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Sweet characterisation of prostate specific antigen using electrochemical lectin-based immunosensor assay and MALDI TOF/TOF analysis: Focus on sialic acid

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Abstract

The construction of a sensitive electrochemical lectin-based immunosensor for detection of a prostate specific antigen (PSA) is shown here. Three lectins with different carbohydrate specificities were used in this study to glycoprofile PSA, which is the most common biomarker for prostate cancer (PCa) diagnosis. The biosensor showed presence of α-L-fucose and α-(2,6)-linked terminal sialic acid within PSA´s glycan with high abundance, while only traces of α-(2,3)-linked terminal sialic acid were found. MALDI TOF/TOF mass spectrometry was applied to validate results obtained by the biosensor with a focus on determination of a type of sialic acid linkage by two methods. The first direct comparison of electrochemical immunosensor assay employing lectins for PSA glycoprofiling with mass spectrometric techniques is provided here and both methods show significant agreement. Thus, electrochemical lectin-based immunosensor has potential to be applied for prostate cancer diagnosis.

Keywords

Glycan; Glycoproteomics; Impedimetric biosensor; Lectins; MALDI-TOF/TOF; Prostate specific antigen (PSA)

1 Introduction

Sialic acids are carbohydrates heavily expressed on proteins and on the outer cell membrane, they play a vital role in numerous processes such as stabilisation of molecules/membranes, cell-cell recognition and host–pathogen interactions [1]. Moreover, altered sialylation is

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frequently accompanied with various malignancies [2, 3] causing an increased disease aggressiveness and tumour growth [4, 5]. Sialic acids are found on both N- and O-linked glycans, frequently attached to an underlying galactose residue via an α-(2,6) or α-(2,3) glycosidic linkage. Although α-(2,6) sialylation is widely present in healthy tissues, an increased enzymatic expression of ST6Gal-1 (β-galactoside α-2,6-sialyltransferase 1, the enzyme responsible for adding α-(2,6) sialic acid to terminal galactose) was reported in various types of cancer [6, 7]. Furthermore, α-(2,3)-linked sialic acid is rarely found in healthy tissues, but is greatly elevated in cancer [8, 9]. The expression of sLe^x tumour antigen (sialyl $Lewis^x$, a tetrasacharide containing an α-(2,3)-terminal sialic acid and α-(1,3)-fucose) is related to metastatic spread of cancer with a significantly poorer patient's prognosis in prostatic [10] and colorectal carcinomas [11]. Thus, the ability to determine changes in the microheterogeneity of sialic acid, could improve cancer diagnosis.

In recent years it has been possible to see an increased incidence and mortality of prostate cancer (PCa), what is now the third leading cause of all cancer-related deaths in men in EU (92 000 deaths in 2012) [12, 13]. Symptoms of an early stage PCa can be absent or mild and thus patients can realize presence of the disease only when it develops into an advanced stage with bad prognosis to cure the disease. Gold standard applied for PCa diagnosis for quite a long time has been analysis of a prostate specific antigen (PSA) level in serum, but this can vary considerably with age, ethnicity and other factors, causing the test results to be hard to interpret. Due to low sensitivity, specificity and prognostic value of this biomarker, in 2012 the US Preventive Services Task Force advised not to use analysis of PSA for routine screening of PCa [14].Therefore new and more reliable methods for PCa diagnosis are needed. Increasing clinical and technical capabilities and better characterisation of existing biomarkers might contribute to introduction of novel methods with better diagnostic, monitoring and prognostic performance and to the discovery of new candidate biomarkers [15].

Analysis of altered protein glycosylation as a result of progression of various types of cancers [16–25] and as targets for personalised medicine [26] is gaining an increased attention [21]. Such focus is driven by the interest to understand correlation between cancer disease progression and prognosis on one side and changes in the glycan composition on the other side [27] for development of advanced therapeutic/diagnostic tools to treat various diseases [28–31]. Mass spectrometry combined with a battery of separation approaches is the most frequently applied analytical platform for in depth analysis of glycans, but getting information about the glycan structure can be quite challenging [32]. This issue can be overcome using lectins (natural glycan recognising proteins) which can detect glycans still attached to intact proteins or cells in natural conformation. Moreover, lectins can recognise different linkages between two carbohydrates within a glycan structure (i.e. α-(2,3)- linked *vs.* α-(2,6)- linked sialic acid to galactose), what is quite difficult for instrumental-based approach [33]. Lectin microarray have distinct advantages such as a possibility for extremely high throughput of analysis, low sample/reagent consumption with high reliability of assays, but analysis of low abundant glycans is problematic and such approach require sample labelling, which can negatively affect robustness of analysis [34, 35]. Therefore, in recent years other detection platforms for glycan detection offering high sensitivity of analysis in a label-free mode of operation are being intensively developed [33, 36–39].

Electrochemical methods and especially electrochemical impedance spectroscopy (EIS) can offer analysis of analyte down to a single molecule level in a label-free mode of operation [33, 40]. The method detects change in the charge transfer resistance (R_{ct}) of the electrode after incubation of the biosensor with the analyte and has been effectively applied in the field of glycomics [33]. In our previous work a lectin-based ultrasensitive impedimetric biosensor for PSA glycoprofiling in a sandwich configuration with immobilised antibody was developed (Supporting Information Fig. S1) [41]. This was possible by immobilisation of an antibody against PSA on the electrode surface, then by the incubation of the biosensor with PSA with the final step being an interaction of lectin with the biosensor *via* a glycan moiety present on PSA (Supporting Information Fig. S1). In this work several lectins were applied for qualitative impedimetric assessment of the glycan composition on the PSA surface and in order to effectively validate these findings, matrix-assisted laser desorption/ionization (MALDI) time-of flight (TOF) mass spectrometry (MS) was used with a focus on analysis of sialic acid linkages. The presented biosensor approach was validated by high-throughput mass spectrometric techniques for the first time, what can be further applied in glyco-biomarker research in early-stage cancer diagnosis, especially focusing on cancer-related sialylated glycans.

