Ultrasensitive impedimetric lectin based biosensor for glycoproteins containing sialic acid

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- **Abstract**

We report on an ultrasensitive label-free lectin-based impedimetric biosensor for the determination of the sialylated glycoproteins fetuin and asialofetuin. A sialic acid binding agglutinin from *Sambucus nigra* I was covalently immobilised on a mixed self-assembled monolayer (SAM) consisting of 11-mercaptoundecanoic acid and 6-mercaptohexanol. Poly(vinyl alcohol) was used as a blocking agent. The sensor layer was characterised by atomic force microscopy, electrochemical impedance spectroscopy and X-ray photoelectron spectroscopy. The biosensor exhibits a linear range that spans 7 orders of magnitude for both glycoproteins, with a detection limit as low as 0.33 fM for fetuin and 0.54 fM for asialofetuin. We also show, by making control experiments with oxidised asialofetuin, that the biosensor is capable of quantitatively detecting changes in the fraction of sialic acid on glycoproteins. We conclude that this work lays a solid foundation for future applications of such a biosensor in terms of the diagnosis of diseases such as chronic inflammatory rheumatoid arthritis, genetic disorders and cancer, all of which are associated with aberrant glycosylation of protein biomarkers.

Keywords: *biosensor, electrochemical impedance spectroscopy (EIS), glycoproteins, label-free detection, self-assembled monolayer (SAM), sialic acid*

- **Introduction**

There is a need to switch from studying DNA and protein profiles into analysis of a perturbed glycan (i.e. a saccharide attached to proteins and lipids) composition. The main reason is that the cellular glycome can contain up to 500,000 glycan modified molecules built from 7,000 unique glycan moieties [1]. Only such complex information storing glycan system termed a "sugar code" can be behind a finely tuned mechanism applied in the cell physiology and pathology [2]. It is predicted 50-90% of human proteins are glycosylated with glycans involved in various processes such as inflammation, fertilisation, cell growth/signalling, host-pathogen interaction, immune response and cancer [3,4]. Moreover, glycans serve as a "quality control" indicator during protein synthesis, as a tertiary structure stabilising agent or as a protease resistance factor [5]. A physiological feature such as presence of various blood groups and removal of old erythrocytes from the blood stream [6] are results of changed glycan composition, as well. Further, an addition of a single molecule of sialic acid to N-glycan of immunoglobulin changes this molecule from being a pro-inflammatory into an anti-inflammatory agent [7]. Thus, a better understanding of glycan recognition can help to develop more efficient strategies for disease treatment with some success stories so far, i.e. "neutralisation" of HIV viruses [8], more efficient vaccines against autoimmune diseases [9] and a better diagnostics and therapy of various diseases [3]. Many previously established or commercially successful strategies how to treat diseases are currently revisited in light of glycan recognition in order to lower side effects, enhance serum half-life or to decrease cellular toxicity [10]. Various techniques such as a high throughput mass spectroscopy, liquid chromatography and nuclear magnetic resonance have been used for glycan determination requiring *prior* release of glycans and appropriate labelling [11]. Contrary, lectins have been extensively used in a more direct manner for cell typing, histochemical staining, and glycoprotein fractionation [12]. Lectins are proteins able to recognise either free sugars or glycans attached to biomolecules or present on the surface of cells. Lectins are natural decipherers or translators of the sugar code [12], thus it is practical to use them for glycan determination. The most popular is an enzyme-linked lectin assay derived from a better known ELISA [13], but in order to enhance assay throughput with lowering consumption of reagents/samples, lectin biochips were recently introduced [3,14]. Biochips usually require to have fluorescently labelled either a biorecognition element or a sample, what can cause unwanted variability in labelling and biorecognition [14]. Thus, other formats of analysis working in a label-free mode of detection are vital. Surface plasmon resonance (SPR) or quartz crystal microbalance (QCM) are working well in a label-free mode of operation [15], but their sensitivity is not sufficient for analysis of low-abundant glycoproteins in a complex sample. New label-free methods are based on microcantilevers [11], single-walled carbon nanotubes emitting near-infrared fluorescence [16] and an electrochemical detection platform. Electrochemical methods working in a sensitive, reproducible and label-free mode of analysis [6,17] are either based on a field-effect sensing or on a more frequently applied electrochemical impedance spectroscopy (EIS) [1,11].

