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Mixed Zwitterion-Based Self-Assembled Monolayer Interface for Impedimetric Glycomic Analyses of Human IgG Samples in an Array Format

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Abstract

An impedimetric lectin biosensor for the detection of changes in the glycan structure of antibodies isolated from human serum is here correlated with the progression of rheumatoid arthritis (RA). The biosensor was built up from a mixed self-assembled monolayer (SAM) on gold consisting of two different thiolated zwitterionic derivatives, carboxybetaine and sulfobetaine, to resist nonspecific interactions. The carboxyl-terminated one was applied also for the covalent immobilization of lectin *Ricinus communis* agglutinin I (RCA-I). The process of building a bioreceptive layer was optimized and characterized using a diverse range of techniques. Impedimetric assays were integrated on a chip consisting of eight gold working electrodes, which is an important step toward the achievement of a moderate level of multiplexing for the analysis of human serum samples. At the end, the results obtained by the impedimetric analysis of immunoglobulins G (IgGs) isolated from serum samples were compared with those of two other standard bioanalytical methods employing lectins, that is, lectin microarrays (MAs) and enzyme-linked lectin binding assays (ELLBAs). The impedimetric results agreed very well with the DAS28 index (RA disease activity score 28), suggesting that impedimetric assays could be used for the development of a new diagnostic procedure sensitive to glycosylation changes in human IgGs and thus RA progression.

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Notes

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1 Introduction

Glycosylation is the most common co- and posttranslational modification of cytosolic and membrane-bound human proteins, providing huge glycoproteome diversity.^{1,2} Glycans (complex carbohydrates) play an important role in viral infection, cancer development, cell signaling/adhesion, and cell–cell interactions. They also increase the solubility and stability (thermal and proteolytic) of glycoproteins and regulate their function.^{3–6} A single molecule of sialic acid in an N-linked complex glycan may change the nature of human immunoglobulin G (IgG, the most common type of serum antibody) from being an antiinflammatory agent to becoming a proinflammatory agent.^{7,8} Despite these facts, the very first therapeutic antibody with a precisely engineered glycan part was introduced only in 2012, although about 70% of all therapeutic proteins on the market are glycosylated.⁹

Changes in the glycan part of selected glycoproteins can often indicate a progressing disease, and changed glycosylation of a particular protein can be employed as a disease biomarker.^{10–12} A glycan–lectin binding approach can thus be used in the early diagnostics of various diseases, including cancer,^{13–15} congenital disorders,¹⁶ or autoimmune diseases (immunoglobulins with a changed glycan structure, targeting their own tissues)^{17,18} or even whole influenza viral particles and free agglutinins.^{19,20}

For “glycocode” deciphering, different labeled lectins (glycan-recognizing proteins) are often applied as an effective tool in many clinical diagnostic procedures, mostly in a microarray (MA) format of analysis, offering an extremely high throughput, simplicity, and high reproducibility of analysis with low sample consumption.^{21–23} The main advantage of using lectins in the search for new biomarkers in complex samples is the fact that it is not needed to know the biomarker’s structure in advance, as in the case of bioanalytical protocols using highly specific antibodies.²⁴

Faradaic electrochemical impedance spectroscopy (EIS) provides a powerful tool for the evaluation of surface characteristics (solution and charge transfer resistance, capacitance, and diffusion characteristics) based on the application of an alternate voltage with a small amplitude at different frequencies. It also allows us to detect various analytes down to the aM range^{25,26} and even whole cells^{27,28} in a label-free format of analysis. Most recently, some new variations of this method were presented, namely, impedance-derived electrochemical capacitance spectroscopy for the lectin-glycoprotein binding affinity evaluation²⁹ and immittance electroanalysis.³⁰ For glycoprofiling of various analytes, it is of high importance to analyze a biomarker using a panel of different lectins to clearly distinguish between different glycan structures or to involve negative controls. An array of differently modified disposable electrodes would be thus an ideal platform for such an analysis for point-of-care diagnostics.^{31,32}

