Supporting Information

Ultrasensitive impedimetric lectin biosensors with efficient antifouling properties applied in glycoprofiling of human serum samples

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Experimental section

Electrode pre-treatment and SAM preparation

Planar polycrystalline gold electrodes (1.6 mm diameter, Bioanalytical systems, USA) were cleaned according to a valid protocol.[1] At first, a process of reductive desorption of previously bound thiols was employed using the laboratory potentiostat/galvanostat Autolab PGSTAT 128N (Ecochemie, Netherlands) in a three electrode cell with Ag/AgCl reference and a counter Pt electrode (Bioanalytical systems, USA) by applying a cyclic potential scanning from - 1,500 mV to -500 mV in 100 mM NaOH under N_2 atmosphere with a rate of 1 V s^{-1} for about 50 scans (until a stable scan was obtained). Then a mechanical polishing of the electrodes for about 5-10 min on a polishing pad using 1 μm particles (Buehler, USA) was performed, followed by two sonications in DW for 5 min. In the following step, the electrodes were left in hot piranha solution (a mixture of concentrated H_2SO_4 and concentrated H_2O_2 in 3:1 ratio, *handle with special care, because piranha solution is very energetic and potentially explosive due to violent reaction with organics; avoid storage in a closed container; solution can become hot, more than 100 °C and it is recommended to wear the full protection)* for 20 min and after washing sonicated in DW for 5 min. Right before the SAM preparation, an electrochemical polishing and stripping procedures of the electrodes (from -200 mV to 1,500 mV at a scan rate of 100 mV s^{-1} up to 25 scans, and 10 cycles starting from +750 mV to +200 mV at a scan rate of 100 mV s^{-1} for gold oxide stripping, respectively) in 100 mM H_2SO_4 were performed. The electrodes were washed with DW and ultra pure ethanol and were left to dry in the dustless environment for several minutes. Right after that, they were immersed in a 1 mM solution of a thiol (or a mixture of two thiols) and incubated in the dark at room temperature for 1.5 h (a single component SAM) or for 24 h (a mixed SAM), in order to obtain a highly dense monolayer. The data obtained during this procedure was also used for evaluation of the thiol monolayer density on the gold electrode surface, taking into account peaks from reductive desorption of thiols and from gold electrochemical polishing (real surface area).[1] The 1 mM stock solution of MUA was prepared in ultra pure ethanol and stored at -20 °C until use and DPS thiol

was stored dry at the same temperature and its 1 mM stock solution in DW was always freshly prepared just before incubation.

Patients and samples

Three (N=3) female patients fulfilling the 2010 ACR-EULAR classification criteria for RA were included in the study.[2] The patients were recruited from a local outpatient rheumatology clinic. Three (N=3) healthy female subjects served as controls. All the studied subjects were non-smokers, had negative history of endocrine disorders, diabetes or impaired glucose tolerance. The RA patients had the following basic clinical characteristics: patient 4 (42 years old, disease duration 31 years, seronegative RA, treatment with methotrexate and hydroxychloroquine), patient 5 (72 years old, disease duration 10 years, seropositive RA, treatment with methotrexate), patient 6 (52 years old, disease duration 1 year, seropositive RA, treatment with prednisone, methotrexate, hydroxychloroquine, nimesulide). All subjects gave informed written consent and the study was approved by the Ethics Committee of the National Institute of Rheumatic Diseases, Piestany, Slovakia in agreement with the ethical guidelines of the Declaration of Helsinki as revised in 2000. Blood samples were taken into standard serum tubes with silicone-coated interior (BD Vacutainer, Franklin Lakes, NJ USA), clotted at room temperature for approximately one hour, and subsequently the serum was transferred into separate tubes and stored at -20°C. Before analyses, the human serum samples were diluted by 10 mM PBS buffer pH 7.4. All stock solutions (lectins, standard glycoproteins and human sera) were stored at -20 °C in aliquots.

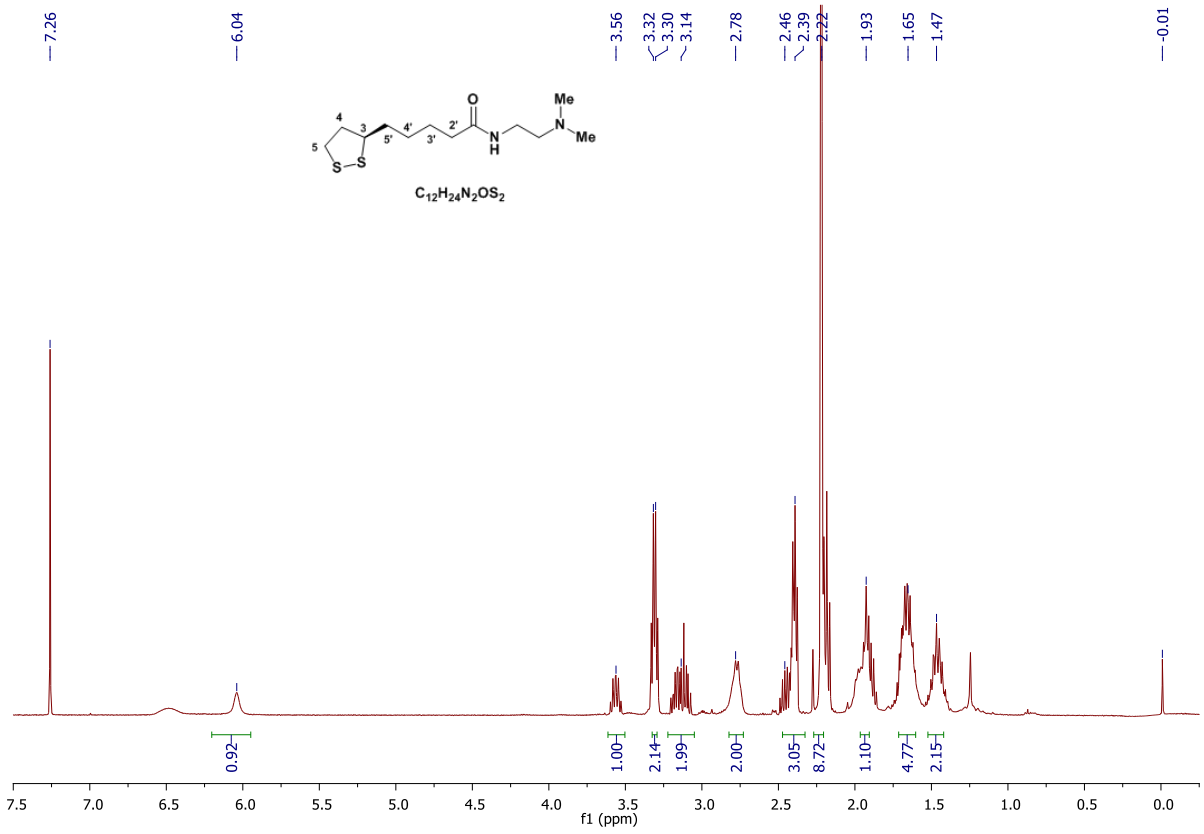
Synthesis of (R)-3-((2-(5-(1,2-dithiolan-3-yl)pentanamido)ethyl)dimethylammonio)propane-1-sulfonate