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EIS is based on an electric perturbation of a thin layer on the conductive surface utilisable in sensing. When a biorecognition took place, the double layer is modified and this change can be used for analysis (mainly resistance R_{ct} and capacitance) [6,18]. There are only two groups led by Prof. Joshi [19] and Prof. Oliveira [20,21,22] involved in detection of glycoproteins by changes in R_{ct} obtained from EIS using lectins as biorecognition probes. In this work, a glycoprotein detection platform employing lectin and EIS was optimised in order to develop a sensitive biosensor device working in a label-free mode of operation. Lectin *Sambucus nigra* agglutinin (SNA I) was chosen due to well-established specificity towards sialic acid containing glycoproteins such as fetuin (FET, Fig. 1) [18,23] and asialofetuin.

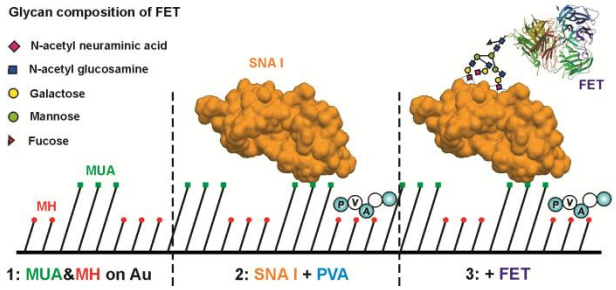

Figure 1: *A scheme representing the biosensor preparation by formation of the mixed SAM consisting of 6-mercaptohexanol (MH) and 11-mercaptoundecanoic acid (MUA) on the bare gold electrode (**1**) and immobilisation of the SNA I lectin via activated carboxylic group and blocking of the surface by poly(vinylalcohol) (PVA) (**2**). Finally, an interaction of the biosensor with fetuin (FET) via glycan recognition is shown (**3**). Glycan composition of FET is shown, as well.*

- **Experimental**

Materials

11-mercaptoundecanoic acid (MUA), 6-mercaptohexanol (MH), potassium hexacyanoferrate(III), potassium hexacyanoferrate(II) trihydrate, potassium chloride, N-hydroxysuccinimide (NHS), *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC), bovine serum albumin (BSA, fatty acid free), poly(vinylalcohol) (PVA, Mowiol® 4-88), sodium periodate, ethylene glycol, cysteamine, acetonitrile, fetuin (FET, 8.7% of sialic acid), asialofetuin (ASF, ≤ 0.5% of sialic acid) and N-acetylneuraminic acid (sialic acid, Type IV-S) were purchased from Sigma Aldrich (St. Louis, USA, www.sigmaaldrich.com). SNA I lectin from *Sambucus nigra* was purchased from Gentaur (Kampenhout, Belgium, www.gentaur.com). Ethanol for UV/VIS spectroscopy (ultra pure) was purchased from Slavus (Bratislava, Slovakia, www.slavus.sk). Zeba™ Spin Desalting Columns (40k MWCO) for protein purification were purchased from Thermo Scientific. All buffer components were dissolved in deionised water (DW).

Electrode cleaning and patterning by SAM

Planar polycrystalline gold electrodes (d=1.6 mm, Bioanalytical systems, USA) were cleaned using mechanical, chemical and electrochemical procedures according to previous report [24] with details provided in the Supporting information file. All electrochemical procedures were run on a potentiostat/galvanostat Autolab PGSTAT 128N (Ecochemie, Utrecht, Netherlands) in a cell with Ag/AgCl reference and a counter Pt electrode. Finally, the electrodes were washed by DW and absolute EtOH, left to dry and subsequently immersed in the mixture 1+1 (if not specified otherwise) of 1 mM MUA and 1 mM MH, both in absolute EtOH, for 30 min. After incubation, the electrodes were washed by absolute EtOH and finally by DW.

Oxidation of glycan in asialofetuin

Glycan of ASF was chemically oxidised using sodium periodate according to a modified protocol [25]. Shortly, 10 μM stock solution of ASF was oxidised by 10 mM sodium periodate in 50% acetonitrile for 2 h in the dark. The reaction was stopped by addition of ethyleneglycol to the final concentration of 15% (v/v) and incubated for additional 1 h in the dark. Further, free aldehyde groups formed by the glycan oxidation were blocked by addition of 1 mM cysteamine. The mixture was incubated for 1 h in the dark and finally the ASF with glycan oxidised was recovered with a desalting column.