In our previous studies, we aimed to prepare different electrochemical biointerfaces for a simple and sensitive detection of different glycans present on the IgG molecules isolated from human sera of healthy individuals and patients with an autoimmune disease, as well.^{17,18,33} For this purpose, different thiolated betaine derivatives were synthesized for the formation of the self-assembled monolayer (SAM) with sufficient antifouling properties for

the real samples analysis because the main challenge for these interfaces is proper blocking of nonspecific interactions.³⁴ Such molecules provide a high resistance to any nonspecific binding and high stability of the prepared surfaces.^{35,36} This effect is caused by the strong binding of water molecules around the SAM, as in the case of commonly used oligoethylene glycol (OEG) moieties.²⁰ The mercaptoundecanoic acid/sulfobetaine mixed SAM used for the human sera analysis provided a highly resistive layer; however, different solubilities of both substances (mercaptoundecanoic acid soluble in ethanol and sulfobetaine soluble in water) led to a limited solubility of both thiols in a mixture of ethanol and water after mixing with a poor reproducibility in the preparation of such an interface as a result.¹⁷ A SAM composed of a single component (a carboxybetaine derivative), offered a dual role, that is, resistivity toward nonspecific interactions and availability of a functional group ($-\text{COOH}$) used for lectin immobilization, could be prepared with an increased patterning reproducibility, but low control over the immobilization protocol was an issue with limited resistivity toward nonspecific interactions after $-\text{COOH}$ group activation and subsequent lectin immobilization.¹⁸

Here, we wanted to address both problematic issues by designing and preparing a mixed SAM composed of two different betaine derivatives, both soluble in water, a carboxybetaine derivative (5-((11-mercaptoundecyl)-dimethylammonio)pentanoate for covalent lectin immobilization) and a sulfobetaine derivative ((R)-3-((2-(5-(1,2-dithiolan-3-yl)-pentanamido)ethyl)dimethylammonio)propane-1-sulfonate, SB) employed as diluting thiols, providing resistivity toward nonspecific interactions for the first time. Both thiols are soluble in the same solvent (water); thus, an enhanced surface patterning reproducibility to design interfaces with resistivity toward nonspecific interactions should be achieved. The other beneficial feature of the application of a mixed SAM is a better control over immobilization of lectin while preserving the resistivity of the SAM toward nonspecific interactions by a sulfobetaine derivative. Poly- and oligozwitterionic molecules are not an optimal choice for the modification of an impedimetric biosensor surface because such surfaces exhibit a high initial R_{CT} and very often a Nyquist plot is very difficult to fit and interpret properly. Thus, even though these molecules are becoming more widely used, for EIS-based biosensors, it is reasonable to apply one zwitterionic unit per molecule.

Moreover, on the basis of a previous study,³⁷ in collaboration with an industrial partner (BVT Technologies, Inc., Czech Republic), we managed to develop an impedimetric lectin-based eight-electrode array to enhance assay throughput, to shorten electrode cleaning steps, and to increase the reproducibility of the surface preparation compared with commercially available and standardly used gold disk electrodes. A moderately high assay throughput by the impedimetric analysis in an array format allowed us to measure significantly more samples than previously described.^{17,18} This array was subsequently used for successful electrochemical glycoprofiling of human IgGs isolated from human sera, confirming the presence of desialylated and degalactosylated (i.e., G0 glycan without galactose or G1 glycan with one galactose within conserved N-glycans on Asn-297 of the Fc fragment of the IgG) IgGs during rheumatoid arthritis (RA) progression.³⁸ A degalactosylation process makes a GlcNAc residue in the N-glycan more accessible to a biorecognition by a mannosebinding protein (MBP), activating a lectin complement activation pathway and thus initiating the inflammation process.³⁹ In this study, besides the control group, we divided

the RA patients into two different groups, seronegative and seropositive individuals (according to the presence or absence of two different RA biomarkers in serum, namely rheumatoid factor antibodies and antibodies against cyclic citrullinated peptides), to distinguish possible effects of these biomarkers on a complex glycoprofile of isolated IgGs.