A new thioctic derivative (*R*)-3-((2-(5-(1,2-dithiolan-3-yl)pentanamido)ethyl) dimethylammonio)propane-1-sulfonate used in the SAM preparation was synthesized in two steps (Scheme 1). The first step is preparation of (*R*)-*N*-(2-(dimethylamino)ethyl)-5-(1,2-dithiolan-3-

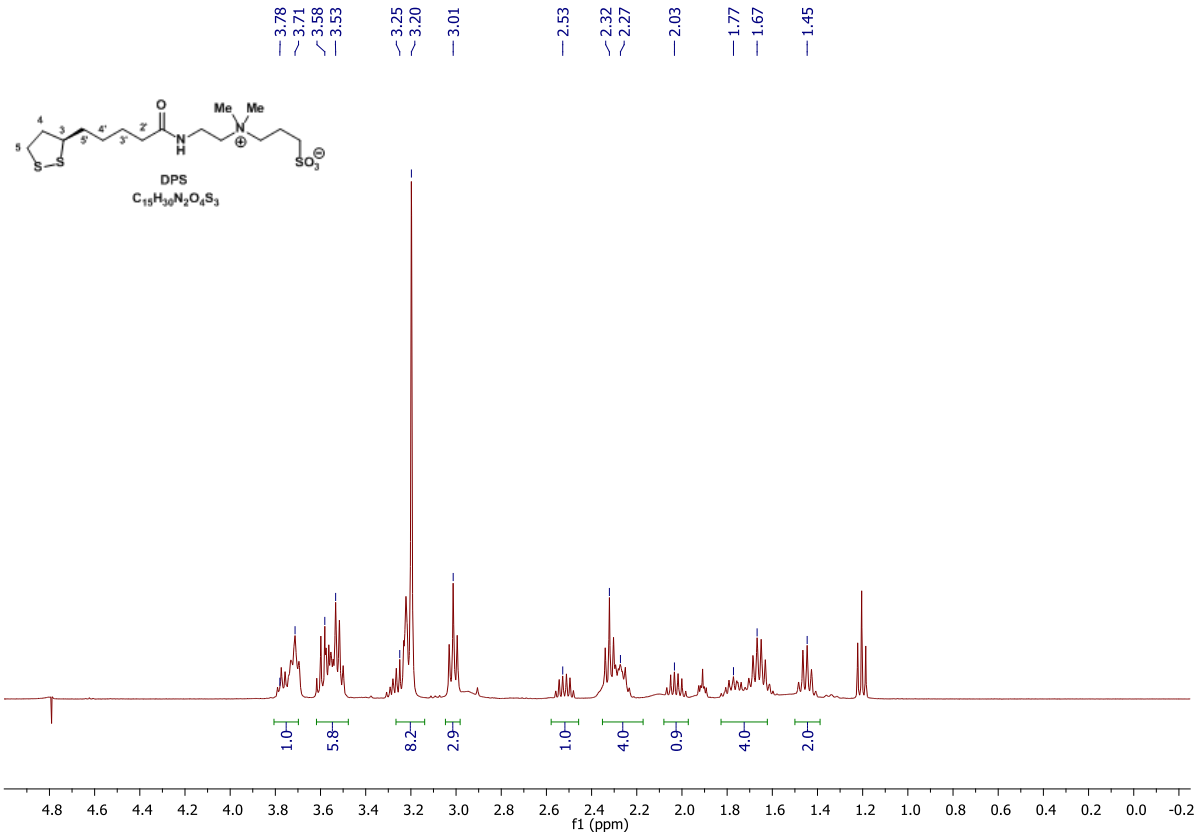
yl)pentanamide[3] as follows: To a stirred solution of (*R*)-Lipoid acid (2.00 g, 9.69 mmol) in CH_2Cl_2 (80.0 mL) was added dropwise DCC (2.40 g, 11.60 mmol, 1.2 equiv) dissolved in dried dichloromethane (4.0 mL). The resulting mixture was stirred for 30 min at room temperature. A solution of *N*-hydroxysuccinimide (NHS, 1.34 g, 11.60 mmol, 1.2 equiv) in acetonitrile (2.0 mL) was added. After stirring for 30 min at room temperature, *N*,*N*-dimethylethylene diamine (3.20 mL, 29.10 mmol, 3.0 equiv) was added dropwise and the reaction was stirred overnight at room temperature. Reaction mixture (yellowish fine suspension) was filtered with the aid of CH_2Cl_2 (15 mL). The filtrate was washed three times with an aqueous solution of 1 M NaOH (3 x 35 mL) and 1 M NaCl (3 x 35 mL), dried (Na_2SO_4), filtered and evaporated under a reduced pressure. The resulting dense oil was dissolved in 1 M HCl (80 mL) and stirred for an hour, filtered and the aqueous solution was added to chloroform ($CHCl_3$, 100 mL). The pH was adjusted between 12 and 13 with NaOH and the two phase system was mixed thoroughly for 30 min. The phases were separated, and the aqueous layer was twice extracted by $CHCl_3$ (2 x 40 mL). Cysteine (10 mg) was added to a combined organic extracts. Organic extracts were dried (Na_2SO_4), filtered and concentrated under a reduced pressure, to give titled compound as a dense yellow oil (2.00 g, 75%). Note: The product can be stored at -20 °C. The product was characterised by IR and NMR with the following characteristics: **IR** (ATR) 3297, 2933, 2769, 1643 (s, NCO), 1550, 1458, 1252, 1042, 752 cm^{-1}; **^{1}H NMR** (400 MHz, $CDCl_3$) δ 6.04 (br s, 1H, NH), 3.56 (m, 1H, H-C3), 3.31 (2 x t, *J* = 5.5 Hz, 2H, HN-C*H*$_2$-CH_2), 3.24 – 3.02 (m, 2H, CH_2-5), 2.90 – 2.68 (m, 2H, CH_2-*CH*$_2$-NMe_2), 2.56 – 2.34 (m, 3H, H^a-4 and CH_2-2'), 2.22 (s, 6H, NMe_2), 1.93 (3, 1H, H^a-4), 1.80 – 1.54 (m, 4H, CH_2-3' and CH_2-5'), 1.54 – 1.34 (m, 2H, CH_2-4') ppm.