2 Materials and methods

2.1 Materials and reagents

Recombinant peptide-N-Glycosidase F (PNGase F) from *Flavobacterium meningosepticum* was purchased from Roche (Basel, CH). Unspecific α-(2,3,6,8)-neuraminidase from *Clostridium perfringens* was obtained from New England BioLabs (Ipswich, MA, USA) and GlycoSialidase S from *Streptococcus pneumoniae,* specific for α-(2,3)-linked sialic acid was obtained from ProZyme (Hayward, CA, USA). Sequencing grade modified trypsin was purchased from Promega (WI, USA). Free-prostate specific antigen (PSA) purified from human seminal fluid was obtained from Fitzgerald Industries International (Acton, MA, USA) and anti-PSA monoclonal antibody (Ab10187) was purchased from Abcam (Cambridge, UK). 2,5-Dihydroxyacetophenone (2,5-DHAP) and 4-HCCA (α-Cyano-4-hydroxycinnamic acid) were obtained from Bruker Daltonics (Billerica, MA, USA). 11-mercaptoundecanoic acid (MUA), 6-mercapto-1-hexanol (MH), 6-aza-2-thiothymine (ATT), diammonium hydrogen citrate, hydrogen peroxide solution 30% (w/w), phosphate buffered saline (PBS) tablets, potassium chloride, potassium hexacyanoferrate(III), potassium hexacyanoferrate(II) trihydrate, 1-hydroxy-benzotriazole (HOBt), N-hydroxysuccinimide (NHS), 1-ethyl-3-(3-(dimethylamino)propyl) carbodiimide (EDC), sodium hydroxide, iodacetamide (IAA), dithiotreitol (DTT), Dowex 50Wx8 (hydrogen form), ENVI-Carb non-porous graphitised carbon (nPGC), 2,5-dihydroxybenzoic acid (DHB), ammonium acetate, ammonium bicarbonate and sulphuric acid (95.0–98.0%) as well as acetonitrile (ACN), methanol and water in HPLC qualities were purchased from Sigma Aldrich (St. Louis, MO, USA). Acetic acid and LiChroprep RP18 sorbent were purchased from Merck (Darmstadt, DE). *Sambucus nigra* agglutinin type I (SNA, specific for α-(2,6)-terminal sialic acid) from elderberry was obtained from EY Laboratories (San Mateo, CA, USA), *Lotus tetragonolobus* agglutinin (LTA, recognising α-L-Fuc) and *Maackia amurensis* agglutinin II (MAA, recognising α-(2,3)-terminal sialic acid) was purchased from Vector Laboratories

(Burlingame, CA, USA). Ultrapure ethanol (for UV/VIS spectroscopy) was purchased from Slavus (Bratislava, SK). Phosphate buffer saline (PBS) solution (10 mM, pH 7.4) was prepared by dissolving 1 tablet in 200 mL of ultra-pure deionized water (DW). All solutions for electrochemical analysis were filtered *prior* to use (0.2 μm sterile filters) and working solutions of PSA and anti-PSA antibody were freshly prepared in 10 mM PBS, pH 7.4.

2.2 Electrochemical lectin-based immunosensor assay

2.2.1 Biosensor surface preparation—Gold disk electrodes with d = 1.6 mm (BASi, IN, USA) were initially cleaned by electrochemical reductive desorption under N_2 atmosphere using cyclic voltammetry (CV) measurements in 0.1 M NaOH. Electrodes were then polished carefully for 5 min each with a micropolish alumina slurry with the particle size of 1.0 and 0.3 μm (Buehler, USA) and afterwards they were rinsed with DW and cleaned ultrasonically for 5 min to eliminate residual alumina particles. Polished electrodes were dipped into a freshly prepared piranha solution (H_2SO_4:H_2O_2, v/v 3:1) for 15 min to remove any possible surface contaminants. As such, piranha solution is reactive, extremely corrosive and can be explosive, a special handling care is needed. After sonication in DW for 5min, the electrodes were electrochemically polished in 0.5 M H_2SO_4 by running 50 scans in the potential window from –200 to +1500 mV followed by the gold oxide stripping procedure (20 scan run from +750 to +200 mV at a scan rate of 100 mV/s). Finally, the electrodes were rinsed thoroughly with DW and ultra-pure ethanol and then dried in a stream of a pure nitrogen gas [42]. Cleaned electrodes were immediately exposed to 400 μL of a mixed ethanolic solution of 1 mM MUA and 1 mM MH in a ratio 1:3 v/v and kept overnight at room temperature (RT) in the dark to form a dense self-assembled monolayer (SAM). After SAM modification, the electrodes were rinsed in ultra-pure ethanol and allowed to dry.

2.2.2 Fabrication of the PSA immunosensor—Thereafter, the SAM-modified electrodes were placed in an aqueous solution of 0.2 M EDC and 0.05 M NHS for 15 min to activate the terminal carboxylic groups of MUA. The antibody was covalently immobilised from a 40 μL stock solution (20 ng/mL in 10mMPBS,pH7.4) directly on the NHS-coated electrodes by incubation for 30 min at RT. Subsequently, the electrodes were immersed in a 0.1% gelatine solution for 30 min to block the non-specific adsorption with further incubation of 10 ng/mL PSA analyte (~30 min). Finally, 40 μL lectin solution (0.5 mg/mL in 10 mM PBS, pH 7.4) was incubated for 30 min followed by gentle rinsing with 10 mM PBS solution. Prior to EIS analysis, all the electrodes were thoroughly rinsed with 10 mM PBS solution.

2.2.3 Assay procedure—All electrochemical measurements were performed on a laboratory potentiostat/galvanostat PGSTAT 128N (Metrohm Autolab, NL) controlled by NOVA software 1.10. A conventional three-electrode system with a gold disk working electrode, auxiliary platinum electrode and Ag/AgCl reference electrode was used in all experiments. All potentials reported are relative to the Ag/AgCl-3 M KCl reference. The EIS measurements were recorded at 50 different frequencies (from 0.1 Hz up to 100 kHz) applying a 200 mV ac voltage in a freshly prepared and filtered redox probe containing 5 mM ferri/ferrocyanide ([Fe(CN)6]3−/4−) and 0.1 M KCl. Data acquired were shown in the complex plane Nyquist plot with standard Randles-Erschler equivalent circuit (R(C[RW])

employed for data fitting with an error not exceeding 5%. All measurements were performed at RT (~25 °C). Each experiment was measured at least in triplicate (±SD) with an independent biosensor device.

2.3 Quartz crystal microbalance (QCM) experiments

All QCM measurements were performed using Autolab PGSTAT 128N (Ecochemie, NL) equipment using an EQCM module. The changes per mass were evaluated using Sauerbrey´s equation:

$$\Delta f = -\frac{2f_0^2}{\mathrm{A}\sqrt{\rho_q \mu_q}} * \Delta \mathrm{m} \quad (1)$$

where Δf is the frequency change (Hz), f_0 is the nominal resonant frequency of the crystal (6 MHz), Δm is the change in mass (g/cm^2) and μ_q is the shear modulus of a quartz crystal (g/(cm s^2)), A is the surface area and ρ_q is the density of quartz in g/mL. For a 6 MHz crystal, the whole equation can be simplified to:

$$\Delta f = -C_f.\Delta m, \quad (2)$$

where C_f is the frequency constant 0.0815 Hz/(ng cm^2). The measurements were monitored and evaluated using Nova 1.10 software and all measurements were carried out at ambient temperature.