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2 Materials and Methods

2.1 Chemicals

Potassium hexacyanoferrate(III), potassium hexacyanoferrate(II) trihydrate, phosphate buffer saline (PBS) tablets, KH_2PO_4 , and K_2HPO_4 (1 M solutions for the preparation of phosphate buffer (PB), pH 7.4), sulfuric acid, sodium hydroxide, o-phenylenediamine (OPD), N-hydroxysuccinimide (NHS), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), *Ricinus communis* agglutinin (RCA-recognizing β -D-galactose, caution: handle with a special care because it is a biological toxin), fetuin (contains 8.7% of sialic acid), asialofetuin (contains 0.5% of sialic acid), and human serum albumin (HSA) were purchased from Sigma Aldrich (USA). Biotinylated lectins (*Griffonia simplicifolia* II, *Lotus tetragonolobus*, *Lycopersicon esculentum*, *R. communis* I, and *Sambucus nigra* agglutinin), a streptavidin–peroxidase (STR–HRP) conjugate, and a carbo-free blocking solution for lectin MA experiments were purchased from Vector Laboratories (USA). A CF647-streptavidin fluorescent label was purchased from Biotium (USA). All solutions were prepared using 0.055 μS ultrapure deionized water (DW) and were subsequently filtered before use using 0.2 μm sterile filters. A sulfobetaine derivative ((R)-3-((2-(5-(1,2-dithiolan-3-yl)-pentanamido)ethyl)dimethylammonio)propane-1-sulfonate, SB was synthesized according to a published protocol,¹⁷ and a CB derivate (5-((11-mercaptoundecyl)dimethylammonio)pentanoate was synthesized according to a protocol, and the scheme has been described in the Supporting Information together with ¹H NMR and FTIR spectra. The chemical structures of CB and SB are depicted in Scheme 1.

2.2 Human Serum Samples

From a pool of 100 samples of human sera available, 28 samples were included in this study (10 healthy individuals with mean age = 49.0 ± 9.7 , 7 seronegative patients with mean age = 59.0 ± 13.6 , and 11 seropositive RA patients with mean age = 56.0 ± 8.8). Samples excluded from this study were samples from individuals suffering from other (inflammatory) diseases, which could negatively affect the correlation between IgG glycosylation and RA progression. All RA patients met the 2010 ACR-EULAR classification criteria for RA.⁴¹ The RA patients were recruited from the National Institute of Rheumatic Diseases in Piestany, Slovak Republic. Control subjects were recruited from the Institute of Experimental Endocrinology, Slovak Academy of Sciences, Bratislava, Slovak Republic. All subjects gave their informed written consent, and 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 samples were collected into polyethylene tubes with a clotting activator (S-Monovette, Sarstedt AG & Co., Germany). After centrifugation, the serum aliquots were stored at -80°C until they were subsequently used for IgG isolation. For IgG purification, a

MelonGel IgG Spin Purification Kit (Thermo Scientific, U.S.) was used, offering results comparable to those using protein A or G. After the isolation procedure, IgG concentration was measured using UV spectrophotometry at 280 nm; all samples were diluted to the same protein concentration and were stored at -80°C until they were analyzed. C-reactive proteins (CRPs) were measured using an ELISA kit (IBL Hamburg, Germany). Disease activity score 28 (DAS28) was calculated from a number of swollen (SJC) and tender joint counts (TJC) and CRP concentration using the formula

$$DAS28 = \left[0.56 \sqrt{TJC28} + 0.28 \sqrt{SJC28} + 0.36 \ln(CRP+1) * 1.10 + 1.15 \right] \quad (\text{eqn. 1})$$