Preparation of a biorecognition surface and assays

A covalent coupling of SNA I lectin on the SAM modified gold surface was performed *via* standard amine coupling chemistry starting with an activation of a carboxylic group of MUA by a mixture of 200 mM EDC and 50 mM NHS (1+1) for 15 min. The surface was washed by DW and followed by incubation with a 10 μM lectin solution in a 50 mM phosphate buffer pH 6.5 for 20 min in an inverted position. In some cases active esters on the electrode surface were deactivated by 1 M ethanolamine solution pH 8.5. In order to block non-specific interactions of the biosensor either 5% PVA in DW (if not mentioned otherwise) or 10 μM BSA in DW were incubated with the electrode surface for 30 min. Glycoproteins were incubated with the biosensor surface with concentrations spanning 9 orders of magnitude (from 1 fM up to 1 μM). The electrode surface was washed by DW after incubation with a glycoprotein and the EIS measurement was performed in an electrolyte containing 5 mM potassium hexacyanoferrate(III), 5 mM potassium hexacyanoferrate(II) and 100 mM KCl. The EIS measurement was run at 50 different frequencies (from 0.1 Hz up to 100 kHz) under Nova Software 1.8. Data acquired were evaluated using the same software represented in a Nyquist plot with a circuit R(C[RW]) employed for data fitting with a typical standard deviation of measurement not exceeding 3.7%. When standard deviation of an automatic fitting procedure exceeded 5.0%, data obtained were not

included in the study. The process of biosensor construction was characterised by Atomic force microscopy (AFM) and X-ray photoelectron spectroscopy (XPS) with experimental details provided in the Supporting information file.

- **Results and discussion**

Formation and characterisation of the interface

Formation of the SAM layer on the gold surface by a chemisorption of thiols is a robust and reproducible modification protocol allowing a high degree of patterning flexibility by tuning the thickness of the layer, while allowing to deposit chosen functional groups in a simple and controllable manner [26].

Formation of the interface

The mixed SAM layer consisting of MUA and MH allowed a carboxylic acid to be freely available for coupling from the solution phase even for bulky protein molecules, since the length of MUA of 1.5-1.7 nm was considerably longer compared to the length of 0.7 nm for MH [27]. The mixed SAM layer not only allowed a –COOH group to be available for protein coupling, but was applied to finely tune density of –COOH groups in the SAM layer employed for covalent immobilisation of a lectin. The biosensor´s interface was modified step by step starting with formation of the SAM film on the gold electrode, followed by a chemical activation of –COOH group by EDC/NHS with subsequent covalent attachment of the SNA I lectin and finishing with blocking of unoccupied surface spots by PVA (Fig. 1).

Characterisation of the interface

EIS was initially employed to see differences in the charge transfer resistance R_{ct} on differently modified gold surfaces. R_{ct} was read from a Nyquist representation of EIS data with a semicircle indicating R_{ct} of the layer present on the electrode surface. A detailed analysis of EIS data showed R_{ct} increased from (310 ± 20) Ω on the bare gold electrode to (5,500 ± 840) Ω upon incubation with a mixture of MUA and MH. When a carboxylic acid present in the SAM surface was activated by NHS/EDC followed by a covalent coupling of SNA I lectin and blocking of the surface by PVA, the R_{ct} dropped to an average value of (1,900 ± 380) Ω (Fig. 2A). Finally, the biosensor with the biorecognition interface completed by incubation with PVA was tested in the ability to selectively detect fetuin (FET) and asialofetuin (ASF). The EIS investigation really proved interaction of the biosensor with FET resulted in a substantial increase of R_{ct}, while incubation with ASF produced only a minor change of R_{ct} (Fig. 2B).

