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Langmuir, **Just Accepted Manuscript** • DOI: 10.1021/acs.langmuir.6b04021 • Publication Date (Web): 01 Mar 2017

Optimization of small glycan presentation for binding of a tumor-associated antibody. Application for construction of an ultrasensitive glycan biosensor

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ABSTRACT: The main aim of the study was to optimize interfacial presentation of a small antigen – a Tn antigen (*N*-acetylgalactosamine) for binding to its analyte anti-Tn antibody. Three different methods for interfacial display of a small glycan are here compared including two methods based on immobilization of the Tn antigen on a mixed self-assembled monolayer (SAM) (2D biosensor) and the third one utilize a layer of a human serum albumin (HSA) for immobilization of a glycan forming a 3D interface. Results showed that the 3-D interface with immobilized Tn antigen is the most effective bioreceptive surface for binding its analyte. The 3D impedimetric glycan biosensor exhibited a limit of detection of 1.4 aM, a wide linear range (6 orders of magnitude) and high assay reproducibility with an average relative standard deviation of 4%. Build-up of an interface was optimized using various techniques with visualization of the glycans on the biosensor surface by atomic force microscopy. The study showed that the 3D biosensor is not only the most sensitive compared to other two biosensor platforms, but that the Tn antigen on the 3D biosensor surface is more accessible for antibody binding with better kinetics of binding ($t_{50\%}$=137 s, $t_{50\%}$=a time needed to attain 50% of a steady-state signal) compared to the 2D biosensor configuration with $t_{50\%}$=354 s. The 3D glycan biosensor was finally applied for analysis of human serum sample spiked with an analyte.

INTRODUCTION

Carbohydrates belong to four fundamental classes of biomolecules along with proteins, nucleic acid and lipids.[1] Complex carbohydrates (glycans) are really important for fundamental understanding of biology and thank to them we can develop new therapeutic and diagnostic strategies for major diseases.[2] Glycans forming a large group of biomolecules with an enormous structural complexity exist mainly in the form of glycoconjugates with proteins and lipids. Cell walls of all living cells are covered by glycans, which mediate the first contact in the host-pathogen interactions.[3] Weak, but highly specific protein-glycan interactions play important roles in many cellular processes e.g. cell signaling, molecular recognition, immunity, tumor metastasis, leukocyte recruitment to sites of inflammation, *etc*.[4]

A big group of glycoconjugates is formed by attachment of glycans to proteins *via* an asparagine residue (*N*-linked glycoproteins) or *via* serine/threonine (*O*-linked glycoproteins).[5] In recent years the importance of changes in the glycan composition of *O*-linked glycans with quite a prominent role of the Tn antigen (i.e. *N*-acetylgalactosamine (GalNAc) linked to threonine or serine) in cancer development and progression was revealed with its presence in 70-90% of cancers.[6] Moreover, the Tn antigen and its derivatives have a significant prognostic potential, since their expression correlates well with a metastatic potential of cancer with poor prognosis for cancers of colon, lung, bladder, cervix, ovary, stomach and prostate.[6]

While expression of the Tn antigen is without any doubt tumor-associated, the molecular mechanisms for its expression are still unclear, but most likely linked to lowered activity of glycosyltransferases or increased activity of glycosidases.[6, 7] Thus, a direct detection of the Tn antigen attached to proteins circulated in the blood stream might be an option for detection of various types of cancer, since this antigen is present in healthy individuals at low level.[6] The level of Tn antigen increases in an early stage of cancers (stage I and II patients), when it might be quite challenging to detect it. Due to an amplification of the antigenic signal by the immune system, autoantibodies against the Tn antigen and its derivatives could be detected in sera long before the Tn antigen.[8] Thus, an analysis of autoantibodies against the Tn antigen using glycan arrays with immobilized Tn antigen and its derivatives can be utilized for identification of an early stage of breast cancer with a prognostic potential.[8] Moreover, analysis of autoantibodies against the Tn antigen and its derivatives could be applied for assays of other types of cancer, as well.[6] Furthermore, the Tn antigen based vaccines can be applied to elicit immune response with production of antibodies against various types of cancer.[9] Analysis of a panel of anti-Tn antibodies showed that while IgM antibodies preferentially bind to termi-

nal GalNAc residue, IgG antibodies recognize both GalNAc epitope and a peptide sequence associated with GalNAc.[10] Recent studies suggested that peptide sequences associated with the Tn antigen could affect also binding of lectins with K_D value, which can be changed from 7 μM to 800 μM, indicating that lectin binding to short glycans involve also interactions with a peptide backbone.[11, 12, 13, 14] This is why it is worth investigating immobilization of the Tn antigen on a peptide/protein backbone for construction of the glycan biosensor.