2.3 Enzyme-Linked Lectin Binding Assay (ELLBA) Measurements

MaxiSorp plates (obtained from Nunc A/S, Roskilde, Denmark) were incubated with a solution of an analyte in PBS ($87.5 \mu\text{g}\cdot\text{mL}^{-1}$) overnight. All following washing and incubation steps were performed with PBST (PBS with the addition of 0.05% Tween 20) buffer. The plate was emptied and washed 3 times with 200 μL of a washing buffer. To emptied and washed wells, a solution of biotinylated *Ricinus communis* agglutinin I (RCA-I) ($0.1 \mu\text{g}\cdot\text{mL}^{-1}$) was added and incubated for 1 h and afterward washed out. Then, STR-HRP ($0.33 \mu\text{g}\cdot\text{mL}^{-1}$) was added into each well and incubated for 1 h. After washing, STR-HRP bound to lectins was determined with substrate OPD dissolved in a citrate-phosphate buffer pH 4.6 in the presence of hydrogen peroxide (incubation for 15 min in the dark). After the addition of 3.6 M H_2SO_4 , the absorbance at 490 nm was measured using a microplate reader Synergy HT (BioTek, USA). The total volume of liquids present in wells was 100 μL , and each sample was measured in triplicate.

2.4 Lectin MA Measurements

Lectin MA experiments were performed with PBS as a printing buffer, PBST as a washing buffer, and PBST containing a $10\times$ diluted carbo-free blocking solution as a blocking buffer. Shortly, at least three different concentrations of diluted IgG samples ($87.5 \mu\text{g}\cdot\text{mL}^{-1}$) were spotted using SpotBot3 Microarray Protein edition (Arrayit, USA) on epoxide-coated slides Nexterion E (Schott, Germany) using a previously optimized protocol. The spotting temperature was set to 10°C and humidity to 60%. Subsequently, the slide was placed in a humidity chamber for 1 h at room temperature with a humidity of 80–90%, blocked using a blocking buffer at room temperature for 1 h and with slow shaking, rinsed under a gentle stream of a printing buffer in a Petri dish, and drained. Then, 70 μL of $25 \mu\text{g}\cdot\text{mL}^{-1}$ biotinylated lectin in a binding buffer was applied to the slide surface and incubated for 1 h. After lectin incubation, the slide was incubated with the Biotium CF647-streptavidin solution ($1 \mu\text{g}\cdot\text{mL}^{-1}$ in a printing buffer) for 15 min. After a washing procedure, the slide was scanned using an InnoScan710 scanner (Innopsys, France) at a wavelength of 635 nm. The slide image was evaluated using the Mapix 5.5.0 by the evaluation of the intensity of fluorescence and intensity of all independent array spots on the array (normalized to the background).

2.5 Electrode Pretreatment and SAM Formation

Gold electrodes on an array chip (BVT Technologies, Czech Republic) were treated by electrochemical reductive desorption and electrochemical polishing and stripping procedures according to a slightly modified, previously described protocol.⁴² In the first step, any bound thiol molecules were desorbed from the surface using cyclic voltammetry (CV) in 10 mM NaOH under anaerobic conditions (10 scans, run under a N₂ atmosphere from -500 to -1500 mV at a scan rate of $100 \text{ mV}\cdot\text{s}^{-1}$). After that, CV was performed once again in 10 mM H₂SO₄ (5 scans, run from -200 to $+1500$ mV at a scan rate of $100 \text{ mV}\cdot\text{s}^{-1}$) for electrochemical polishing and gold oxide stripping (10 scans, run from $+750$ to $+200$ mV at a scan rate of $100 \text{ mV}\cdot\text{s}^{-1}$), respectively. All steps were performed using an Electrochemistry Kit and PalmSens3 potentiostat/galvanostat (BVT Technologies, Czech Republic; PalmSens BV, The Netherlands) in a three-electrode cell system, using auxiliary Pt and reference Ag/AgCl (3 M KCl) electrodes (BASi, USA). Immediately after a gold oxide stripping procedure was completed, the electrodes were washed with DW and left to dry in a dustless environment and subsequently used for SAM formation from a 1 mM thiol stock solution prepared freshly before use.