The second step was preparation of (R)-3-((2-(5-(1,2-dithiolan-3-yl)pentanamido)ethyl)dimethylammonio)propane-1-sulfonate (DPS), as follows. To a stirred solution of (*R*)-*N*-(2-(dimethylamino)ethyl)-5-(1,2-dithiolan-3-yl)pentanamide (1.10 g, 4.00 mmol) in dried THF (6.0 mL) was added dropwise solution of 1,3-propane sultone (540 mg, 4.40 mmol, 1.10 equiv) in dried THF (2.0 mL) under argon atmosphere and the reaction mixture was stirred overnight at room temperature. Formed white suspension was centrifuged (3045 g, 5 min) and a supernatant was

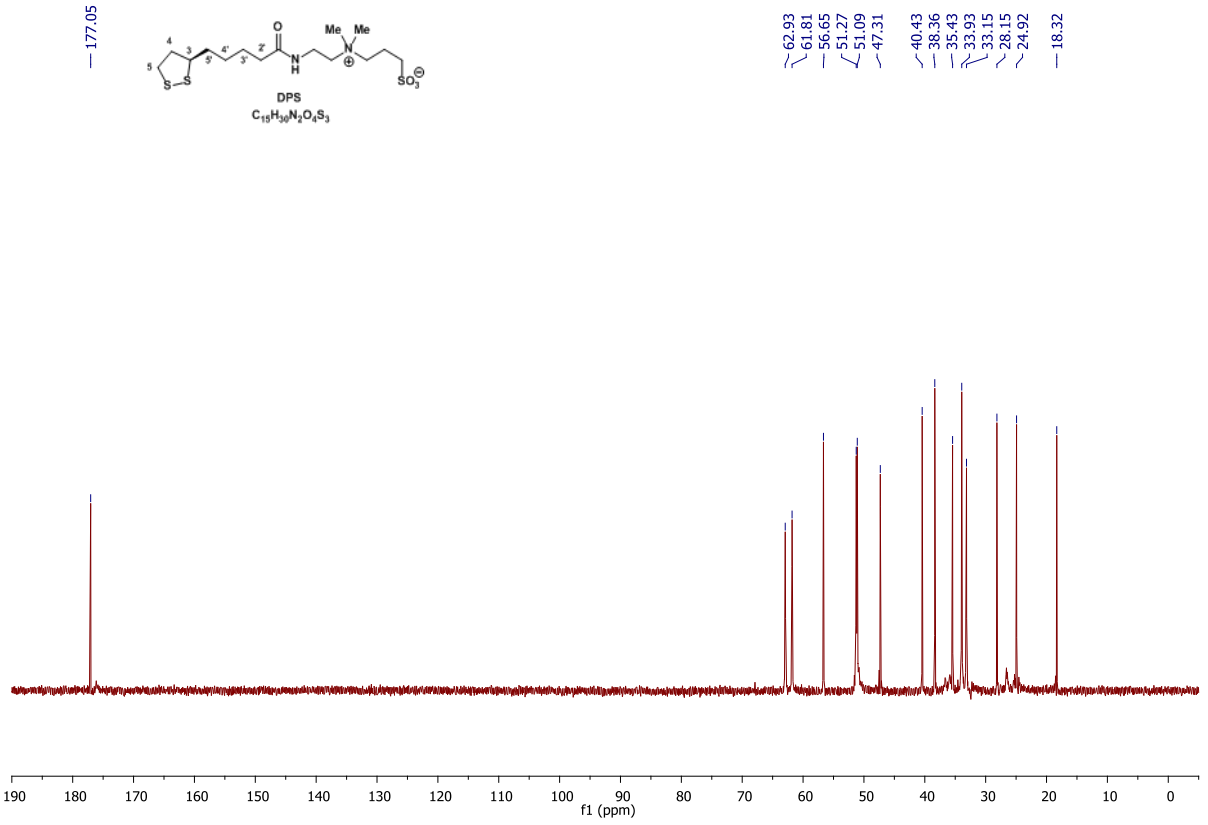
discarded. A residue was triturated with diethyl ether, acetone and the product was dried under a reduced pressure, to give DPS (see Scheme 1) as a white solid (1.10g, 70%). The product was characterised by IR and NMR with the following characteristics: **IR** (FTIR) 3460, 3296, 2978, 2868, 1655 (s, NCO), 1544, 1445, 1184, 1037, 730 cm^{-1}; **^{1}H NMR** (400 MHz, D_2O) δ 3.75 (m, 1H, NH-C*H*$_2$-), 3.65 – 3.46 (m, 6H, NH-C*H*$_2$- and H-3 and C*H*$_2$-N^+ and N^+-C*H*$_2$), 3.35 – 3.21 (m, 2H, H-5), 3.20 (s, 6H, N^+Me_2), 3.01 (t, *J* = 7.2 Hz, 2H, C*H*$_2$-SO_3^-), 2.53 (m, 1H, H-4), 2.44 – 2.18 (m, 4H, H-2' and CH_2-C*H*$_2$-$CH_2SO_3^-$), 2.03 (m, 1H, H-4), 1.83 – 1.56 (m, 4H, H-3'and H-5'), 1.54 – 1.37 (m, 2H, H-4') ppm; **^{13}C NMR** (100.6 MHz, D_2O) δ 177.05 (NCO), 62.93 (CH_2-*C*H_2-N^+), 61.81 (CH_2-*C*H_2-N^+), 56.65 (C-3), 51.27 (Me-N^+), 51.09 (Me-N^+), 47.31 (*C*H_2), 40.43 (*C*H_2, C-4), 38.36 (*C*H_2, C-5), 35.43 (*C*H_2), 33.93 (*C*H_2), 33.15 (*C*H_2), 28.15 (*C*H_2), 24.92 (*C*H_2), 18.32 (*C*H_2) ppm.