Traditional glycan printed arrays although allowing extremely high throughput and flexible assays are not particularly sensitive with a typical concentration of protein analytes needed for analysis of 1 nM.[15] This is why alternative assay protocols able to provide analysis with an improved sensitivity of detection are sought.[16] Since our recent work showed that impedimetric assays could be applied for an ultrasensitive analysis of a wide range of analytes[17, 18], in this work we focused on application of an impedimetric assay protocol for analysis of anti-Tn antibody with the glycan biosensor built with immobilized Tn antigen. For this purpose gold electrode was applied, which can be easily patterned by thiols forming a SAM layer with tunable density of functional groups[19, 20] applied for immobilization of a glycan. SAMs can offer control of density and orientation of immobilized glycan, which is beneficial for investigation of a structure-activity relationship of multivalent interactions.[21] Since the Tn antigen is small i.e. formed by attachment of one carbohydrate to one amino acid, in the study we optimized the way the Tn antigen is displayed on the biosensor interface. Three different strategies were compared i.e. immobilization of the Tn antigen either on two types of mixed SAMs (2D biosensor) or to HSA protein attached to a mixed SAM layer (3D biosensor).

MATERIAL AND METHODS

Chemicals. A complete list of abbreviations used for chemicals and instrumentation is in the Supporting information file. Thiols HS-$(CH_2)_3$-EG_2-OCH_2-COOH (OEG-COOH, where EG= -(O-CH_2-CH_2)- and HS-$(CH_2)_3$-EG_2-OH (OEG-OH) were purchased from Prochimia (Poland). 11-mercaptoundecanoic acid (MUA), 6-mercapto-1-hexanol (MH), potassium hexacyanoferrate (III), potassium hexacyanoferrate (II) trihydrate, potassium chloride, sodium hydroxide, sulphuric acid, *N*-hydroxysuccinimide (NHS), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), phosphate buffered saline tablet (PBS, one tablet dissolved in 200 mL of deionized water yields 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4, at 25 °C.), lectin from *Dolichos biflorus* (DBA), Concanavalin A (Con A) and human serum albumin (HSA) were purchased from Sigma Aldrich (USA). Goat anti-mouse antibody (ab6708) applied as a non-specific binding probe was purchased from Abcam (UK). Antibody GOD-2C4, a mouse IgG1κ antibody not cross-reacting with the GalNAc-β-O epitope or the blood group A antigen and specific to both synthetic Tn antigens and mucin-associated Tn antigen, was produced using a procedure as published by Jansson and co-workers.[22] Antibody GOD-2C4 binds to the Tn antigen expressed by cancer of breast, colon, lung, ovary, and pancreas and was the first anti-Tn antibody showing anti-tumor activity on a solid tumor *in-vivo*[22] and recently the antibody was applied to identify possible carrier of the Tn antigen in samples from patients having breast cancer[23]. Binding specificity towards various glycans, glycoprotein and proteins showed no binding of GOD-2C4 antibody to BSA or HSA proteins with a biospecific binding towards the Tn antigen[22]. The Tn antigen (GalNAcα1-*O*-serine) was provided from Carbosynth (United Kingdom). Ethanol for UV/VIS spectroscopy (ultra-pure) was purchased from Slavus (Slovakia). All buffer components were dissolved in deionized water (DW). Proteins were dissolved in 10 mM PBS solution with pH 7.4 at concentration of 1 mg ml^{-1} and were stored at -20°C in aliquots.

Electrode pre-treatment and SAM preparation. The cleaning of planar polycrystalline gold electrodes (d=1.6 mm, Bioanalytical systems, USA) was made by already established protocol[24], with a laboratory potentiostat/galvanostat Autolab PGSTAT 128N (Ecochemie, Netherlands), in a three electrode cell with an Ag/AgCl reference and a counter Pt electrode (Bioanalytical systems, USA). The first step was reductive desorption of previously bound thiols in 0.1 M solution of NaOH, by applying a cyclic potential scanning from -1,500 mV to -500 mV under N_2 atmosphere with a scan rate of 1 V s^{-1} for 50 cycles (until a stable scan was obtained). The second step was mechanical polishing of the electrodes on a polishing pad using 1 μm particles for 5 min and 0.3 μm particles for 5 min (Buehler, USA). After mechanical polishing, the electrodes were sonicated two times in DW for 5 min. The third step was immersion of the electrodes into the solution of piranha (H_2O_2:H_2SO_4 at ratio 1+3) for 20 min, *handle with a special care since it is a strongly oxidizing agent*. After piranha treatment, the electrodes were again sonicated in DW for 5 min. The fourth and fifth steps were electrochemical polishing and stripping in 0.1 M solution of H_2SO_4. Electrochemical polishing was made by cyclic voltammetry, from -200 mV to 1,500 mV at a scan rate of 100 mV s^{-1} for 25 cycles, and stripping was realized by 10 cycles starting from +750 mV to +200 mV at a scan rate of 100 mV s^{-1}. The electrodes were washed with DW and ultrapure ethanol. After the cleaning process, the electrodes were immersed into the 1 mM solution of thiols (OEG-COOH:OEG-OH ratio of 1+1, 1+3, 1+5, 1+10) or (MUA:MH ratio of 1+3, 1+5, 1+10) and incubated overnight in dark at room temperature. The 1 mM stock solutions of thiols were prepared in ultrapure ethanol and stored at -20 °C.