The real gold electrode surface area was calculated from the 25th scan of CV assays performed in 10 mM H₂SO₄ at a sweeping potential from -0.2 to $+1.5$ V at a sweep rate of $100 \text{ mV}\cdot\text{s}^{-1}$ by the integration of a peak attributed to the reduction of the Au/ α gold oxide layer as described previously.⁴² Moreover, reductive desorption to strip the SAM from the gold electrode by application of a negative voltage could be applied to calculate the density of thiols present on the electrode surface¹⁴ by performing CV at cathodic potentials (-0.5 to -1.5 V) in 10 mM deaerated NaOH (at least 15 min of nitrogen purging) at a sweep rate of $10 \text{ mV}\cdot\text{s}^{-1}$, as described previously.⁴²

2.6 EIS Measurements

On a chip/electrode modified with a mixed SAM by a simple passive incubation at room temperature overnight, the biorecognition element (previously selected RCA-I lectin from 5 different lectins based on ELLBA and protein MA experiments) was immobilized using a standard amine coupling with carboxylic groups of carboxybetaine derivative CB, being activated by a 1 + 1 mixture of 0.2 M EDC and 0.05 M NHS for 15 min (the SB derivative remained intact). Lectin was covalently immobilized on the activated SAM by 30 min of incubation in a 2 μL stock solution ($0.1 \text{ mg}\cdot\text{mL}^{-1}$ in PB). All EIS measurements were performed in a filtered electrolyte containing 5 mM potassium hexacyanoferrate(III), 5 mM potassium hexacyanoferrate(II), and 0.01 M PB. The analysis was run at a potential of $+200$ mV at 50 different frequencies (ranging from 0.05 Hz to 50 kHz) using PSTrace 4.7.1 software (PalmSens BV, The Netherlands). Data acquired were evaluated with the EIS Spectrum Analyzer software using a Nyquist plot with an R(Q[RW]) equivalent circuit employed. Change in charge transfer resistance (R_{CT}) relative to a reference surface (biosensor surface after lectin immobilization and stabilization in a sterile 10 mM PB solution for at least 10 min) expressed in % was used as a signal from measurements. Each analyte/sample was measured at least in triplicate with an independent biosensor device, and results are shown with a standard deviation ($\pm\text{SD}$) calculated using Excel. Human samples were diluted in a sterile and filtered 10 mM PB, pH 7.4, to prevent any significant ionic

strength changes during the experiment. All stock solutions (lectins, standard glycoproteins, and human samples) were freshly prepared before analysis.

2.7 Atomic Force Microscopy (AFM) Measurements

Peak force tapping mode AFM (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 using ScanAsyst in air mode. Square-shaped gold chips (Arrandee, Germany) and BVT Au chips (BVT Technologies, Czech Republic) were modified as previously described and scanned using a SCANASYST-AIR silicon tip on a nitride lever (Bruker, USA, with $f_0 = 50\text{--}90\text{ kHz}$ and $k = 0.4\text{ N}\cdot\text{m}^{-1}$) sharpened to a tip radius of 2 nm.

2.8 Quartz Crystal Microbalance (QCM) Measurements

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

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

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 ($\text{g}\cdot\text{cm}^{-2}$), μ_q is the shear modulus of quartz ($\text{g}\cdot\text{cm}^{-1}\cdot\text{s}^{-2}$), A is the surface area and ρ_q is the density of quartz in $\text{g}\cdot\text{mL}^{-1}$. For a 6 MHz quartz crystal covered by Au, the whole equation can be simplified to

$$\Delta f = - C_f \Delta m, \quad (\text{eqn. 3})$$

where C_f is the frequency constant, $0.0815\text{ Hz}\cdot\text{ng}^{-1}\cdot\text{cm}^2$. The measurements were monitored and evaluated using the Nova 1.10 software, and all measurements were carried out at room temperature.