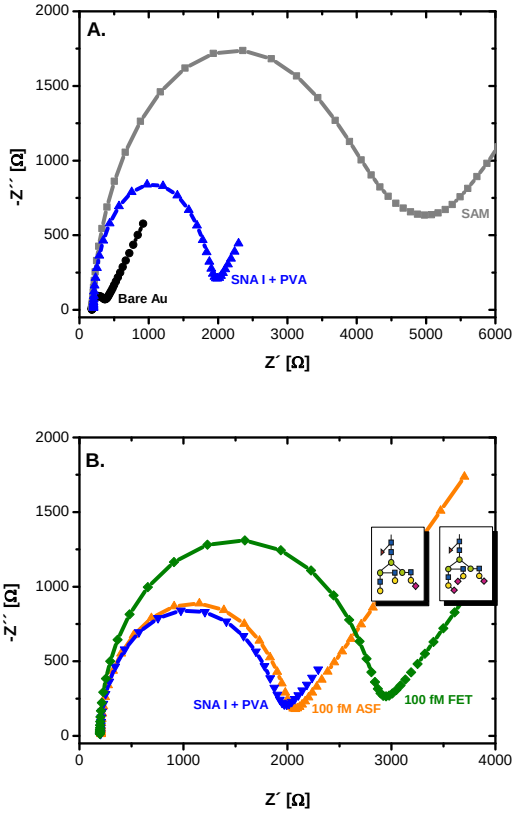

Figure 2: *The EIS data represented in a Nyquist plot showing a charge transfer resistance for various stages of the surface patterning i.e. bare gold, gold modified by the mixed SAM and after immobilisation of the SNA I lectin and blocking by PVA (**A**); the biosensor response in the absence of any protein (SNA I + PVA) and in the presence of 100 fM FET or 100 fM ASF (**B**). Moreover, structures of glycans present either in FET or ASF are shown, as well. If 3 sialic acid residues are present per FET then on average 6 molecules of ASF should contain 1 sialic acid.*

AFM was employed to see differences in the topology of the gold surface as a result of the interface patterning. The bare gold surface exhibited a root mean square roughness R_q of 0.9 nm, a value which increased to 1.1 nm after deposition of the mixed SAM. This really confirmed presence of the SAM layer on the gold surface enhancing an overall roughness of the interface. A value of R_q increased to 3.1 nm after SNA I lectin was covalently immobilised on the electrode surface and after the final step of surface blocking by PVA was completed its roughness R_q dropped to value of 2.4 nm (Fig. 3). An increase of a surface roughness after lectin immobilisation indicated a substantial amount of the lectin was covalently attached to the modified gold surface. A final drop of surface roughness by applied PVA can be explained by a preferential deposition of PVA into interfacial spots not occupied by the SNA I lectin, leading to decrease of the surface roughness. We tried to examine the interface by AFM after incubation

with FET, but this attempt was not successful. Most likely reason was detachment of loosely bound FET to SNA I by the AFM tip with a lots of artefacts seen in AFM images acquired by a contaminated AFM tip.

Activation of –COOH group by EDC/NHS introduced an active ester containing nitrogen and two carbonyl groups. Indeed, XPS provided evidence for presence of nitrogen signature in the spectra after activation of –COOH when EDC/NHS took place (Fig. S1A). Moreover, an increase in the amount of oxygen functionalities present on the surface after activation were clearly seen (Fig. S1B). XPS showed that with an increase of the amount of MUA in a mixture of two thiols applied for surface patterning, an increased amount of –COOH groups present on the modified surface was found, as well (data not shown).

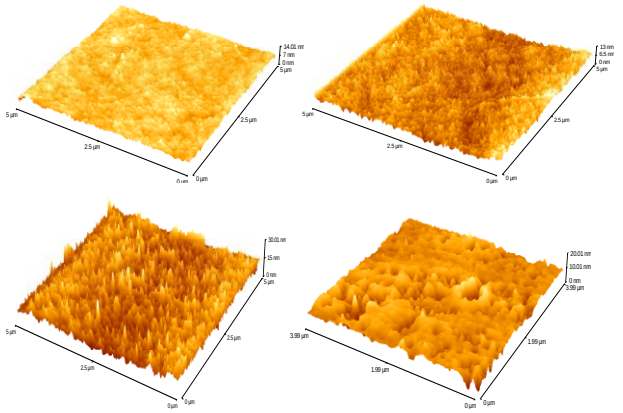

Figure 3: *The AFM images of the gold surfaces during a patterning procedure starting with the bare gold (upper left, R_q=0.9 nm), the gold surface modified by the 1+1 mixture of MUA and MH (upper right, R_q=1.1 nm), the surface with covalently attached the SNA I lectin (lower left, R_q=3.1 nm) and the surface after being blocked by PVA (lower right, R_q=2.4 nm). Scale of z-axis was adjusted in a way to clearly see topological features on the surface after each modification step.*

Optimisation of the biosensor´s interface

Two important parameters were extensively optimised during modification of a gold electrode surface, such as a fraction of -COOH group present in the mixed SAM layer and the concentration of PVA applied for blocking of unoccupied spots on the electrode surface. During optimisation sensitivity of detection, a linear range, a FET/ASF sensitivity ratio and a relative standard deviation of the biosensor were considered for evaluation of the device performance. Moreover, an applicability of PVA to block non-specific interactions was compared to the ability of BSA to resist non-specific protein binding.