Preparation of the glycan biosensors. After incubation of the electrodes in the solution of thiols they were washed with ultrapure ethanol and DW. Carboxyl groups on SAM layer were activated with 40 μl solution of 200 mM EDC and 50 mM NHS at ratio 1+1 (solution of EDC and NHS were previously prepared in DW and stored at -20°C in aliquots) for 12 min. After activation, the electrodes were washed with DW and 100 μM Tn antigen was added to the biosensor surface (glycan was previously dissolved in 10 mM PBS pH 7.4 and stored at -20°C in aliquots). The glycan immobilization was made at room temperature for 1.5 h.

In case of a 3D glycan biosensor, after activation of –COOH group within a mixed SAM (OEG-COOH and OEG-OH) by EDC/NHS, the surface was incubated with HSA (10^{-4} - 10^{-1} mg ml^{-1}) for 30 min. After immobilization of HSA, the protein was activated with 40 μl solution of 200 mM EDC and 50 mM NHS at ratio 1+1 for 12 min and then the activated surface was incubated with the Tn antigen (100 μM) for 1.5 h.

Electrochemical impedance spectroscopy (EIS) measurements. EIS can provide interfacial characteristics of a bioreceptive layer using a redox robe. The result of EIS analysis is presented in a Nyquist plot, from which such characteristics can be obtained. The method is sensitive to thickness and

density of biomolecules attached to the surface, resulting in resistivity change of the biosensor surface.[16, 25]

EIS was measured in an electrolyte containing 5 mM potassium hexacyanoferrate (III), 5 mM potassium hexacyanoferrate (II) and 0.01 M PBS, pH 7.4. The analysis was run at 50 different frequencies (ranging from 0.1 Hz up to 100 kHz) under Nova Software 1.10 (Ecochemie, Netherlands). The results were presented in a form of a Nyquist plot, with an equivalent circuit R(Q[RW]) applied for data fitting. The charge transfer resistance (R_{CT}) parameter was used as the output signal for calibration of the biosensor. Each analyte was measured at least in triplicate on three independent biosensor devices (electrodes) and results are shown with a standard deviation (± SD) or relative standard deviation (RSD) calculated in Excel. It is worth noting that such RSDs are not relative standard errors of analyte detection, but rather represent reproducibility of the biosensor construction, since each calibration curve was constructed by an independent biosensor device. Measurements of a particular analyte were performed on the same day.

Serum sample analysis. The human serum sample was collected from a control subject recruited from the Institute of Experimental Endocrinology, Slovak Academy of Sciences, Bratislava, Slovak Republic. The subject gave their informed written consent, the study was approved by the Ethics Committee of the National Institute for Rheumatic Diseases, Piestany, Slovak Republic, in accordance with the ethical guidelines of the Helsinki Declaration as revised in 2000. Blood sample was collected into polyethylene tube with a clotting activator (S-Monovette, Sarstedt AG & Co., Germany). After the centrifugation, the serum aliquot was stored at -80 °C until use. The serum sample was diluted in 10 mM PBS pH 7.4 with a dilution ratio 1+1000.

Quartz crystal microbalance (QCM) measurements. QCM is a mechanical detection method to measure the change in mass on a surface through change in frequency of an oscillating quartz crystal. A decrease of the frequency of a QCM device is linearly proportional to increased amount of species attached to the surface and can be applied for calculation of density of species present on the surface.[16, 25]

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

$$\Delta f = -\frac{2f_0^2}{A\sqrt{\rho_q \mu_q}}.\Delta m \qquad \text{(eqn. 1)},$$

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 (g cm^{-1} s^{-2}), A is the surface area and ρ_q is density of quartz in g ml^{-1} . For a 6 MHz crystal, the whole equation can be simplified to:

$$\Delta f = -C_f.\Delta m \qquad \text{(eqn. 2)},$$

where C_f is a frequency constant of 0.0815 Hz ng^{-1} cm^2. Assays were monitored and evaluated using the Nova 1.10 software and all measurements were run at room temperature.

Surface plasmon resonance (SPR) measurements. SPR is frequently used optical detection method for following a binding event in real time providing kinetics of an interaction.[16, 25]

Experiments were performed using a dual channel Reichert SR7000DC SPR system controlled by the SPR Autolink System software (AMETEK, Reichert Technologies, USA). For the SPR measurements bare gold SPR sensor chips (XanTec Bioanalytics GmbH, Germany) were used. The bare gold SPR sensor chip was cleaned three times with piranha (H_2O_2:H_2SO_4 at ratio 1+3, *handle with a special care since it is a strongly oxidizing agent*) for 2-3 s, then washed with DW and ultrapure ethanol. Finally, the SPR chip was modified with thiols as described previously for the gold electrodes (Section 2.3).

The amount of the Tn antigen immobilized on the surface of the 2D and 3D biosensor was investigated using the modified SPR sensor chip (i.e. OEG-OH/OEG-COOH SAM for the 2D biosensor and HSA immobilized on OEG-OH/OEG-COOH SAM for the 3D biosensor). The modified sensor chip was activated for 10 min (both working and reference channels of the SPR flow cell using a fresh mixture of 0.2 M EDC and 0.05 M NHS at the flow rate of 20 μL min^{-1}). Then, the Tn antigen (100 μM in 10 mM sodium acetate buffer pH 4.5 was injected into the working channel for 48 min (an association phase). At the end of the SPR experiment a dissociation phase was induced by injection of 0.01 M PBS for 20 min at the flow rate of 20 μL min^{-1}. Finally, the SPR response in the working channel was subtracted from the SPR signal read in the reference channel. For calculation of the total amount of bound Tn antigen, the conversion 1 mRIU= 1 pg mm^{-2} (according to the manufacturer) was applied.