^{1}H NMR spectrum of *(R)*-*N*-(2-(dimethylamino)ethyl)-5-(1,2-dithiolan-3-yl)pentanamide.



^{1}H NMR spectrum of DPS.



^{13}C NMR spectrum of DPS.

Oxidation of the glycan moieties on glycoproteins

Glycan structures in INV were chemically oxidised using sodium periodate according to a protocol described earlier.[4] Briefly, 10 μM of stock solution of INV was oxidised by 10 mM sodium periodate in 50% acetonitrile solution for 2 h in the dark at ambient temperature. The reaction was stopped by an addition of ethyleneglycol to a final concentration of 15% (v/v) and incubated for an additional hour in the dark. Free aldehyde groups formed by glycan oxidation, verified by FTIR (data not shown), were blocked by incubation with 1 mM ethanolamine for 1 h in the dark and finally the oxidised INV (oxINV) was recovered with a desalting column.

Lectin microarray assays

For lectin microarray experiments, a printing buffer (11.3 mM NaH_2PO_4, 9 mM KOH, 137 mM NaCl, pH 7.4), a washing buffer (printing buffer containing 0.05% (v/v) Tween-20, pH 7.4), and a blocking and binding buffer (washing buffer + 1 M ethanolamine) were used. Five different concentrations of four different glycoproteins (INV, oxINV, FET and TRF) were spotted using SpotBot3 Microarray Protein edition (Arrayit, USA) on an epoxide coated slides Nexterion E (Schott, Germany) using a previously optimised protocol at temperature of 10 °C and humidity of 50 %.[5] The highest glycoprotein concentration investigated was 0.1 mg ml^{-1}. After glycoprotein printing, the slide was incubated with a blocking buffer in the humidity chamber for 1 h at a room temperature with humidity of 80 % with slow shaking. The slide was gently rinsed by a printing buffer in a Petri dish, drained to remove excess buffer and then, 70 μl of a 50 μg ml^{-1} biotinylated lectin solutions (Con A and SNA) in a binding buffer were dropped on the slide surface and incubated for 1 h. After incubation, the slide was incubated with the CF555-streptavidin solution (5 μg ml^{-1} in a binding buffer) for 15 min. After washing, the slide was scanned using InnoScan710 scanner (Innopsys, France) at the wavelength of 532 nm. The slide image was evaluated using the Mapix 5.5.0 software and the slide image was evaluated in Photo-PAINT X5 software (Corel, USA) by evaluation of the

intensity of green spots (484 pixels) with a black colour having a value of 0 and a green one having a value of 255. Intensity of eight independent array spots was evaluated for every glycoprotein analysed.

Quartz crystal microbalance (QCM) measurements

All QCM measurements were performed with an Autolab PGSTAT 128N (Ecochemie, Netherlands) equipment using an optional EQCM module. The changes in mass were evaluated using 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 0.0815 Hz ng^{-1} cm^2. The measurements were monitored and evaluated using the Nova 1.9 software and all measurements were run at room temperature.

X-ray photoelectron spectroscopy (XPS) measurements

The XPS signals on square shaped gold chips (Litcon, Sweden) of 12x12x0.3 mm modified as previously described[6] for SAM formation on 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.

Fourier-transform infrared (FTIR) spectroscopy

Fourier-transform infra red (FTIR) spectra were measured with Nicolet 6700 (Thermo Fisher Scientific, USA) spectrometer equipped with DTGS detector and Omnic 8.0 software. The spectra were collected in the middle region from 4,000 to 400 cm^{-1} at a resolution of 4 cm^{-1}, the number of scans was 128. Diamond Smart Orbit ATR accessory was applied for measurement in a solid state.

Atomic force microscopy (AFM)

Peak force tapping mode atomic force microscopy (Scan Asyst, Bruker, USA) in air was carried out on a Bioscope Catalyst instrument and Olympus IX71 microscope in conjunction with NanoScope 8.15 software at a scan rate of 0.5 line s^{-1} with the tip set of 200 pN. Square shaped gold chips (12x12x0.3 mm) were modified as previously described for the planar gold electrodes and scanned using SCANASYST-AIR silicon tip on nitride lever (Bruker, USA, with f_0=50-90 kHz and k=0.4 N m^{-1}), sharpened for a tip radius of 2 nm.

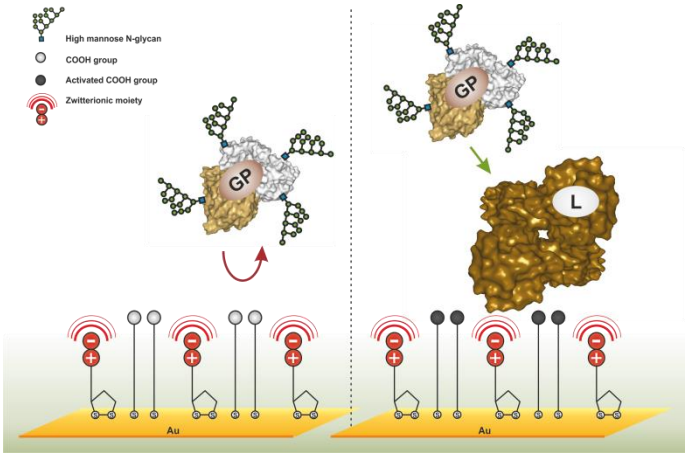


Fig. S1.: Schematic representation of a mixed self-assembled monolayer formed on gold surface from 11-mercaptoundecanoic acid with sulfobetaine with antifouling properties (left) and subsequent activation of –COOH group and immobilisation of a lectin for preparation of the lectin biosensor (right); L –Con A lectin with an affinity to mannose residues, GP – glycoprotein.