A mixed SAM film

The first optimised parameter was a composition of the thiol solution consisted of MUA and MH applied on the gold electrode surface to form the mixed SAM layer (Table 1). Pure MH layer did not immobilise enough lectin since SNA I could be attached to this SAM layer only *via* non-specific adsorption. Although some response for FET or ASF could be observed i.e. (44 ± 3) Ω decade^{-1} or (50 ± 3) Ω decade^{-1}, respectively, this can be attributed rather to a non-specific interaction of both glycoproteins with the interface. A pure MUA layer could not be employed for EIS signal reading due to extremely high R_{ct} of the interface and atypical Nyquist plot. A detailed analysis of the performance of the biosensor constructed with the interface having different amount of –COOH group in the mixture with MH revealed sensitivity and linear range of the device increased almost linearly with increasing the amount of MUA in the mixed thiol solution. On the other hand, the FET/ASF specificity ratio was the highest on the interface prepared from 1+1 mixture of MUA and MH. This really suggests, at 3+1 ratio of MUA and MH, an increase in the sensitivity of FET detection is triggered by a non-specific protein binding to the interface most likely *via* electrostatic forces. Thus, for subsequent optimisation, a ratio of 1+1 for MUA and MH was chosen for the surface modification.

Table 1: The effect of changed composition of the mixed thiol solution on the performance of the biosensor

MUA:MH ratio in solution	Sensitivity [Ω decade^{-1}]	Linear range [orders of magnitude]	FET/ASF signal ratio [a]
1+0 [b]	-	-	-
1+3	110 ± 20	4	0.84
1+1	550 ± 20	7	3.5
3+1	870 ± 50	9	1.3
0+1	44 ± 3	5	0.88

[a] – calculated by dividing the sensitivity of the device for FET to the sensitivity of the biosensor for ASF,

[b] – high resistivity of the biosensor with atypical shape of a Nyquist plot showing minimal Z changes compared to other assays

All measurements were performed with three independent biosensor devices

PVA as a blocking agent

The second optimised parameter was a concentration of PVA employed for blocking of unoccupied spots on the surface, after covalent immobilisation of SNA I took place. PVA was applied in the study because its beneficial anti-fouling properties in designing lectin-based device were recently demonstrated [28]. Analysis of the performance of the biosensor as a result of changed concentration of PVA applied for surface blocking revealed the best concentration of PVA was 5.0%, since such the biosensor provided a linear range spanning 7 orders of

magnitude and the highest specificity (Table 2), while showing a moderate sensitivity. Higher biosensor´s sensitivity at surfaces covered by PVA from 0.5 or 2.5% PVA solutions could be again ascribed to a non-specific interaction of the proteins with the surface not being sufficiently blocked. Thus, for further assays the concentration of PVA solution of 5.0% employed for the surface blocking was selected.

Table 2: The effect of changed concentration of PVA solution used as a blocking agent on the performance of the biosensor

PVA [%]	Sensitivity [Ω decade^{-1}]	Linear range [orders of magnitude]	FET/ASF signal ratio [a]
0.5	680 ± 50	3	1.8
2.5	810 ± 40	4	1.6
5.0	550 ± 20	7	3.5
7.5	280 ± 30	4	1.4

[a] – calculated by dividing the sensitivity of the device for FET to the sensitivity of the biosensor for ASF
All measurements were performed with three independent biosensor devices

Characterisation of the biosensor device

Finally, the performance of the biosensor with optimally constructed interface was compared to the performance of the biosensor device having BSA as a blocking agent (Table 3). The main reason for choosing BSA as a comparative blocking agent is its almost exclusive application to suppress non-specific interactions in biorecognition-based biosensors and bioanalytical devices, including ELISA [29]. Even though, a higher sensitivity of the BSA treated biosensor was observed compared to the PVA blocked biosensor, a narrower linear range from 1 fM to 1 pM (3 orders of magnitude) for BSA blocked one in comparison to a linear range from 1 fM to 10 nM (7 orders of magnitude) for PVA treated one was observed. At higher FET concentration above 1 pM the signal of the biosensor based on the BSA blocked electrode surface started to level off with ΔR_{ct} of (5,100 ± 230) Ω at FET concentration of 10 nM. A similar value of ΔR_{ct} of (4,600 ± 80) Ω was observed for PVA treated biosensor incubated with the same concentration of FET of 10 nM. Thus, a bulky BSA with a hydrodynamic diameter of 7.0 nm lowered accessibility of the SNA I lectin for its analyte binding. Moreover, the PVA blocked biosensor outperformed the one based on BSA in terms of selectivity and assay precision (Table 3).