Atomic force microscopy (AFM) measurements. AFM is applied to visualize interfacial features at nanoscale.[17] For AFM visualization a Bioscope Catalyst instrument and an Olympus IX71 microscope in conjunction with NanoScope 8.15 software were run at a scan rate of 0.5 line s^{-1} with the tip set to 200 pN (Scan Asyst, Bruker, USA). Measurements were made using a peak force tapping mode in air. Bare gold SPR sensor chips (XanTec bioanalytics GmbH, Germany) were cleaned three times with piranha (H_2O_2:H_2SO_4 at ratio 1+3, *handle with a special care since it is a strongly oxidizing agent*) for 2-3 s, then with DW and ultrapure ethanol, and the chips were modified with thiols and glycans as it was described previously. The SCANASYST-AIR silicon tip on nitride lever (Bruker, USA, with f_0=50-90 kHz and k=0.4 N m^{-1}), sharpened to a tip radius of 2 nm was used in the assays.

RESULTS AND DISCUSSION

The glycan biosensor based on MUA/MH SAM (biosensor 1). In the previous study, we prepared an ultrasensitive glycan biosensor based on a mixed SAM composed of MUA and MH with immobilized sialyllactose for detection of glycan binding proteins.[17] Here we wanted to test the same interfacial SAM for immobilization of the Tn antigen for subsequent detection of an anti-Tn antibody (GOD-2C4) and DBA lectin.

The results showed that the optimal composition of a mixed SAM for construction of the Tn biosensor contain 16.7% of MUA in the mixture (**Fig. 1** and **Fig. S1**). Taking into an account distance between neighboring thiols of 0.5 nm[20], an average distance between neighboring MUA thiols in a mixed SAM composed of 16.7% MUA would be 1.4 nm. Length difference between MUA (~1.7 nm) and MH (~1.1 nm) of 0.6 nm might be sufficient to partially accommodate the Tn antigen (~ 1.0 nm x 1.0 nm) within a mixed SAM composed of MUA and MH (**Fig. 2A**), what would negatively influence binding performance between glycan and antibody. Examination of the selectivity of the glycan biosensor showed that the sensitivity of detection of GOD-2C4 was (8.3 ± 1.0) % decade^{-}

[1] (R^2=0.961) in the concentration window 14 aM – 14 fM with an average RSD of assays of 18% (12-23%). The biosensor sensitivity for DBA of (4.4 ± 0.2) % decade^{-1} (R^2=0.991) and HSA of (4.4 ± 0.1) % decade^{-1} (R^2=0.994) was similar to drift of the biosensor signal, when incubated in PBS buffer, so it can be concluded that DBA and HSA did not interact with the glycan biosensor.

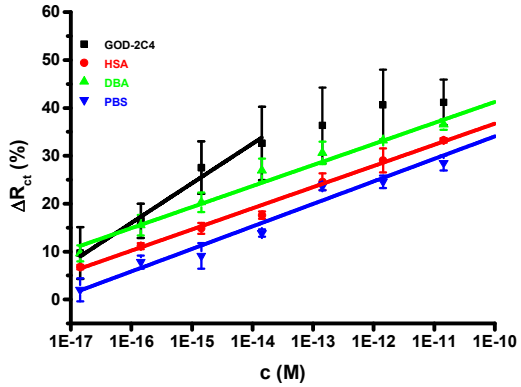

Figure 1. Selectivity of the glycan biosensor constructed on a mixed SAM containing 16.7% of MUA in a thiol mixture was probed by analysis of two analytes (GOD-2C4 and DBA) and a non-specific binding probe (HSA). Moreover, a drift of the signal of the biosensor incubated only with PBS measured at the same time intervals as for GOD-2C4, HSA and DBA is shown, as well. At least three independent electrodes were used for data generation. EIS assays were performed in an electrolyte containing 5 mM potassium hexacyanoferrate (III), 5 mM potassium hexacyanoferrate (II) and 0.01 M PBS, pH 7.4. The analysis was run at 50 different frequencies (0.1 Hz - 100 kHz) at room temperature.

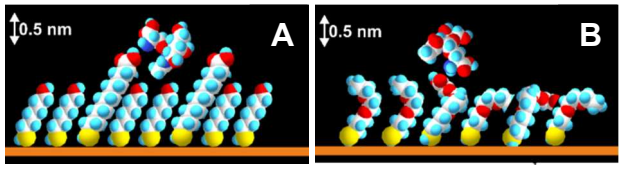

Figure 2. A mixed SAM composed of MUA and MH attached to gold with the Tn antigen shown above a mixed SAM layer (**A**) and a mixed SAM composed of OEG-COOH and OEG-OH with the Tn antigen shown above a mixed SAM layer, as well (**B**). Both figures are drawn to scale.