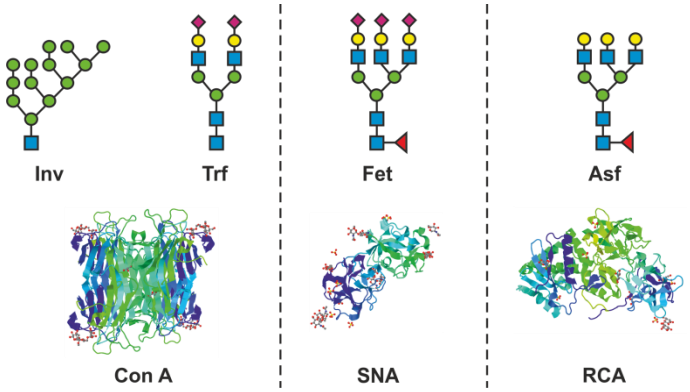


Fig. S2: Graphical representation of typical glycan determinants on glycoproteins (Inv: invertase, Trf: transferrin, Fet:fetuin, Asf-asialofetuin) applied in the study with a structure of three lectins (Con A: concanavalin A, SNA: *Sambucus nigra* agglutinin, RCA: *Ricinus communis* agglutinin) utilised for the biosensor construction.

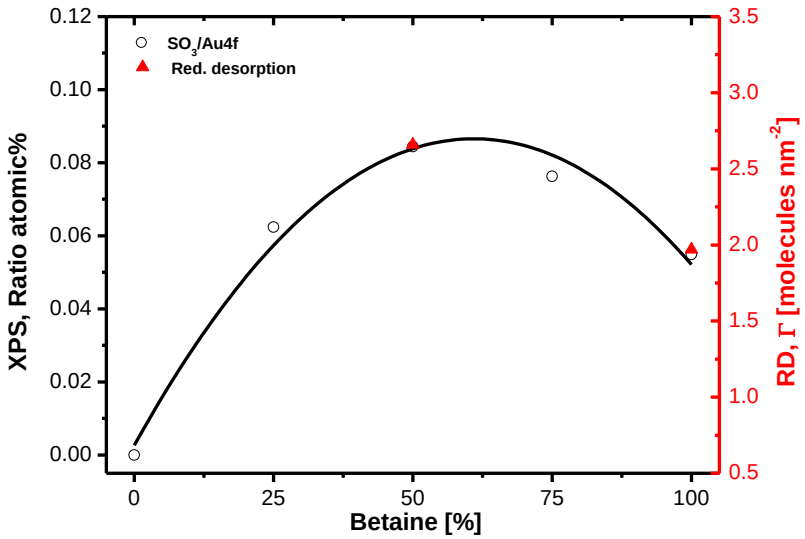


Fig. S3.: Composition of SAM layers on the gold surface prepared from different mixtures of sulfobetaine derivative and 11-mercaptoundecanoic acid as determined by XPS in the form of atomic% for SO_3^{2-} group and the absolute surface coverage for sulfobetaine derivative obtained from a reductive desorption (RD) experiment.

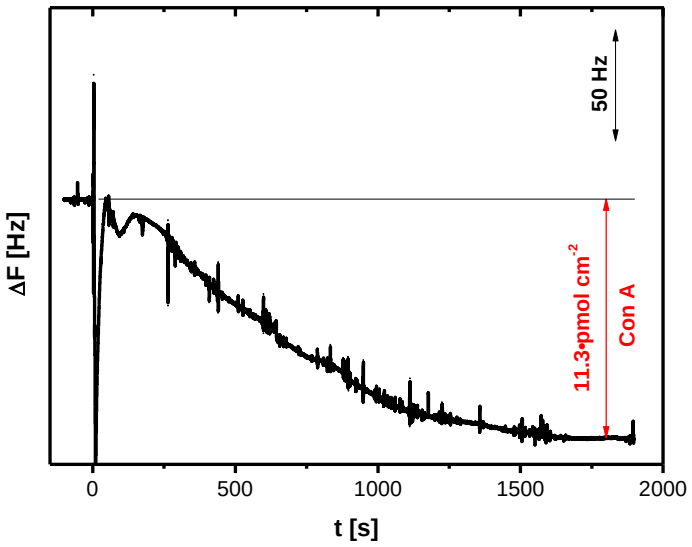


Fig. S4.: Quartz crystal microbalance experiment showing immobilisation of Con A lectin injected at time t=0 s on a SAM layer deposited from a 1+1 mixture of sulfobetaine and 11-mercaptoundecanoic acid.