Table 3: Comparison of the performance of the biosensor modified either with PVA or BSA as a blocking agent

Blocking agent	Sensitivity [Ω decade^{-1}]	Linear range [orders of magnitude]	FET/ASF signal ratio [a]	RSD [%]
BSA	790 ± 40	3	1.5 ± 0.2	8.4 ± 5.8
PVA	550 ± 20	7	3.5 ± 0.5	4.6 ± 3.7

[a] – calculated by dividing the sensitivity of the device for FET to the sensitivity of the biosensor for ASF
All measurements were performed with three independent biosensor devices

Performance of the optimised biosensor in the presence of FET (8.7% of sialic acid), ASF (≤ 0.5% of sialic acid) and oxidised ASF is shown in Fig. 4. The detection limit for both glycoproteins were calculated to be (0.33 ± 0.10) fM for fetuin and (0.54 ± 0.14) fM for asialofetuin (S/N=3, where N is an average response of the device in the absence of a glycoprotein) with a linear range spanning 7 orders of magnitude. An experiment performed with asialofetuin having a glycan chemically oxidised, being a control without a recognisable sialic acid, gave a consistently lower sensitivity of detection of (135 ± 17) Ω decade^{-1} compared to intact asialofetuin with sensitivity of (160 ± 15) Ω decade^{-1} and fetuin of (559 ± 12) Ω decade^{-1}. When a subtracted specific sensitivity for fetuin of (424 ± 21) Ω decade^{-1} was divided by a subtracted specific sensitivity for asialofetuin of (25 ± 23) Ω decade^{-1} a sensitivity ratio of 17.0 was calculated in an agreement with 17.4-fold higher amount of sialic acid present in fetuin compared to asialofetuin. Non-specific protein binding on the biosensor surface under optimised conditions is still an issue to work on, in order to further increase robustness and overall performance of the biosensor. The performance of the biosensor was examined by addition of unbound sialic acid with a negligible response of 14 Ω decade^{-1} in the concentration range from 1 pM to 100 pM, suggesting the biosensor is sensitive mainly to sialic acid bound to the protein backbone.

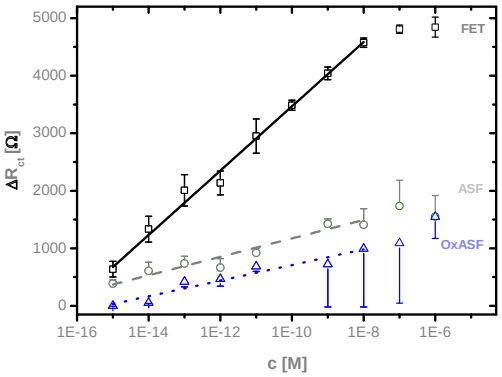

Figure 4: *A calibration plot of the optimised biosensor device for FET, ASF or oxidised ASF (OxASF) showing error bars from 3 independent biosensor devices. For clarity only upper part of error bars for ASF and a lower part of error bars for oxidised ASF are shown. Higher error bars for all proteins investigated might be a result of a repeated use of a particular biosensor device for assay of all 10 protein concentrations including several washing steps and repeated incubations.*

Glycoprotein detection has been previously performed with instrumental tools including HPLC and capillary electrophoresis coupled with mass spectrometry or using a battery of bioanalytical tools based on integration of lectins for a glycoprotein biorecognition. Combination of capillary electrophoresis with mass spectrometry offered quite high detection limit (DL) of 1.8 μM [30]. Liquid chromatography combined with tandem mass spectrometry was much more sensitive with DL for serum glycoproteins down to 200 pM level offering linear range within 3 orders of magnitude with a quite complex sample pre-treatment with several hours needed for analysis [31].

