, Yoneyama T, Tobisawa Y, Ohyama C. Recent progress and perspectives on prostate cancer biomarkers. *Int J Clin Oncol*. 2017; 22:214–221. [PubMed: 27730440]
- [15]. Pihikova D, Kasak P, Kubanikova P, Sokol R, Tkac J. Aberrant sialylation of a prostate-specific antigen: electrochemical label-free glycoprofiling in prostate cancer serum samples. *Anal Chim Acta*. 2016; 934:72–79. [PubMed: 27506346]
- [16]. Belicky S, Tkac J. Can glycoprofiling be helpful in detecting prostate cancer? *Chem Pap*. 2015; 69:90–111.
- [17]. Dosekova E, Filip J, Bertok T, Both P, Kasak P, Tkac J. Nanotechnology in Glycomics: Applications in Diagnostics, Therapy, Imaging, and Separation Processes. *Medicinal Research Reviews*. 2017; 37:514–626. [PubMed: 27859448]
- [18]. Bertok T, Dosekova E, Belicky S, Holazova A, Lorencova L, Mislovicova D, Paprckova D, Vikartovska A, Plicka R, Krejci J, Ilcikova M, et al. Mixed zwitterion-based self-assembled monolayer interface for impedimetric glycomic analyses of human IgG samples in an array format. *Langmuir*. 2016; 32:7070–7078. [PubMed: 27311591]
- [19]. Labib M, Sargent EH, Kelley SO. Electrochemical methods for the analysis of clinically relevant biomolecules. *Chem Rev*. 2016; 116:9001–9090. [PubMed: 27428515]
- [20]. Bertok T, Šedivá A, Filip J, Ilcikova M, Kasak P, Velic D, Jane E, Mravcová M, Rovenský J, Kunzo P, Lobotka P, et al. Carboxybetaine modified Interface for electrochemical glycoprofiling of antibodies isolated from human serum. *Langmuir*. 2015; 31:7148–7157. [PubMed: 26048139]
- [21]. Klukova L, Bertok T, Petrikova M, Sediva A, Mislovicova D, Katrlík J, Vikartovska A, Filip J, Kasak P, Andicsová-Eckstein A, Mosná ek J, et al. Glycoprofiling as a novel tool in serological assays of systemic sclerosis: a comparative study with three bioanalytical methods. *Anal Chim Acta*. 2015; 853:555–562. [PubMed: 25467503]
- [22]. Jolly P, Tamboli V, Harniman RL, Estrela P, Allender CJ, Bowen JL. Aptamer–MIP hybrid receptor for highly sensitive electrochemical detection of prostate specific antigen. *Biosens Bioelectron*. 2016; 75:188–195. [PubMed: 26318788]
- [23]. Tzouavadaki I, Jolly P, Lu X, Ingebrandt S, de Micheli G, Estrela P, Carrara S. Label-free ultrasensitive memristive aptasensor. *Nano Lett*. 2016; 16:4472–4476. [PubMed: 27341189]
- [24]. Pihíková D, Belický Š, Kasák P, Bertok T, Tkac J. Sensitive detection and glycoprofiling of a prostate specific antigen using impedimetric assays. *Analyst*. 2016; 141:1044–1051. [PubMed: 26647853]
- [25]. Trefulka M, Palecek E. Can voltammetry distinguish glycan isomers? *Chem Pap*. 2015; 69:241–244.
- [26]. Strme ki S, Trefulka M, Zatloukalová P, Durech M, Vojtesek B, Pale ek E. Immunoassays of chemically modified polysaccharides, glycans in glycoproteins and ribose in nucleic acids. *Anal Chim Acta*. 2017; 955:108–115. [PubMed: 28088277]