2.4 MALDI TOF/TOF analysis of intact PSA glycoprotein

A 15.9 μg of PSA glycoprotein sample was dissolved in 500 μL of 0.1% TFA using VivaSpin 500 column (molecular weight cut-off limited to 10 kDa) (Sartorius Stedim, DE). Briefly, 7.6 mg of 2,5-DHAP was dissolved in 375 μL of ethanol and 125 μL of 18 mg/mL aqueous solution of diammonium hydrogen citrate was added. The intact glycoprotein sample (1 μL) was spotted onto an AnchorChip 384 BC MALDI target plate (Bruker Daltonics, MA, USA), pre-mixed with 1 μL 0.1% TFA, overlaid with 1 μL of matrix 2,5-DHAP solution and left to dry by air to enable the crystallisation. The mass spectrometric measurement of intact PSA was performed on an UltrafleXtreme (Bruker Daltonics, MA, USA) in a linear positive ion mode. Calibration of the mass spectra was carried out with a Protein calibration standard II from Bruker Daltonics. 3000 laser shots were collected into one spectrum and the ions were recorded in the range between 20 000 and 50 000 *m/z*. The acquired raw spectra were processed by the FlexAnalysis and ProteinScape 3.0 software (Bruker Daltonics, MA, USA).

2.5 NanoLC-MALDI TOF/TOF analysis

2.5.1 Reduction, alkylation and in solution tryptic digestion—Prior to reduction and alkylation of PSA, PBS buffer (pH 7.4) was exchanged to 50 mM ammonium bicarbonate buffer (pH 8.5) with VivaSpin 500 column (molecular weight cut-off limited to 10 kDa) (Sartorius Stedim, DE) at 3 °C. A 75 μg aliquot of PSA was dissolved in the final

volume of 100 μL of 50 mM ammonium bicarbonate buffer containing 10 mM DTT and was reduced at 60 °C for 40 min. These reduced samples were then alkylated with the addition of a 15 μL aliquot of 100mMIAA dissolved in 50 mM ammonium bicarbonate buffer and the reaction was allowed to proceed at RT for 30 min in the dark. Reduced and alkylated PSA sample was digested with 3.75 μg of sequencing grade modified trypsin (added from stock solution with trypsin concentration of 20 μg/mL in 20 mM ammonium bicarbonate buffer) using an enzyme/substrate ratio of 1:20 (w/w), and the sample was subjected to overnight incubation at 37 °C.

2.5.2 NanoLC-MALDI TOF/TOF analysis of tryptic peptides—Peptides from tryptic digestion of 2 μg of PSA were loaded onto a trap column (Acclaim PepMap100 C18, 75 μm x 20 mm, Dionex, CA, USA) and separated with a C18 column (Acclaim PepMap C18, 75 μm x 150 mm, Dionex) on UltiMate 3000 RSLCnano system (Dionex, CA, USA). A sample was injected and a linear 30 min gradient (10–55% B) was used for peptide separation. Two mobile phases were used where A was 0.05% TFA (v/v) and B was 80% ACN (v/v) with 0.05% TFA. Fractions were spotted on AnchorChip 384 BC MALDI target plate (Bruker Daltonics, MA, USA) together with 4-HCCA solution as a MALDI matrix. The fractionated samples were analysed with a MALDI TOF/TOF (UltrafleXtreme, Bruker Daltonics, MA, USA) instrument operated in the positive ion mode. Fragmentation data were searched by the Mascot search engine (Version 2.4.1, Matrix Science, UK) against SwissProt protein sequence database (version 2015_09) limited to *Homo sapiens* taxonomy with allowed oxidation of methionines and carbamidomethylation of cysteines as variable modifications and expected semitrypsin cleavage.

2.6 MALDI TOF/TOF analysis of released N-glycans

2.6.1 N-glycan release, enrichment and purification—The PSA sample (75 μg) was reduced, alkylated and digested with trypsin as described above. The digested sample was acidified with 10% acetic acid to a final 2% concentration and incubated with 2 mL of freshly regenerated Dowex 50Wx8 resin (hydrogen form) for 1.5 h. The mixture was poured into empty 25 mL polypropylene column (Biorad, CA, USA) and glycopeptides were eluted with 0.5 M ammonium acetate (pH 6.0). Glycopeptide fractions were pooled and concentrated in a vacuum centrifuge prior to a Zip Tip (C18) purification (Millipore, MA, USA) to remove any residual contaminants. The dried sample was dissolved in 100 μL of DWand the trypsin residues were denatured by incubation at 95 °C for 5 min with subsequent addition of 100 μL of 0.1 M ammonium bicarbonate (pH 8.5). N-glycans of reduced and alkylated tryptic glycopeptides were released by an addition of a 3 μL of PNGase F with an overnight incubation at 37 °C. Consequently, the solution was treated with second round of 2mL of ion-exchange resin (Dowex 50Wx8, hydrogen form) in a favour of N-glycan separation from peptides. The N-glycans of the interest were eluted with two column volumes of 2% acetic acid followed by lyophilisation.

The freeze-dried sample was dissolved in 150 μL of DW and subjected to a non-porous graphitised carbon ENVI-Carb solid phase extraction to fractionate the isolated N-glycans and remove salts and other small molecules [43]. Briefly, after conditioning with 40% ACN, the column was equilibrated with DW followed by re-applying of N-glycans. Then, the

column was washed with DW and the subsequent elution and fractionation were performed with 40% ACN (neutral, non-sialylated glycans) and with 40% ACN containing 0.1% TFA (charged, sialylated glycans). Lastly, each fraction was freeze dried.

The final N-glycan purification was carried out with LiChroprep RP 18 (25–40 μm) reversed phase resin that was preconditioned with methanol and DW prior to the application of a reaction mixture into the cartridge. Column was sequentially washed with DW, 15, 40 and 100% of MeOH and each fraction was dried in a vacuum centrifuge. The DW and 15% MeOH fractions containing purified *N*-glycans were re-suspended in 6 μL of DW.