Integration of lectins with analytical instrumentation proved to have distinct advantages compared to pure instrumental way of analysis of glycoproteins such as short time of analysis and a simplified sample pre-treatment. Typical DL for SPR are down to nM level [32], for SPR imaging (an array format of analysis) down to 20 nM [33], for enzyme-linked lectin assays down to low nM or sub nM level [13,18], for QCM down to μM or nM level [34] and for reflectometric interference spectroscopy down to 100 nM [35]. Even though a lectin microarray technology offers to detect 0.5 pg of a glycoprotein *per* spot, this is feasible due to a low volume of a sample applied rather than due to a low concentration of a glycoprotein analysed. Typical DL for a glycoprotein analysis by a lectin microarray technology is down to 20 nM or 200 nM [36]. An improvement in the DL for the lectin microarrays was possible by utilisation of an evanescent field effect sensing with DL down to sub nM level [37] or on nanoparticle decorated surfaces with DL down to 0.4 nM [38]. Bioanalytical devices based on lectins are able to detect glycoproteins in the concentration range spanning 2 orders of magnitude.

Electrochemical detection platform in combination with immobilised lectins offered lower DL so far compared to other detection platforms mentioned above. Two glycoproteins labelled with quantum dots were electrochemically detected with DL down to 34 nM or 3 pM [39] and a biosensor integrated with concanavalin A labelled with daunomycin detected ovalbumin down to 100 pM [40] with utilisable concentration range spanning 2-3 orders of magnitude. EIS-based electrochemical detection platform offered DL of 150 fM for two glycoproteins using two immobilised lectins [19] or low nM range for ovalbumin with a concanavalin A integrated biosensor [20]. There is only one report relying on change of capacitance from EIS assays after biorecognition took place with DL down to 20 fM [18]. Thus, the biosensor device presented here offered the lowest DL for any lectin-based bioanalytical device or any analytical instrumentation for analysis of glycoproteins published so far. Moreover, the constructed biosensor device offered 2-3 orders of magnitude lower DL compared to other EIS-based devices integrated with lectins. The main reason for preferential use of R_{ct} compared to capacitance as a transducing signal with EIS is a less demanding formation of the interface [6]. Further, reliability of EIS as an analytical tool can be enhanced by employing other EIS circuit elements besides R_{ct} to make a 3-D map resembling results obtainable from principal component analysis [6,20,21,22].

The constructed biosensor can analyse a sample within 30 min with 20 min needed for sample incubation, 6 min for EIS measurements and a couple of minutes necessary for the electrode washing and the electrode integration within a measurement set-up. An overall analysis time of the biosensor is considerably shorter compared to ELISA-like lectin assays with response time at least 4 h [18]. Moreover, EIS-based biosensors can be integrated into an array format of analysis, what can significantly enhance assay throughput. Assay precision of the biosensor expressed as a relative RSD is within values previously published e.g. 2.9% – 5.8% [21,22].

Lectin are less specific for target analytes compared to antibodies, but this feature can be advantageous in cases a target molecule or a biomarker of some disease or a physiological change is not known. A true potential of lectin based bioanalytical devices was revealed in a study, where panel of lectins clearly showed that it was possible to distinguish between patients having bacterial infection and healthy individuals [3,14]. The authors claim that extended lectin panels have the potential to even distinguish between types of bacterial infection and identify specific disease stages. Another study confirmed differences in glycan patterns between healthy patients and patients having chronic pancreatitis and pancreatic cancer [14]. These two

studies confirmed lectins can be effectively used for clear resolution between distinct samples even in case the nature of a biomarker present in the sample from sick people is not known. Further, a powerful combination of lectin bioanalytical devices with mass spectrometry will presumably become one of the driving forces for development in glycomics. Lectins have been successfully used in affinity pre-treatment of low abundance glycoproteins with subsequent use of mass spectrometry for identification of glycoproteins [4,14]. Such a strategy can be used to identify new disease biomarkers, a key challenge in biomedical/clinical diagnosis in order to increase the survival rate of patients. Once a biomarker is validated and approved, an array of more specific antibodies can be used for high-throughput screening of clinical samples.

- **Conclusions**

An extensive optimisation of an interfacial layer of the biosensor resulted in a highly sensitive and robust biosensor device able to detect glycoproteins fetuin and asialofetuin *via* recognition of terminal sialic acid by an immobilised SNA I lectin. The biosensor offered a wide linear range spanning 7 orders of concentration magnitude, a feature important for analysis of real samples with proteins present at significantly different concentrations. A linear range of the biosensor starting from 1 fM level (e.g. 24,000 glycoprotein molecules in 40 µL of a sample) will allow the device to detect even low-abundant proteins, what is a key element for detection of various disease markers present at extremely low levels in samples at initial stages of a particular disease. Moreover, the biosensor was able to detect changed amount of sialic acid on glycoproteins in a quantitative way. An assay time of 30 min might not be sufficient for analysis of large number of real samples, but performance of the biosensor can be enhanced using an array format of analysis, enhancing assay throughput dramatically. Selectivity of detection is of special interest, in case measurements of real samples are considered, and has to be improved by suppressing non-specific interactions, what is currently on the way in our laboratory.