The glycan biosensor based on OEG-COOH/OEG-OH SAM (biosensor 2). Optimization experiments showed that the optimal SAM for construction of the glycan biosensor should contain 25% of OEG-COOH thiol in a mixture (**Fig. S2**), but the glycan biosensor built on such a mixed SAM exhibited rather high average RSD of assays of 39%, while the glycan biosensor built on a mixed SAM containing 16.7% of OEG-COOH exhibited an average RSD of assays of 18% (1%-28%). The glycan biosensor constructed on a SAM containing 16.7% of OEG-COOH could detect GOD-2C4 with a sensitivity of (4.7 ± 0.5) % decade^{-1} (R^2=0.938), limit of detection (LOD) of 110 aM and a linear concentration range from 110 aM to 140 pM (~6 orders of magnitude). Moreover, DBA could be effectively detected with this glycan biosensor with the sensitivity of (6.2 ± 0.3) % decade^{-1} (R^2=0.987), LOD of 270 aM and a linear concentration range from 270 aM to 140 pM (~6 orders of magnitude). It is interesting that for DBA lectin the Tn antigen exposed on the SAM composed of OEG-COOH and OEG-OH is accessible for binding, most likely since the Tn antigen cannot be easily buried within this SAM layer (**Fig. 2B**) compared to a previous case (MUA/MH SAM, **Fig. 2A**). DBA binding is more effective compared to GOD-2C4 binding to the immobilized Tn antigen with the sensitivity ratio of 1.3 (=6.2/4.7).

Most importantly the glycan biosensor is not sensitive towards a non-selective binding probe HSA and no signal drift was observed, when the glycan biosensor was incubated with PBS (**Fig. 3** and **Fig. S2**) making the glycan biosensor stable and selective.

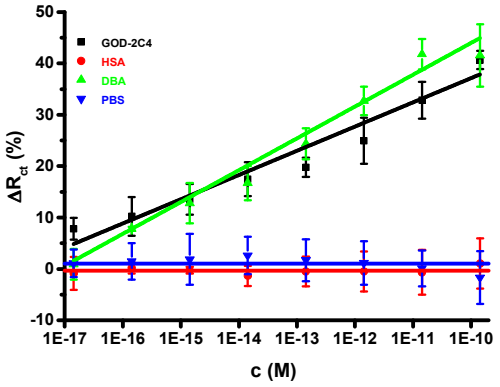

Figure 3. Selectivity of the glycan biosensor constructed on a mixed SAM containing 16.7% of OEG-COOH was probed by analysis of two analytes (GOD-2C4 and DBA) and a non-specific binding probe (HSA). Moreover, a drift of the signal of the biosensor incubated only with PBS measured at the same time intervals as for GOD-2C4, HSA and DBA is shown, as well. For other assay conditions see legend to **Fig. 1**.

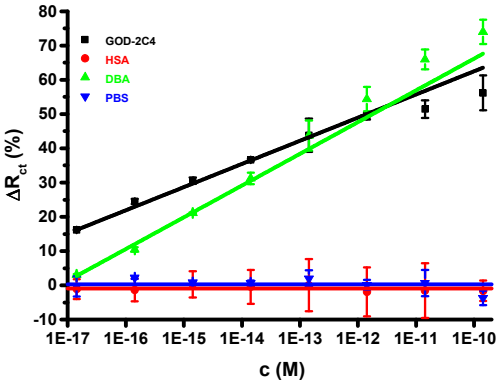

Figure 4. Selectivity of the glycan biosensor constructed on HSA layer was probed by analysis of two analytes (GOD-2C4 and DBA) and a non-specific binding probe (HSA). Moreover, a drift of the signal of the biosensor incubated only with PBS measured at the same time intervals as for GOD-2C4, HSA and DBA is shown, as well. For other assay conditions see legend to **Fig. 1.**

The glycan biosensor based on HSA attached to OEG-COOH/OEG-OH SAM (biosensor 3). The third type of the interfacial layer tested as a support to accommodate the Tn antigen was a layer of HSA protein covalently attached to OEG-COOH/OEG-OH SAM (at ratio OEG-COOH:OEG-OH=1+5). Four different HSA concentrations were applied for HSA immobilization i.e. 0.0001; 0.001, 0.01 and 0.1 mg ml^{-1} with the highest sensitivity of the glycan biosensor achieved with the one built on HSA layer deposited from 0.001 mg ml^{-1}

HSA solution (**Fig. S3**). The glycan biosensor constructed with an optimal HSA composition could detect both analytes – GOD-2C4 and DBA (**Fig. 4**). The antibody could be detected with the sensitivity of (6.7 ± 0.1) % decade^{-1} (R^2=0.998), LOD of 1.4 aM and a linear concentration range from 1.4 aM to 1.4 pM (~6 orders of magnitude). Moreover, assays reproducibility was very high with an average RSD of 4% (1-9%). DBA could be detected with the sensitivity of (9.3 ± 0.3) % decade^{-1} (R^2=0.994), LOD of 36 aM and a linear concentration range from 36 aM to 1.4 pM (~5 orders of magnitude). Again, DBA binding is more effective compared to GOD-2C4 binding to the immobilized Tn antigen with the sensitivity ratio of 1.4 (=9.3/6.8). This value is very similar to the value of 1.3 obtained with the glycan biosensor 2 based on OEG-COOH/OEG-OH SAM layer without HSA layer present.