2.6.2 Linkage specificity on α-(2,3) and on α-(2,6) sialic acid: exoglycosidase digests—Two neuraminidases (an unspecific α-(2,3,6,8)-neuraminidase and a specific α-(2,3)-neuraminidase) were applied (0.5 μL each) to purified sialylated N-glycans derived from nPGC SPE (2 μL) and incubated overnight with 5 μL of 0.1M ammonium acetate buffer (pH 5.0) and 2.5 μL of DW at 37 °C.

For each MALDI TOF analysis (before and after enzymatic digestion), 1 μL of N-glycan sample was applied on a Ground Steel target plate (Bruker Daltonics, MA, USA) and overlaid with 1 μL of ATT matrix solution (5 mg/mL) in 50% ethanol and 20 mM ammonium citrate and dried under vacuum to promote uniform crystallisation.

2.6.3 Linkage specificity on α-(2,3) and on α-(2,6) sialic acid: chemical derivatisation—Determination of the sialic acid linkage was performed as described previously [44]. Briefly, the carboxyl group activator (0.25 M EDC) and the coupling reagent (0.25 M HOBt) were dissolved in ethanol and added to the sample (sialylated N-glycans derived from nPGC SPE). A reaction mixture was incubated for 1 h at 37 °C followed by addition of ACN to its final concentration of 50%. The reaction mixture was then placed for 20 min at −20 °C. Afterwards, chemically derivatised N-glycans were cleaned-up by cotton hydrophilic interaction liquid chromatography solid phase extraction (HILIC)-SPE [45]. Shortly, a home-made pipette tip filled with cotton as stationary phase was sequentially pre-conditioned with DW and 85% ACN, followed by aspirating and dispensing the sample 20 times. The retained analytes were washed with 85% ACN + 0.1% TFA and eluted with 10 μL of DW. 1 μL of purified glycan sample was spotted onto AnchorChip 600/384 MTP (Bruker Daltonics, MA, USA) and pre-mixed on the plate with 1 μL of a DHB matrix (10 mg/mL) in 50% CAN containing 1 mM NaOH and allowed to dry by air at RT.

2.6.4 Processing of MALDI TOF/TOF data—All mass spectrometric measurements of free or derivatised N-glycans were performed on an UltrafleXtreme (BrukerDaltonics, MA, USA) in both reflectron positive and negative ion modes for MS and MS/MS (LIFT) analysis. Calibration of the mass spectra was carried out with a Peptide calibration standard II from Bruker Daltonics. The ions were recorded between 900 and 3500 *m/z* and the laser intensity was optimised to give the best S/N ratio with the best maintenance of monoisotopic resolution for each sample. All acquired raw spectra were processed and analysed by the FlexAnalysis and ProteinScape 3.0 software (Bruker Daltonics, MA, USA).

3 Results and discussion

In a previous pilot study, we showed for the first time that label-free impedimetric detection in a sandwich configuration with an immobilised antibody and lectin applied to complete a sandwich around captured PSA can be applied both for detection of the level of PSA and for glycoprofiling of PSA´s glycan on the same surface [41]. In this work, we aimed to extend such study by application of three different lectins for glycoprofiling of PSA´s glycan with the impedimetric biosensor working in a sandwich configuration and to validate the biosensor results by glycoprofiling of PSA´s with MALDI TOF/TOF. Only such validation can tell us if a simplified lectin-based biosensor can be reliably applied for glycoprofiling of cancer biomarkers.

3.1 Glycoprofiling of PSA by the impedimetric biosensor

In this work we adopted optimal conditions for preparation of the biosensor interface found in a previous paper [41] i.e. by forming a mixed SAM prepared from MUA and MH at ratio of 1:3 and immobilisation of an anti-PSA antibody from a solution with concentration of 20 ng/mL. Then the surface of the biosensor was blocked by gelatine to resist non-specific interactions since Haab´s group found out in a pioneering study that a particular attention has to be paid to block interactions between an immobilised antibody and lectins applied to complete a sandwich configuration [46]. Gelatine was chosen as the best blocking agent from a preliminary testing. The results presented in Supporting Information Fig. S2 indicate that while a substantial interaction of SNA with immobilised antibody on the biosensor surface not blocked by gelatine was observed (Supporting Information Fig. S2A), the biosensor surface blocked by gelatine SNA could not interact with immobilised antibody (Supporting Information Fig. S2B). Antifouling ability of the biosensor with immobilised anti-PSA antibody and blocked with gelatine is shown in Fig. 1. It can be seen that non-specific interactions decreased 23-fold, when gelatine was applied as a blocking agent using HAS as a non-specific binding probe and PSA was still detectable (Fig. 1). In this manuscript, the biosensor interface with immobilised antibodies was exposed to PSA at concentration of 10 ng/mL, what is a PSA level indicating PCa. Then, three different lectins were used to glycoprofile PSA´s glycan. In this work lectins at concentration of 0.5 mg/mL were applied to complete a sandwich biosensor configuration. For example a typical Rct of the interface with immobilised anti-PSA antibodies was (20 ± 7) kΩ and R_{ct} increased to a value of (29 ± 13) kΩ upon blocking of the surface by gelatine and further to a value of (42 ± 19) kΩ after incubation of the surface with 10 ng/mL of PSA (Fig. 2A). Since there is a natural variation in the response from individual electrodes applied in the study, it is better to express change of the output signal as a relative signal change in %.When the absolute R_{ct} of the biosensor after addition of 10 ng/mL of PSA was normalised to the biosensor response before addition of PSA, an average relative response change ΔR_{ct} of (47 ± 9)% was observed. In the control experiment, the biosensor with immobilised antibody and blocked by gelatine was incubated with a plain buffer to test stability of the biosensor surface with an average response of the device of (28.3 ± 0.3) kΩ with four consecutive buffer incubations for 20 min, indicating an excellent short term stability of the interfacial biosensor layer.

QCM analysis was performed to quantify density of proteins on the biosensor interface upon formation of protein layers (data not shown). Antibody was immobilised on the mixed SAM with a density of 0.99 pmol/cm^2, what is 39% of a theoretical coverage of a full antibody monolayer, when a hard sphere model is taken into account [47]. When PSA was incubated with such a surface from a stock solution having concentration of 1 ng/mL its density reached 0.40 pmol/cm^2, indicating that five molecules of immobilised anti-PSA bound one molecule of PSA on average (taking into account two binding pockets per one antibody). Furthermore, after injection of SNA lectin a surface density of 0.23 pmol/cm^2 was reached for lectin indicating that 58% of PSA proteins were occupied by lectin.