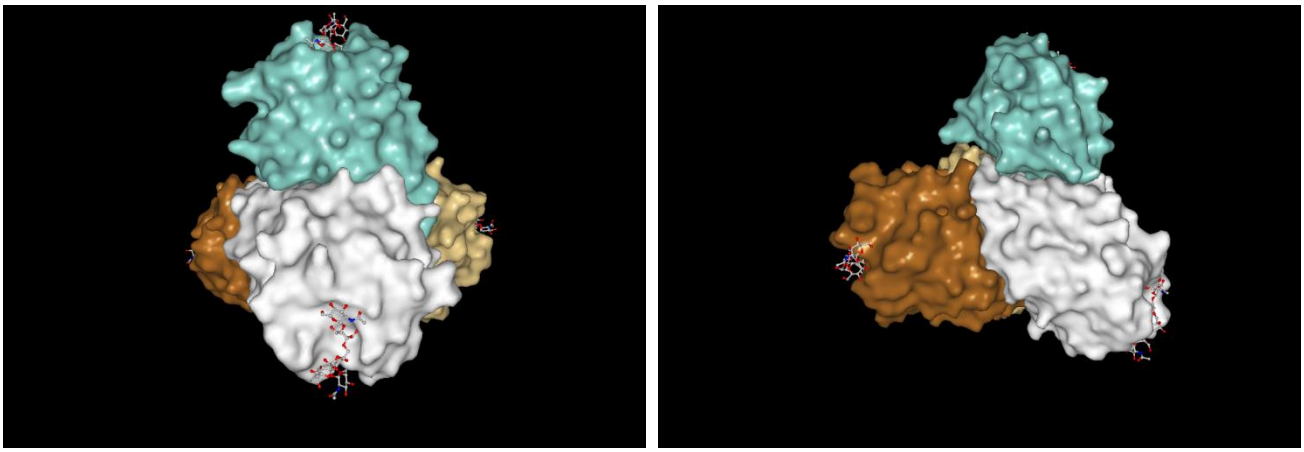


Fig. S5.: Structure of Con A lectin (PDB file 1TEI) showing composition from four identical subunits, which are represented in different colours.

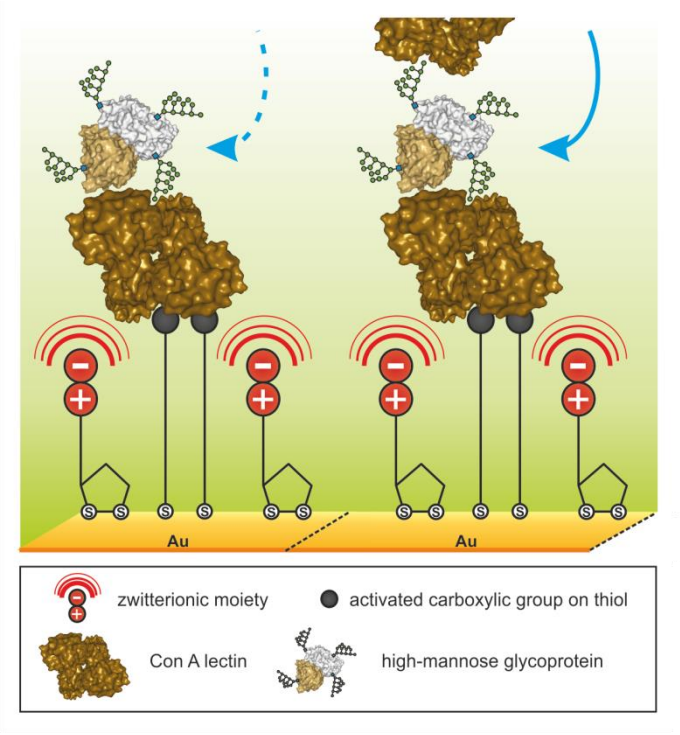


Fig. S6.: Schematic representation of the Con A biosensor after biorecognition took place (left) and the sandwich configuration of the Con A biosensor with additional outer layer of Con A lectin (right).

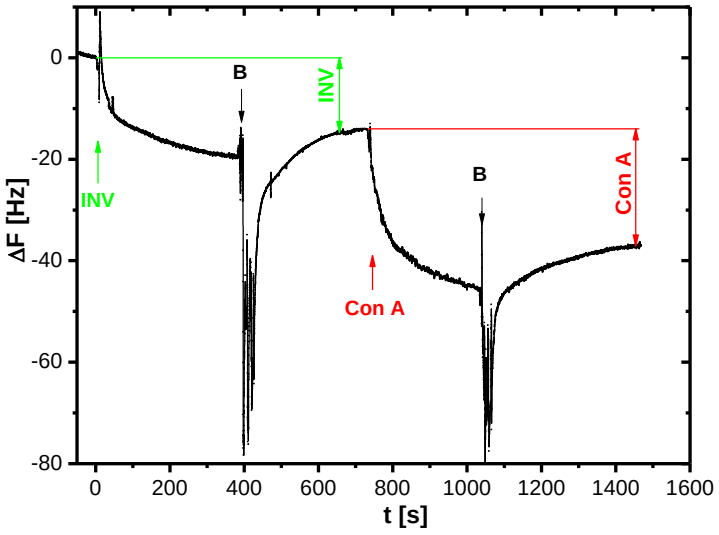


Fig. S7.: Quartz crystal microbalance assay showing that the Con A biosensor after interaction with its analyte invertase (INV) is able to interact with Con A lectin forming additional outer lectin layer. B – injection of plain buffer.