2.9 Synthesis of CB

11-Bromo-1-undecene, ethanol, NaOH, N,N-dimethyl amine solution, ethyl bromovalerate, NaHCO_3 , diethyl ether, methanol, azobisisobutyronitrile (AIBN), Amberlite IRA 400, thioacetic acid, and acetone were purchased from Aldrich and used as received. Ultrapure water was obtained from a Millipore system. ^1H NMR spectra were recorded on a Varian 400 MR spectrometer instrument at 298 K. Chemical shifts are reported in ppm downfield to the solvent standard. The solvent was used as a reference. The working frequency was 400 MHz. Coupling patterns were designated as s-singlet, d-doublet, t-triplet, q-quartet, and m-multiplet. The concentration of samples was around 10 mg of a sample dissolved in 0.7 mL of the solvent.

ATR-FTIR data were recorded using an FTIR 760 (Nicolet) infrared spectrophotometer with 32 scans. The spectra were characterized in wavenumbers.

The synthesis of 5-((11-mercaptoundecyl)dimethylammonio)-pentanoate, CB, was performed in four steps according to Scheme S1. The reaction of 11-bromo-1-undecene with dimethylamine resulted in a tertiary amine. The quaternization of the tertiary amine group with ethyl bromoacetate yielded a carboxybetaine ester. The addition reaction onto the terminal double bond was carried out with thioacetic acid with a catalytic amount of AIBN under UV irradiation and inert conditions, and the final product CB was obtained after deprotection under strict inert conditions.

3 Results and Discussion

3.1 SAM Optimization to Resist Nonspecific Interactions

It is of the highest interest to prepare highly resistive interfaces, allowing covalent binding of a biorecognition element when we want to prepare affinity-based biosensors. For this purpose, a sulfobetaine derivative (SB)¹⁷ and a carboxybetaine derivative (CB) were synthesized (see Supporting Information describing the synthesis and characterization of a newly synthesized CB) and subsequently used for SAM preparation. Mixed SAM formation from two zwitterionic betaine derivatives applied for the construction of EIS-based biosensors is an elegant method, allowing us to tune initial charge transfer resistance of the surface with an ability to resist nonspecific protein binding. In our case, both derivatives prevent nonspecific protein interaction due to zwitterionic character, but they differ in negatively charged groups. SB is sulfobetaine bearing a terminal sulfate group, and CB is carboxybetaine with a terminal carboxylate group. CB is applied for formation of a carboxylic ester via NHS/EDC coupling chemistry with carboxylate functional group for immobilization of sensing lectin.⁴³ Moreover, SB molecules with a shorter length serve also as a diluent for CB. The CB derivative has to have a length (i.e., to be longer than that of SB) that allows the -COOH group of CB to be available for subsequent activation and covalent lectin immobilization.⁴⁴ This is why a distance with four methylene units between positive and negative groups within a CB molecule was chosen. In a previous study,¹⁸ a carboxybetaine derivative with one methylene distance between charged moieties, which is shorter than that of SB, was applied. This derivative was not employed herein because it is anticipated that the densely packed mixed SAM would result in difficult access and less reactivity of carboxylate to the reagent and lectin during the subsequent immobilization step.

Both derivatives prevent nonspecific protein adsorption, with CB applied for the immobilization of lectin via NHS/EDC amine coupling chemistry. Moreover, the length of CB molecules was designed in a way to achieve a high immobilization yield.⁴³ The ratio of both molecules in the solution from which the SAM was formed on the gold surface was optimized to prepare an interface, resisting nonspecific protein binding investigated using HSA, (as a nonspecific binding probe) and EIS (Figure 1).

As can be seen from the 3D graph (Figure 1), the SAM with the lowest nonspecific interactions was the one with a CB + SB ratio of 1 + 3 (i.e., containing 25% of CB within a CB/SB mixture). This SAM was used for all further experiments.