In a preliminary test four lectins (SNA, MAA, LTA, LCA) were tested for their applicability to glycoprofile PSA glycan and finally three lectins – SNA (detection of α-(2,6)-terminal sialic acid), MAA (detection of α-(2,3)-terminal sialic acid) and LTA (determination of α-L-fucose) were applied. When the immunosensor with PSA attached was incubated with LTA lectin an increase of Δ*R*ct of (22 ± 6)% was observed (Fig. 2B), suggesting presence of α-L-fucose in the PSA glycan. When the biosensor with attached PSA was incubated with SNA lectin, an increase of Δ*R*ct of (35 ± 2)% was observed, a response which is much higher compared to response observed with MAA of (5 ± 2)% (Supporting Information Fig. 2A and B). Higher EIS response with SNA lectin applied compared to application of MAA lectin can be either due to higher amount of α-(2,6)-terminal sialic acid compared to α-(2,3)-terminal sialic acid or due to higher affinity of SNA towards glycan compared to MAA. A K_D value in the range 1.13–1.15 μM for SNA lectin and 2.49–3.60 μM for MAA lectin were described [48], suggesting similar binding strength of both lectins. Such results then indicate that PSA glycan contains mainly α-(2,6)-terminal sialic acid and fucose and lower amount of α-(2,3)-terminal sialic acid. Such results are in agreement with glycan composition of PSA from healthy individual with major amount of fucose and α-(2,6)-terminal sialic acid determined with only a minor presence of α-(2,3)-terminal sialic acid [49].

3.2 Analysis of released N-linked glycans

Identification of PSA together with analysis of molecular weight of 28,332 Da (Supporting Information Figs. S3 and S4) is shown in Supp info file. N-glycans, released from tryptic glycopeptides, were fractionated by nPGC into neutral (eluted by 40% ACN) and charged (including sialylated structures; eluted by 40% ACN with addition of TFA to its final concentration of 0.1%) fraction [50]. Every fraction was analysed by MALDI TOF/TOF and spectra were interpreted manually as well as confirmed by searching against the GlycomeDB tool of ProteinScape software. One of the most abundant structures, the biantennary N-glycan with one sialic acid and one fucose attached ((Hex)2(HexNAc)2(Deoxyhexose)1(Neu5Ac)1 + (Man)3(GlcNAc)2), was selected as a representative structure for further detailed analysis of the linkage of terminal sialic acid. MS/MS (LIFT) spectrum of the representative N-glycan (parent mass 2,076.6 Da) is shown in Fig. 3.

3.2.1 Determination of sialic acid linkage—

Since we wanted to confirm exact composition of the PSA glycan, MALDI TOF/TOF was applied to validate glycoprofiling results obtained by the biosensor. MALDI TOF MS is a broadly applied method for the

differentiation of α-(2,3)-and α-(2,6)-isomers of sialic acid [44, 51–56]. One way for determination of specific sialic acid-linkage is an employment of linkage-specific neuraminidases with a requirement to perform several digestion and purification steps [57]. An alternative strategy for both sialic acid-linkage specific distinction and stabilisation of labile sialic acid in the ion source is involvement of a selective modification of its carboxyl groups [44, 51, 58]. Sialic acid chemical derivatisation was accomplished by treating sialylated glycans with an activator of carboxylic acid moieties, EDC and with HOBt as the coupling reagent (see Supporting Information Fig. S5) [44]. This moderate and fast chemical derivatisation leads to mass shifts allowing a direct discrimination of particular sialic acid linkages in MS spectrum. As such prepared, α-(2,3)-linked sialic acid is able to convert their carboxyl groups into intramolecular cyclic lactones [59], while α-(2,6)-linked sialic acid can form esters and amides [44, 51, 58].

The first strategy for sialic acid linkage discrimination was selective chemical derivatisation of the sialic acid carboxyl groups with EDC/HOBt reagents with ethanol. Whereas the α-(2,6)-linked sialic acid undergoes ethyl esterification reaction, glycan carrying this type of sialic acid yields a mass increment of 28 Da per sialic acid in the MS spectrum. On the contrary, lactonisation of the α-(2,3)-linked sialic acid results in 18 Da mass loss per single sialic acid [44, 60]. As can be seen from the Fig. 4A, the derivatisation of representative, one of the most abundant sialylated N-glycan structures isolated from PSA (*m/z* 2076.6, [M - H] −), resulted in a 28 Da mass increment (*m/z* 2129.4, [M + Na]+) after the selective chemical derivatisation. This result from MALDI TOF analysis after the linkage specific ethyl esterification proved the presence of one terminal α-(2,6)-linked sialic acid in the representative N-glycan structure of PSA. Moreover, Fig. 4B demonstrated the α-(2,6) sialylation in the MALDI TOF/TOF spectrum (parent *m/z* 2129.4) after EDC/HOBt treatment. Importantly, the fragmentation spectra showed a signal at *m/z* 706.99, [M + Na]+ indicating an N-acetyl-lactosamine antenna carrying α-(2,6)-linked terminal sialic acid, that was assigned before the derivatisation (Fig. 3) as the *m/z* 657.01, [M + H]+.

Results obtained from linkage specific derivatisation were confirmed by specific exoglycosidase digests. The nPGC fraction containing sialylated N-glycans was subjected to unspecific α-(2,3,6,8)-neuraminidase and a specific α-(2,3)-neuraminidase according to protocol mentioned in the Experimental section. The treatment with unspecific neuraminidase led to almost absolute loss of signals in a negative MS ion mode, what is in accordance with the loss of negative charge located on the terminal sialic acids. On the other hand, the new signals (after exoglycosidase treatment) corresponding to appropriate asialo-structures were present in the positive ion mode. Thus, removal of all the sialic acids was complete and new respective structures, sialic-acid depleted, were formed. In contrast, addition of a specific α-(2,3)-neuraminidase did not lead to loss of signals in the spectra acquired after the treatment at all. Therefore, absence of sialic acid with α-(2,3)-linkage was assumed. These results, obtained from specific exoglycosidase treatments correspond to the ones from a specific chemical derivatisation as well as the literature [8].