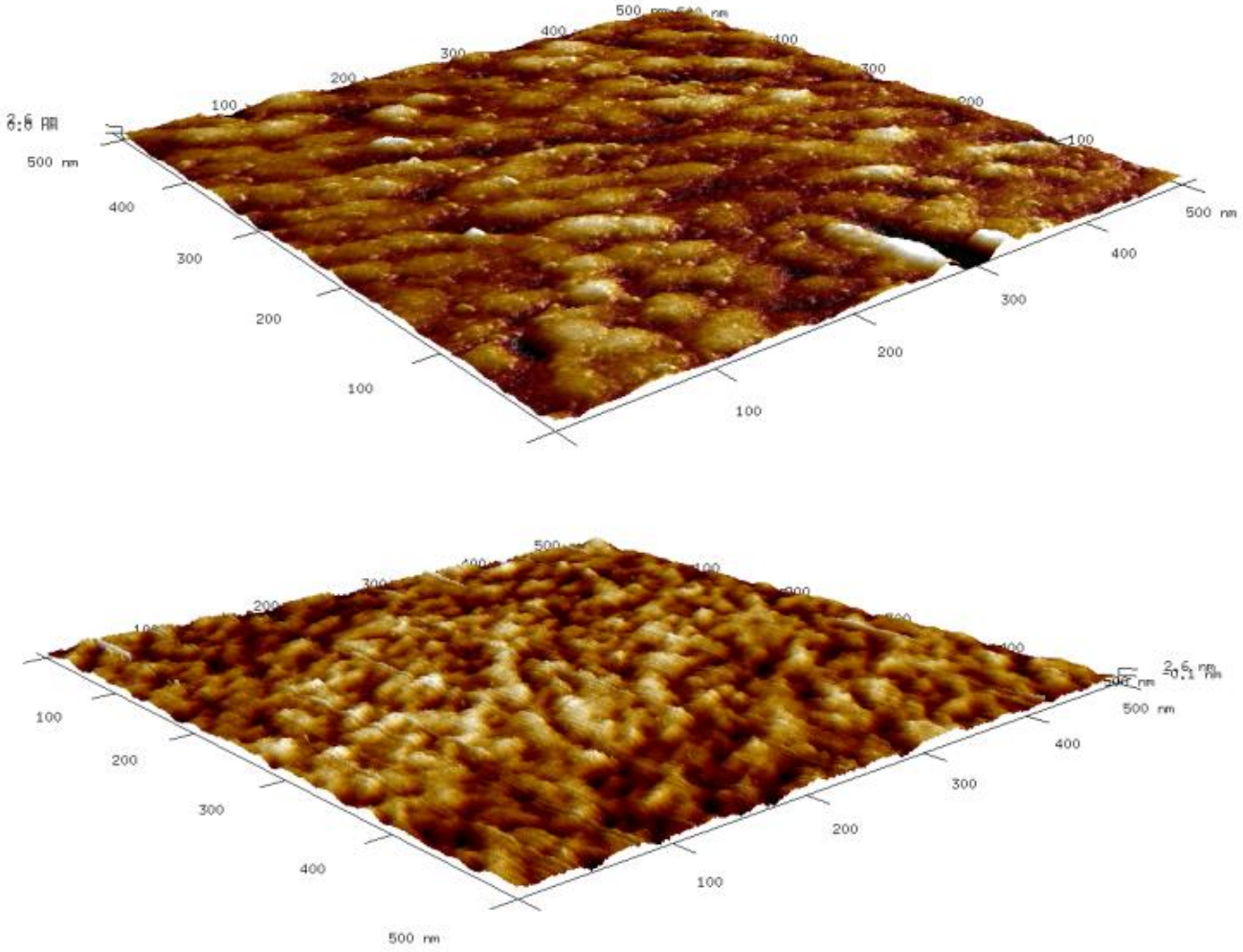
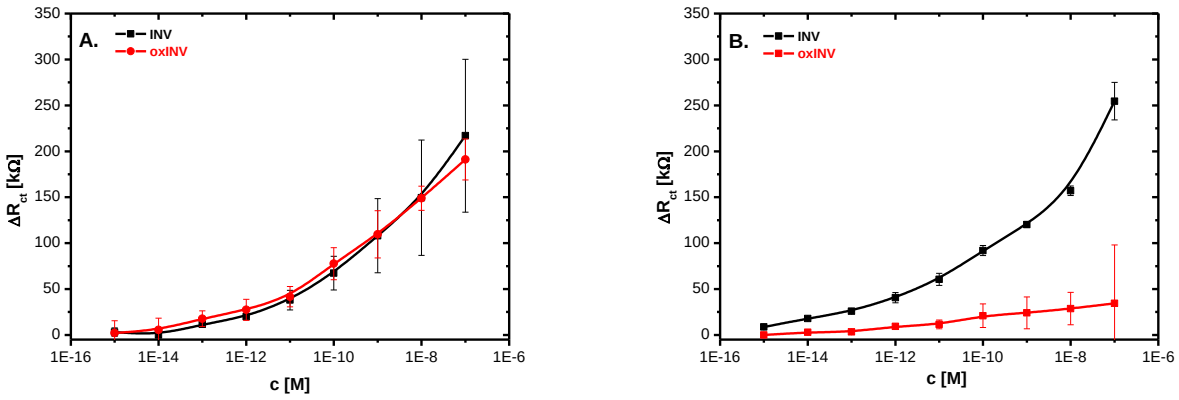


Fig. S8.: AFM images of bare gold surface (upper image) with R_q=0.8 nm and gold surface modified by SAM (image at the bottom) with R_q=1.0 nm. Both images have the same z-axis.



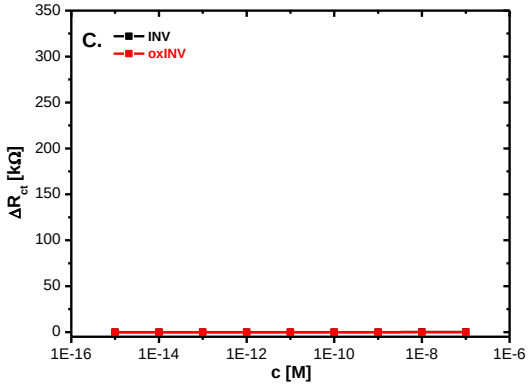


Fig. S9.: Calibration curves for the Con A biosensor built on SAM layer deposited from a mixture 11-mercaptoundecanoic acid:sulfobetaine of A) 3:1, B) 1:3 and C) 0:1 i.e. a pure sulfobetaine SAM layer. Each analyte was measured at least in triplicate with an independent biosensor device and results are shown with a standard deviation (± SD) calculated in Excel.

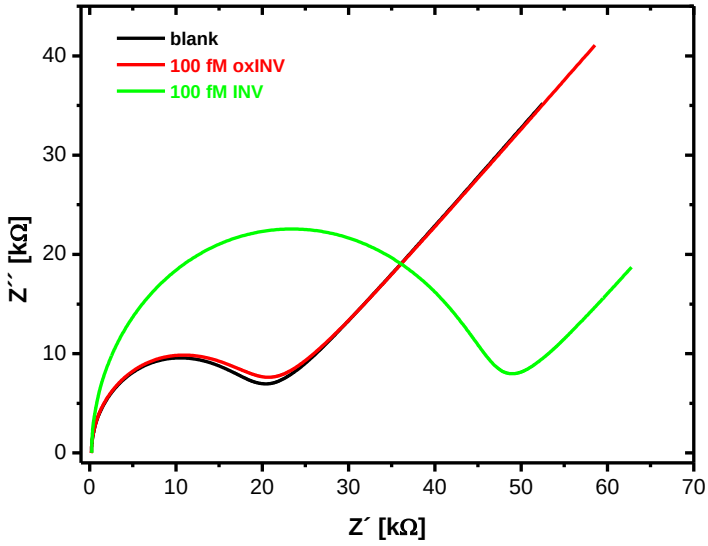


Fig. S10.: A typical Nyquist plot of the Con A biosensor incubated with its analyte invertase (INV) and oxidised invertase (oxINV) applied as a control. Moreover, the response of the Con A biosensor in a plain PBS buffer (blank) is shown, as well.