Selectivity of the biosensor was tested using HSA as a non-selective binding probe (**Fig. 4**), lectin Concanavalin A and goat anti-mouse antibody not recognizing the Tn antigen (**Fig. S4**), showing only a minor binding.

SPR experiments for calculation of the immobilized Tn antigen (biosensor 2 vs. biosensor 3). SPR experiments revealed that the Tn antigen after covalent attachment to the biosensor 2 surface gave a response of 140 μRIU, equivalent to the surface density of 140 pg mm^{-2} (46 pmol cm^{-2}). If we take into account that the Tn antigen has a projected surface area of ~ 1 nm^2, then a theoretical surface coverage for the surface confined Tn antigen would be 170 pmol cm^{-2}, indicating that the Tn antigen on the interface of the biosensor 2 occupies 27% of a theoretical coverage.

In case of the biosensor 3 (i.e. 3D configuration), the SPR response after covalent attachment of the Tn antigen was 870 μRIU, equivalent to the surface density of 870 pg mm^{-2} (290 pmol cm^{-2}). The surface of the glycan biosensor 3 built on molecular HSA scaffolds will have larger (~2-fold) surface area due to HSA molecules present compared to the surface without this protein layer. Thus, it can be anticipated that a theoretical surface coverage for the Tn antigen on HSA layer would be 340 pmol cm^{-2} (*per* geometric surface area), indicating that the Tn antigen on the interface of the biosensor 3 occupies 85% of a theoretical coverage.

When only a geometric surface area is taken into account then 6.3-fold higher surface density of the Tn antigen on the interface of the biosensor 3 was immobilized compared to the interface of the biosensor 2.

QCM experiments – biosensor 2 vs. biosensor 3. QCM experiments were run to understand what amount of analyte – GOD-2C4 can be attached to the Tn antigen modified interfaces of the biosensor 2 and the biosensor 3. Results showed that after injection of GOD-2C4 at concentration of 3.3 nM a change in the frequency of the QCM crystal of -15.7 Hz (193 ng cm^{-2} or 1.3 pmol cm^{-2}, i.e. 51% of a theoretical full monolayer) was observed on the surface of the biosensor 2 (**Fig. S5**). Slightly larger QCM response of -22.0 Hz (270 ng cm^{-2} or 1.8 pmol cm^{-2}, i.e. 36% of a theoretical full monolayer) was observed on the interface of the biosensor 3 (**Fig. S5**). When only a geometric surface area is taken into account then the amount of GOD-2C4 analyte attached to the biosensor 3 interface is 1.4-fold higher compared to the biosensor 2. Results from EIS measurements confirmed that the sensitivity of GOD-2C4 detection is 1.4-fold higher on biosensor 3 compared to biosensor 2. Thus results obtained by QCM and EIS, two independent assay methods, are in an excellent agreement.

It is quite interesting to directly compare kinetics of GOD-2C4 analyte binding to the Tn antigen presented on OEG-COOH/OEG-OH SAM layer (biosensor 2) or to the Tn antigen presented on a monolayer of HSA protein (biosensor 3) (**Fig. S6**). Results from such an experiment showed that binding of GOD-2C4 is more quick ($t_{50\%}$=137 s) on the biosensor 3 compared to the response on the biosensor 2 with $t_{50\%}$=354 s (**Fig. S6**). This confirms better accessibility of the Tn antigen on HSA layer compared to the interface without immobilized HSA layer.

AFM experiments – biosensor 2 vs. biosensor 3. A closer look at the modified biosensor surfaces visualized by AFM revealed that the glycan biosensor 2 with the Tn antigen immobilized on a mixed OEG-COOH/OEG-OH SAM layer exhibits more rough interface (**Fig. 5A** and **Fig. 6A**) due to natural roughness of the gold layer compared to the roughness of an interfacial layer of the biosensor 3 with the Tn antigen presented on a HSA layer (**Fig. 5C** and **Fig. 6B**). Analysis of the profile of features present on both types of biosensors (**Fig. 6**) confirms the fact that the biosensor 2 interface before interaction with GOD-2C4 analyte is more rough (R_q=0.32 nm) compared to the interface of the biosensor 3 (R_q=0.09 nm) (**Fig. 6**). After incubation of GOD-2C4 analyte with both biosensor surfaces it was possible to see individual antibody molecules attached to the Tn antigen (**Fig. 5B** and **Fig. 5D**). The roughness of the biosensor 2 interface increased from R_q=0.32 nm to 1.8 nm after incubation with an analyte and the roughness of the surface of the biosensor 3 increased from R_q=0.09 nm to 0.89 nm. Quite high roughness of the interface of the biosensor 2 before interaction with bound GOD-2C4 analyte having features with height ± 2 nm, might cause that the Tn antigen could be less available for binding to GOD-2C4 compared to the situation on the surface of the biosensor 3 with height of features before incubation of GOD-2C4 of only ± 0.2 nm. Thus, HSA layer can suppress natural nanoscale roughness of the gold film, what is a feature important for effective display of short glycans like the Tn antigen having size of ~ (1.0 x 1.0) nm for subsequent protein binding.