4 Concluding remarks

The results obtained by the lectin-based immunosensor indicate that PSA used in the study contains mainly fucose and α-2,6-linked sialic acid with only small amount of α-2,3-linked sialic acid, in agreement with glycoprofiling of PSA by MALDI TOF/TOF. Further, we can conclude that identification of the type of the linkage of sialic acid present within glycan by MS is quite time/reagent consuming, while analysis of sialic acid by the lectin-based immunosensor is shorter and simpler with a potential to be applied in glycoprofiling of PSA. There are beneficial features of our lectin-based immunosensor, which can be summarised as follows: (i) ability to detect increasing/decreasing amount of α-(2,6)-Neu5Ac/or α-(2,3)-Neu5Ac on the selected biomarker (even tri- and tetraantennary glycans, which can be present on PSA [48]) since lectin biosensor could detect glycans in a quantitative way [61, 62], (ii) high sensitivity of detection with a low limit of detection and (iii) small sample consumption. On the other hand, the main drawback of this method is still low throughput of analysis, what can be addressed by working in an array format of analysis in the future. Glycoprofiling of PSA using lectins could be applied to discriminate between healthy individuals and patients with prostate cancer as suggested previously [8, 63]. Moreover, there is a possibility to extend electrochemical sandwich protocol for analysis of other cancer biomarkers, but in order to discriminate between cancer patients and healthy individuals a substantial change in the glycosylation profile of the selected biomarker is required.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Aa	amino acid
ATT	6-aza-2-thiothymine
CV	cyclic voltammetry
2,5-DHAP	2,5-dihydroxyacetophenone
DHB	2,5-dihydroxybenzoic acid
DW	deionized water

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EDC	1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide
EIS	electrochemical impedance spectroscopy
Fuc	fucose
GlcNAc	N-acetylglucosamine
4-HCCA	α-Cyano-4-hydroxycinnamic acid
Hex	hexose
HexNAc	N-acetylhexoseamine
HILIC SPE	hydrophilic interaction liquid chromatography solid phase extraction
HOBt	1-hydroxy-benzotriazole
IAA	iodacetamide
LCA	*Lens culinaris* agglutinin
LTA	*Lotus tetragonolobus* agglutinin
MAA	*Maackia amurensis* agglutinin
Man	mannose
MH	6-mercapto-1-hexanol
MUA	11-mercaptoundecanoic acid
Neu5Ac	sialic acid; N-acetylneuraminic acid
NHS	N-hydroxysuccinimide
nPGC SPE	non-porous graphitised carbon solid phase extraction
PCa	prostate cancer
PNGase F	peptide-N-Glycosidase F
PSA	prostate specific antigen
QCM	quartz crystal microbalance
$\boldsymbol{R_{ct}}$	charge transfer resistance
RT	room temperature
sLe^x	sialyl Lewisx antigen
SAM	self-assembled monolayer
SNA	*Sambucus nigra* agglutinin
ST6Gal-1	β-galactoside α-2,6- Sialyltransferase

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Significance of the study

There is an increased incidence and mortality of prostate cancer (PCa) in the EU. Gold standard for PCa diagnosis is analysis of a prostate specific antigen (PSA) in serum, but the level of this biomarker in serum is influenced by other factors besides progression or development of PCa. Therefore, the US Preventive Services Task Force recently suggested not to apply analysis of PSA for routine screening of PCa due to low sensitivity, specificity and prognostic value of this biomarker. Thus, new and more reliable methods for PCa diagnosis are needed. Here we show that electrochemical lectin-based immunosensor can be applied both for detection of PSA concentration and for glycoprofiling of PSA´s glycan, which composition changes with PCa development/progression. Such approach has a potential to increase reliability of PCa identification.

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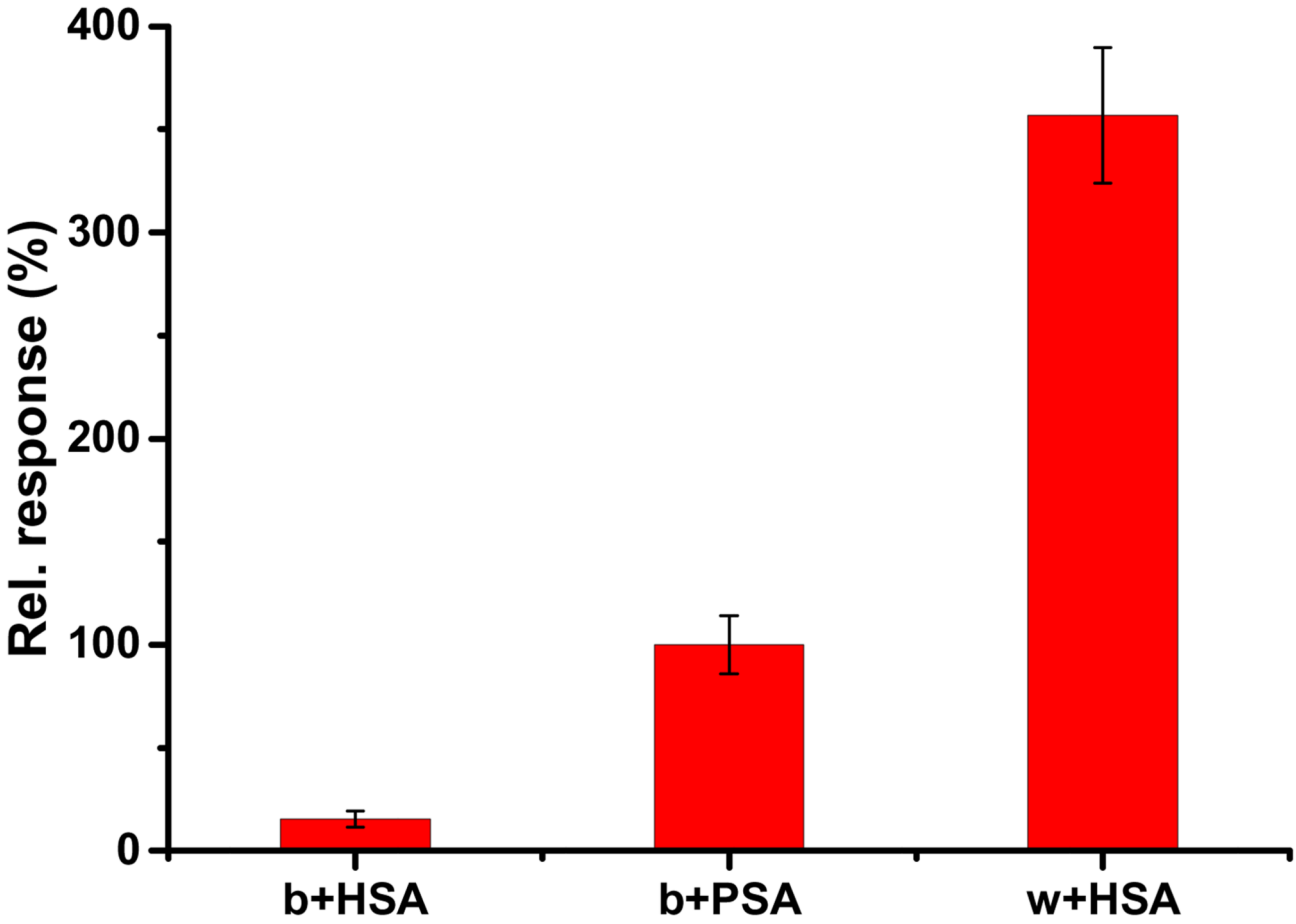

Figure 1.