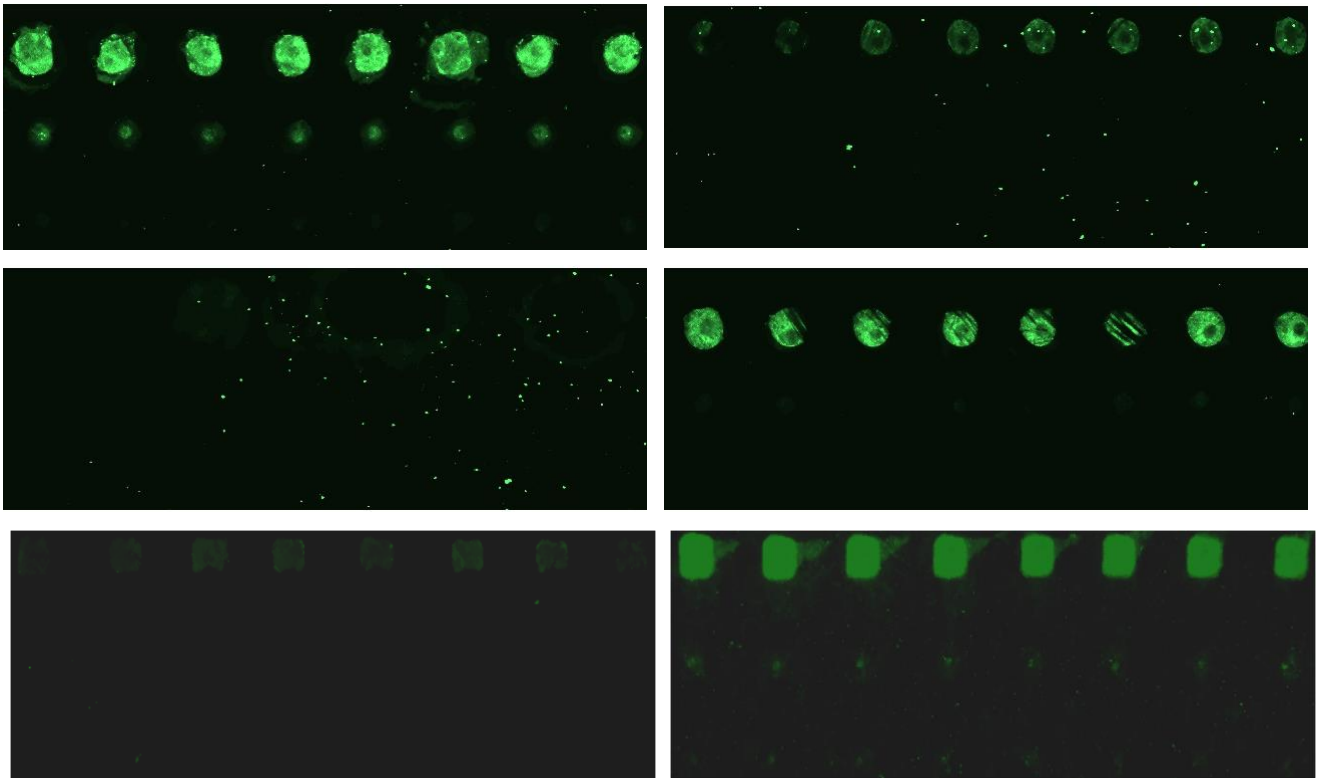


Fig. S11.: Application of the lectin microarray in analysis of various glycoproteins printed at three different concentrations (in every image upper row – 0.1 mg ml^{-1}, middle row – 0.01 mg ml^{-1} and lower row – 0.001 mg ml^{-1}). Upper left image shows interaction of Con A with invertase, upper right image Con A interaction with transferrin, middle left image Con A with oxidised invertase, middle right image interaction of SNA lectin with fetuin, lower left image RCA with fetuin and lower right image RCA with asialofetuin.

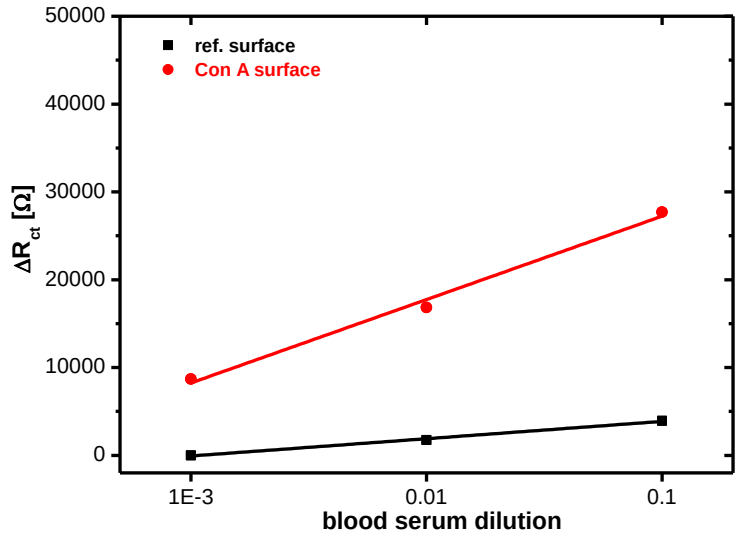


Fig. S12.: Optimisation of a dilution of blood serum in order to get sensitive glycoprofiling by the biosensor, while minimising non-specific interactions.

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