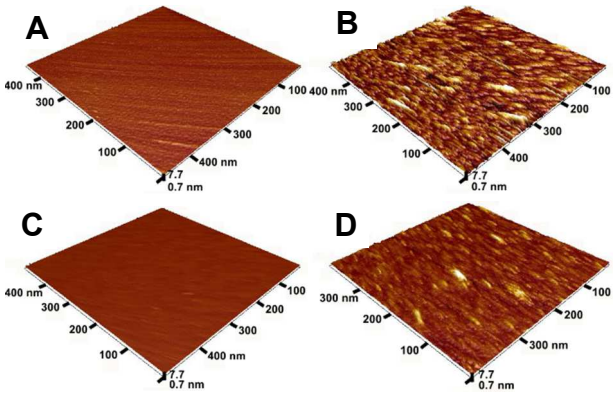

Figure 5. AFM images of the glycan biosensor 2 before (**A**) and after incubation with GOD-2C4 analyte (**B**) and of the glycan biosensor 3 before (**C**) and after incubation with GOD-2C4 analyte (**D**). In all four AFM images z-axis is the same for better comparison of the size of features present on the surface.

When we looked at the structure of HSA molecule we found out that HSA has at least 10 acidic amino acids available for further activation by EDC/NHS from which 6 are glutamic acids (Glu132, Glu266, Glu280, Glu479, Glu492 and Glu501)

and 4 are aspartic acids (Asp269, Asp301, Asp471 and Asp562) (**Fig. S7**) and thus quite a few glycans can be attached to one HSA molecule.

Comparison of the glycan biosensors with published concepts. Recently a comparison study with involvement of 6 different platforms for construction of glycan arrays applied for detection of 4 lectins was performed. The results clearly showed that a microarray with short glycans (GalNAc and other monosaccharide determinants) attached to proteins (bovine serum albumin (BSA) or HSA) exhibited response after incubation with lectins, while response on a glycan microarray with glycans directly printed on the microscope slide did not reveal any binding.[15] This confirms that short glycans/carbohydrate antigens have to be displayed in a way to be accessible for binding, what could be achieved by presentation of glycans on a backbone of a protein. The importance of the Tn antigen presentation on a peptide backbone for effective binding of anti Tn-antibodies was discussed in a recent review, as well.[26] Moreover, glycans attached to the backbone of BSA/HSA protein can be displayed at similar densities as present on natural glycoproteins[27, 28], a feature important for getting physiologically relevant information.

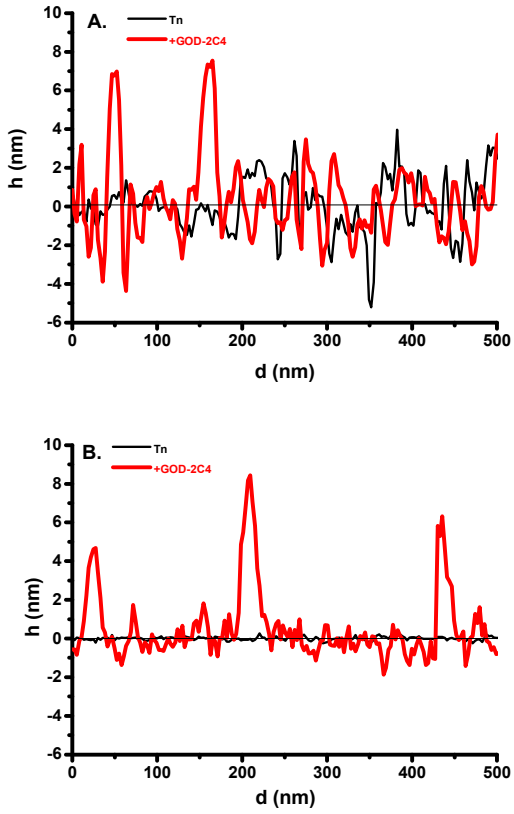

Figure 6. AFM profile of the features present on the biosensor 2 interface before (Tn) and after incubation with GOD-2C4 analyte (+GOD-2C4) (**A**) and the features present on the biosensor 3 before and after interaction with GOD-2C4 analyte are shown, as well (**B**).