Evaluation of the specificity of PSA detection by the biosensor device with anti-PSA antibody immobilised. Human serum albumin (HSA) was applied in the study as a non-specific binding probe. In the figure binding of HSA to the biosensor not blocked by gelatine (w+HSA) and to the biosensor surface blocked with gelatine (b+HSA) is shown. Moreover, a specific interaction of the biosensor with the surface blocked by gelatine towards its analyte PSA (b+PSA) is shown, as well.

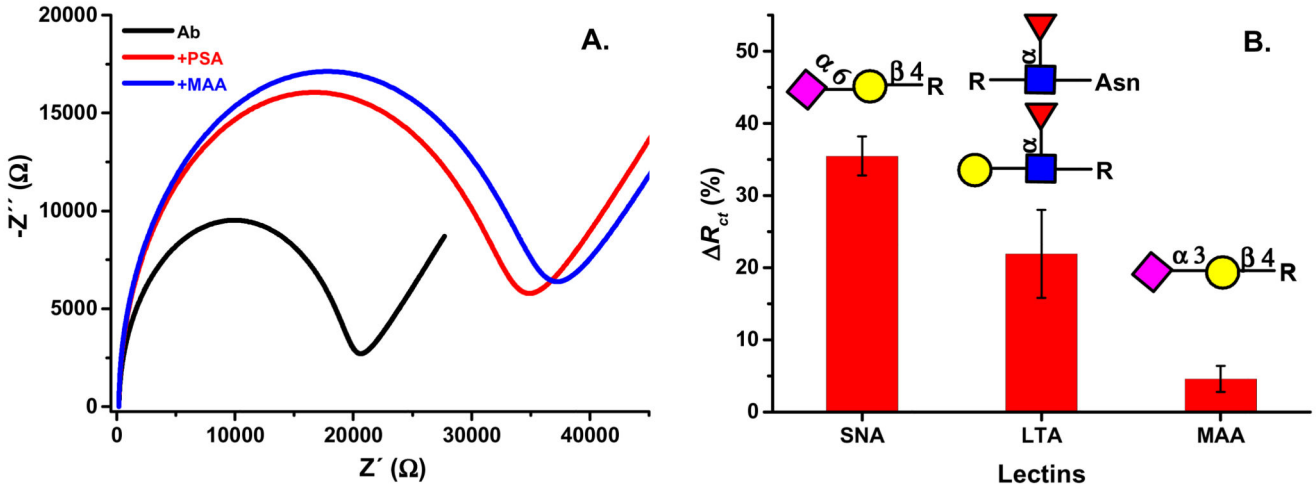

Figure 2.

(A) A Nyquist plot showing change of the R_{ct} (a diameter of a semicircle) upon incubation of the biosensor with various proteins. Ab means the biosensor after immobilisation of anti-PSA antibody, +PSA means the biosensor after incubation with 10 ng/mL of PSA and +MAA means the biosensor with a final incubation with MAA lectin. (B) Glycoprofiling of PSA attached to anti-PSA modified biosensor surface from 10 ng/mL PSA solution. For glycoprofiling of PSA´s glycan three different lectins (SNA, LTA and MAA) were applied by incubation of the biosensor with PSA attached. Typical glycan structures recognised by lectins are shown in the figure according to CFG nomenclature.

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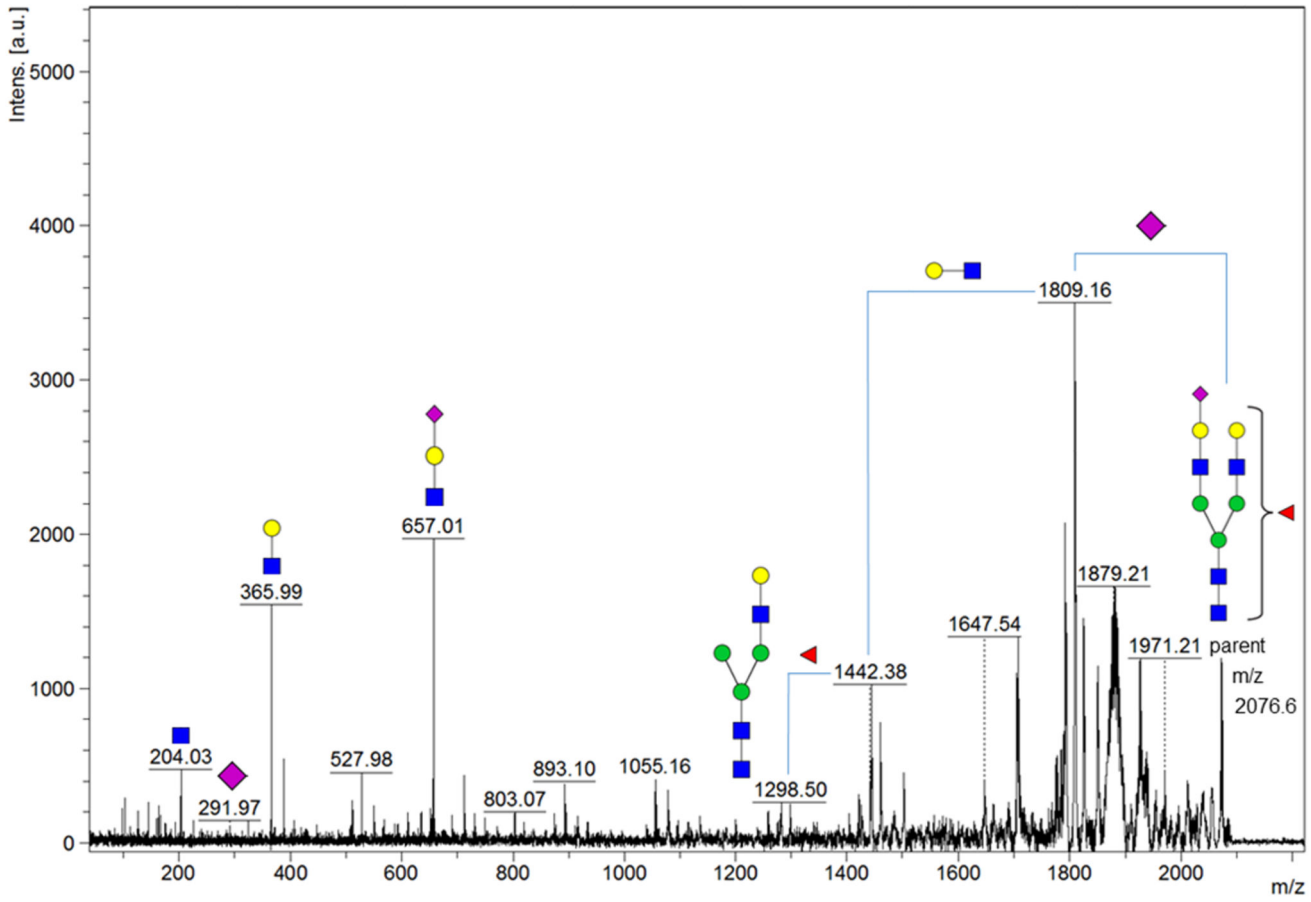