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- **Supporting information**

Experimental procedures

Electrode cleaning

The 1st step was a reductive desorption of previously bound thiols by applying a cyclic potential scanning from -1,500 mV to -500 mV in 100 mM NaOH under N_2 atmosphere with a sweep rate of 1 V s^{-1} until a stable cyclic voltammogram was obtained. The 2nd step was a mechanical polishing of electrodes for 5 min on a polishing pad using 1.0 □m and 0.3 □m particles (Buehler, USA), followed by two sonications in DW for 3 min. In the 3rd step the electrodes were left in hot piranha (a mixture of concentrated sulphuric acid and concentrated hydrogen peroxide in 3+1 ratio) for 20 min and sonicated. Then, CV was employed for an electrochemical polishing of the electrodes (from -200 mV to 1,500 mV at a scan rate of 100 mV s^{-1} until a stable CV was obtained) and gold oxide stripping (10 cycles starting from +750 mV to +200 mV at a scan rate of 100 mV s^{-1}) on the electrodes in 100 mM H_2SO_4.

Atomic force microscopy (AFM)

Ambient contact mode atomic force microscopy imaging was carried out with a Veeco microscope (Di CP-II, Plainview, USA) in conjunction with the integrated Veeco DiProScan control software at a scan rate of 1 line s^{-1} with the tip set to 120 nN. Square shaped gold chips (12x12 mm with a thickness of 0.3 mm, Litcon AB, Sweden) modified as previously described for gold electrodes were imaged with an AFM tip MPP-11123 having a diameter of 10 nm and images were finally processed by the IP AutoProbeImage 2.1.15 software.

X-ray photoelectron spectroscopy (XPS)

XPS signals on square shaped gold chips modified as previously described for gold electrodes were recorded using a Thermo Scientific K-Alpha XPS system (Thermo Fisher Scientific, UK) equipped with a micro-focused, monochromatic Al Kα X-ray source (1486.6 eV). An X-ray beam of 400 μm size was used at 6 mA x 12 kV. The spectra were acquired in the constant analyser energy mode with pass energy of 200 eV for the survey. Narrow regions were collected with pass energy of 50 eV. Charge compensation was achieved with the system flood gun that provides low energy electrons (~0 eV) and low energy argon ions (20 eV) from a single source. The argon partial pressure was $2x10^{-7}$ mbar in the analysis chamber. The Thermo Scientific *Avantage* software, version 4.84 (Thermo Fisher Scientific), was used for digital acquisition and data processing. Spectral calibration was determined by using the automated calibration routine and the internal Au, Ag and Cu standards supplied with the K-Alpha system. The surface compositions (in atomic %) were determined by considering the integrated peak areas of detected atoms and the respective sensitivity factors.

- **Results and discussion**

Characterisation of the interface by XPS

XPS spectra are obtained by irradiating the surface with a beam of X-rays while measuring the kinetic energy and number of electrons that escape from the top 1 to 10 nm of the layer being analysed. The XPS as a semi-quantitative and a surface sensitive analytical technique was quite often applied to verify chemical composition of a SAM interface before and after modifications. Thus, XPS was applied for detection of changed nature of functional groups present on the interface as a result of surface patterning e.g. activation of –COOH group by EDC/NHS coupling agents.

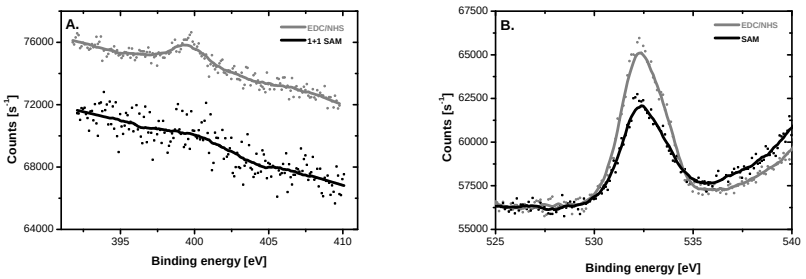

Fig.S1 *The XPS spectra of the SAM modified gold surface before and after activation by EDC/NHS coupling agents with a focus on nitrogen (**A**) or oxygen functionalities (**B**) present on the interface.*

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