Classical ELISA with a glycan immobilized on a BSA backbone showed LOD of 140 nM, while immobilization of a glycan on gold nanoparticles with the size of 2 nm showed LOD of 50 pM for analysis of IgGs.[29] Using the glycan array constructed with the dendrons, LOD for antibody detection was 350 pM[30], while LOD of 4 nM for determination of antibodies was achieved in the conventional ELISA plates[31]. In an another paper, three different analytical platforms based on immobilized glycans for detection of antibodies were compared and although LOD was not quantified, a linear calibration curve started from 10 pM, 250 pM and 700 pM for an ELISA-like format, for a suspension-based array and for a typical glycan microarray, respectively.[32] When the biosensor platforms (SPR, localized SPR, QCM, field-effect sensing - FET, various electrochemical approaches and cantilever-based assays) of detection are taking into account, a typical LOD for detection of various analytes with the glycan biosensors are down to nM-pM range[16]. The only exception was FET sensing of influenza hemagglutinins with LOD down to aM level.[33] Recently we showed that lectins could be detected down to concentration of 8 aM[17, 18] and hemagglutinins down to concentration of 140 aM[17]. This means that the glycan biosensor based on the Tn antigen immobilized on HSA layer is not only the most sensitive Tn-glycan based device for lectin detection with LOD of 36 aM and for antibody detection with LOD of 1.4 aM, but belongs to the most sensitive electrical biosensors for detection of disease biomarkers described so far.[1, 34] High sensitivity of detection by the glycan biosensor could be important in the cases, when the density of Tn antigen in the human body is high with Tn-antibodies mainly bound to such antigens resulting in low circulating concentration of Tn-antibodies.

Analysis of a spiked serum sample. Serum sample from a healthy individual was spiked with three different concentrations of GOD-2C4 antibody (0.1 pM, 1 pM and 10 pM) and such analysis revealed sensitivity for GOD-2C4 antibody detection of 6.0 % decade^{-1}, what is value slightly lower compared to a sensitivity of detection of the same analyte in plain buffer of 6.7 % decade^{-1} (i.e. 90% of the biosensor sensitivity obtained in buffer equal to recovery index of 90%). This can be ascribed to the matrix effect of a complex human serum sample as described in numerous studies, when recovery index of 55%[35], 67%[36], 74%[37], 91%[38] and 97%[39] was determined for various analytes and dilutions. Thus the recovery index of 90% is within the range previously published by others. Obviously, analysis of more samples would be needed in the future to make more general conclusions about performance of the glycan biosensor in analysis of real samples. There are numerous possible applications of the Tn-based glycan biosensor, which can be applied for detection of A) autoantibodies to the Tn antigen present in serum during cancer development and progression[8]; B) antibodies against the Tn antigen injected into humans during passive immunotherapy[22]; C) antibodies elicited against the Tn antigen after treatment by the Tn antigen-based vaccines during active immunization of cancer patients[8, 9]; D) anti-Tn antibodies present in the supernatant from hybridomas cultures during their production[22]; and E) analysis of a refolding efficiency during production of antibody fragments against the Tn antigen by a recombinant technology[40]. There is however a need to employ impedimetric assays into an array format of analysis with a minor degree of multiplexing, what is a feasible task, as shown for lectin biosensors recently by us using a chip with 8 electrodes.[41] Furthermore, efficient approaches designed by Davis´s lab how to deal with matrix effect of serum could be applied for construction of glycan biosensors, as well. Additionally, the effect of peptide envi-

ronment close to immobilized Tn antigen on the biosensor performance should be tested in the future, as well.

CONCLUSIONS

The study showed that the best way for presenting a small glycan such as the Tn antigen for subsequent binding of anti Tn-antibody is immobilization of the glycan on the HSA layer. This study strongly supports conclusions made by a comparative study about the requirement to present small glycans on protein backbone to make glycans accessible to their binders. Moreover, the glycan biosensor based on HSA layer (3D biosensor) offered much better analytical performance compared to the glycan biosensor based on 2D configuration in terms of sensitivity (6.7 % decade^{-1} *vs.* 4.7 % decade^{-1}), linear range (1.4 aM-1.4 pM with R^2=0.998 *vs.* 270 aM-1.4 pM with R^2=0.987), LOD (1.4 aM *vs.* 270 aM), average RSD (4% *vs.* 18%) and kinetics of interactions ($t_{50\%}$=137 s *vs.* $t_{50\%}$=354 s). Moreover, the 3D biosensor exhibits much lower surface roughness of 0.09 nm compared to a value of 0.32 nm observed for the 2D biosensor configuration. This is an important issue for proper interfacial display of a small glycan such as the Tn antigen and also for surface presentation of other small ligands, what is not an issue for detection of auto antibodies produced against protein targets[42, 43, 44].

ACKNOWLEDGEMENT

This publication was made possible by NPRP grant no. 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. Funding from the Slovak research and development agency APVV-14-0753 and VEGA 2/0162/14 is acknowledged. The research leading to these results has received funding from the European Research Council under the European Union's Seventh Framework Program (FP/2007-2013)/ERC Grant Agreement no 311532. 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.

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ASSOCIATED CONTENT

Supporting Information

The file contains List of abbreviations, optimization of the composition of MUA/MH SAM (**Fig. S1**), optimization of the composition of OEG-COOH/OEG-OH SAM (**Fig. S2**), optimization of the composition of the glycan biosensor based on HSA layer (**Fig. S3**), selectivity of analyte detection compared to binding of two non-specific binding probes (Con A and an antibody not binding the Tn antigen) (**Fig. S4**), results from QCM experiments (**Fig. S5** and **Fig. S6**) and 3D structure of HSA with acidic amino acids (**Fig. S7**) accessible for covalent binding of the Tn antigen. The Supporting Information is available free of charge on the ACS Publications website.

TOC graphic