Figure 3.

(C) MALDI TOF/TOF (LIFT) spectrum of representative $((Hex)_2(HexNAc)_2(Deoxyhexose)_1(Neu5Ac)_1 + (Man)_3(GlcNAc)_2)$ N-glycan (with m/z 2,076.6 in a negative ion mode) showing the typical fragmentation pattern of sialylated structure. The highest signal with m/z 1,809.16 is referring to the loss of negatively charged sialic acid, leading to strong ionisation of residual structure in a positive ion mode. Another typical signal is the one with m/z 657.01, representing the N-acetyl-lactosamine residue terminated with sialic acid, which creates the typical mammalian N-glycan antennae.

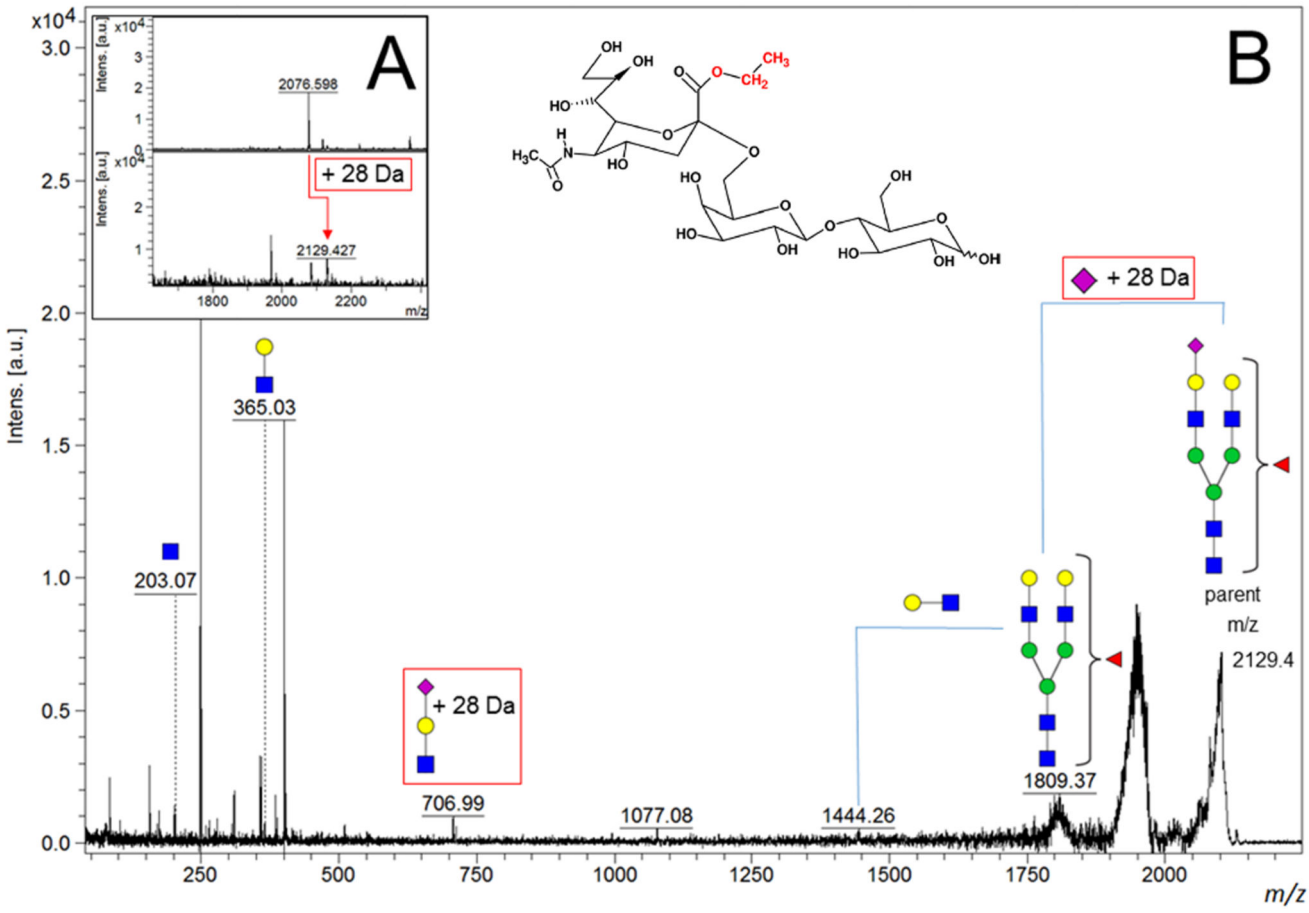

Figure 4.

(A) Mass increment of 28 Da caused by ethyl esterification of a representative $((Hex)_2(HexNAc)_2(Deoxyhexose)_1(Neu5Ac)_1 + (Man)_3(GlcNAc)_2)$ N-glycan (with m/z 2,076.6, $[M - H]^-$ before the derivatisation and m/z 2,129.4 $[M + Na]^+$ after). (B) MALDI TOF/TOF (LIFT) spectrum of the respective structure after the derivatisation. The loss of ethyl ester of sialic acid resulting in the signal at m/z 1,809.4, as well as the presence of the signal at m/z 706.99 (N-acetyl-lactosamine with terminal ethyl ester of sialic acid) proved the selectivity of derivatisation and the presence of α-(2,6)-linked sialic acid. The structure of ethyl esterified glycan is shown, as well.

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