- [27]. Ostatna V, Cernocka H, Palecek E. Protein structure-sensitive electrocatalysis at DTT-modified electrodes. *J Am Chem Soc.* 2010; 132:9408–9413. [PubMed: 20557043]
- [28]. Palecek E, Cernocka H, Ostatna V, Navratilova L, Brazdova M. Detection of p53 protein sequence-specific DNA binding at an electrified interface. *Anal Chim Acta.* 2014; 828:1–8. [PubMed: 24845809]
- [29]. Palecek E, Ostatna V, Cernocka H, Joerger AC, Fersht AR. Electrocatalytic monitoring of metal binding and mutation-induced conformational changes in p53 at picomole level. *J Am Chem Soc.* 2011; 133:7190–7196. [PubMed: 21491862]
- [30]. Palecek E, Ostatna V, Masarik M, Bertoncini CW, Jovin TM. Changes in interfacial properties of alpha-synuclein preceding its aggregation. *Analyst.* 2008; 133:76–84. [PubMed: 18087617]
- [31]. Vargová V, Helma R, Palecek E, Ostatná V. Electrochemical sensing of Concanavalin A and ovalbumin interaction in solution. *Anal Chim Acta.* 2016; 935:97–103. [PubMed: 27543018]
- [32]. Dorcak V, Ostatna V, Palecek E. Electrochemical reduction and oxidation signals of angiotensin peptides. Role of individual amino acid residues. *Electrochem Commun.* 2013; 31:80–83.
- [33]. Cernocká H, Ostatná V, Palecek E. Protein structural transition at negatively charged electrode surfaces. Effects of temperature and current density. *Electrochim Acta.* 2015; 174:356–360.
- [34]. Vetterl, V., Hason, S. Electrochemical Properties of Nucleic Acid Components. *Electrochemistry of Nucleic Acids and Proteins. Towards Electrochemical Sensors for Genomics and Proteomics.* Palecek, E., Scheller, F., Wang, J., editors. Elsevier; Amsterdam: 2005. p. 18-73.
- [35]. Barek J, Fischer J, Navratil T, Peckova K, Yosypchuk B, Zima J. Nontraditional electrode materials in environmental analysis of biologically active organic compounds. *Electroanalysis.* 2007; 19:2003–2014.
- [36]. Yosypchuk B, Heyrovsky M, Palecek E, Novotny L. Use of solid amalgam electrodes in nucleic acid analysis. *Electroanalysis.* 2002; 14:1488–1493.
- [37]. Lövgren J, Valtanen-André C, Marsal K, Liua H, Lundwall Å. Measurement of prostate-specific antigen and human glandular Kallikrein 2 in different body fluids. *J Androl.* 1999; 20:348–355. [PubMed: 10386814]
- [38]. Wang TJ, Rittenhouse HG, Wolfert RL, Lynne CM, Brackett NL. PSA concentrations in seminal plasma. *Clin Chem.* 1998; 44:895–896.
- [39]. Juskova P, Ostatna V, Palecek E, Foret F. Fabrication and characterization of solid mercury amalgam electrodes for protein analysis. *Anal Chem.* 2010; 82:2690–2695. [PubMed: 20210297]
- [40]. Palecek E, Trefulka M. Electrocatalytic detection of polysaccharides at picomolar concentrations. *Analyst.* 2011; 136:321–326. [PubMed: 21072334]

Fig. 1. Schematic presentation of PSA (adapted from pdb code: 3QUM) with glycosylation site (red), showing disulfide bonds between Cys residues (yellow) present mainly on the opposite site of the molecule, suggesting possible explanation of the increase in the peak H after lectin biorecognition; source: www.rcsb.org (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 2.

A, B CPS peak H of 1 μM native (black) and denatured (gray) PSA, lectin *Sambucus nigra* (SNA, red), lectin *Maackia amurensis* (MAA, cyan) and complex between native PSA and 1 μM SNA (magenta), native PSA and 1 μM MAA (blue), denatured PSA and SNA (yellow) at hanging mercury drop electrode (HMDE) in 50 mM Na-phosphate, pH 7 at 20 °C; $I_{\text{str}} = -45 \mu\text{A}$. **C, D.** Dependence of peak area on **C.** stripping current $-I_{\text{str}}$ at 20 °C and **D.** temperature at $I_{\text{str}} = -45 \mu\text{A}$ for PSA (■, black) and PSA complex with SNA (●, magenta) or with MAA (▲, blue). PSA was adsorbed at the working electrode from 5 μL of 50 mM Na-phosphate, pH 7 at open current circuit for 60 s without stirring. The PSA-modified electrode was incubated in 1 μM lectin in 50 mM Na-phosphate, pH 7 for 120 s under stirring at 1500 rpm. The protein-modified electrode was washed and transferred into the blank background electrolyte followed by CPS measurement at given I_{str} and temperature. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 3.

A. Adsorptive transfer stripping C-E curves of PSA (black), SNA (red), MAA (cyan), PSA-SNA complex (magenta) and PSA-MAA complex (blue) in background electrolyte (e). **B.** Dependence of differential capacity on concentration of PSA. **C.** Dependence of peak H area on concentration of lectin SNA (●, magenta) or MAA (■, blue) at 1 μM PSA concentration. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 4.

Fluorescent microarray experiment showing an increased binding of SNA lectin (magenta) to PSA covalently immobilized on an epoxy coated glass slide, compared to MAA lectin (grey). In the figure inset a real fluorescence read at 635 nm for both lectins in a concentration range of PSA from 0.021 to 1 mg mL⁻¹ is shown. Fluorescence intensity was normalized to background. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Schematic 1.

Schematic representation of modification of HMDE by PSA and lectin.