Moreover, the effect of the coincubation of both thiols on the SAM density was investigated using a reductive desorption procedure, employing CV, as described previously.^{17,42} The effect was similar as that in our previous study, where we suggested more dense layers in the case of mixed SAMs compared with SAMs consisting of only one thiol (Figure S3). The main reason is most likely the effect of lowering the electrostatic repulsion between two negatively charged head groups of thiols when two different thiols (with different chain lengths and an additional permanently positively charged functional group) are present. Such effects have been described previously.^{45,46} The density of thiols in the case of the SAM formed from a solution having a CB + SB ratio of 1 + 3 was 1.14 molecules·nm⁻², whereas the most dense SAM was formed from the solution composed of CB + SB having a 3 + 1 ratio (1.87 molecules·nm⁻²), in agreement with our previous study.¹⁷

The nonspecific adsorption of HSA on a mixed SAM could be quite high, and a significant nonspecific HSA binding was observed on single-component SAMs composed of SB or CB, as well. This behavior is not in agreement with previous studies.^{36,47–50} There might be several reasons why the SAM described herein is not that effective in preventing nonspecific protein binding compared with previous studies: thiol coverage is too low (1.87 molecules·nm⁻² compared with 5.5–5.7 molecules·nm⁻² for densely packed single-component SAMs composed of chemisorbed aliphatic thiols⁴²), nonoptimal composition of thiol solutions applied for gold modification because the thiol solvent and thiol concentration can significantly affect resistivity toward nonspecific interactions,⁴⁹ different sensing platforms for the investigation of nonspecific protein binding using EIS in this study compared with ellipsometry^{51,52} or SPR^{36,47,50,53} applied in previous studies, and four carbons between charged groups within CB, making it more hydrophobic compared with CB with a shorter separation between charged groups within CB.⁴³ Despite all this, a proper selection of the composition of a mixed SAM and concentration of proteins in the sample analyzed could provide reasonably selective analysis of IgGs isolated from human serum.

3.2 QCM and AFM Characterization of Prepared Surfaces

To control a surface morphology, we performed AFM experiments with two Au surfaces modified by the same protocol (as described above). BVT Au array chips exhibited higher surface roughness (Figure S4a) compared with square gold chips (Arrandee, Germany) prepared by chemical vapor deposition with clearly defined Au grains on the surface. These structures are rough on a micrometer scale with a morphology at the nanoscale smooth enough to prepare a well-defined SAM for RCA-I immobilization (Figure S4b). This was confirmed by comparing both surfaces after RCA-I immobilization as shown in Figure S5, indicating very similar dimensions of isolated protein islets found on the surface. Any difference between the two structures is very likely caused by a different orientation of the protein on the biosensor surface because the orientation during the immobilization process is random.

QCM experiments were performed using Metrohm EQCM Au chips for the evaluation of RCA-I surface density after the amine coupling procedure. For the mixed SAM (CB + SB = 1 + 3 ratio), optimized before and used in our next experiments, the change in the mass on the surface (after the association and dissociation phases) after lectin immobilization was

calculated to be $196 \text{ ng}\cdot\text{cm}^{-2}$, corresponding to $1.64 \text{ pmol}\cdot\text{cm}^{-2}$. A theoretical surface coverage for this lectin, when a hard sphere model⁵⁴ was taken into account, was calculated to be $4.32 \text{ pmol}\cdot\text{cm}^{-2}$, suggesting that the lectin layer forms 38% of a full monolayer.

3.3 EIS Measurements

All the EIS measurements were performed for the evaluation of the surface R_{CT} , calibration of the biosensor, and real sample measurements. The sample consumption in all cases was 2 μL (incubation for 20 min). For biosensor calibration, three glycoprotein standards were used, namely, fetuin from a bovine serum (8.7% of sialic acid), asialofetuin (less than 0.5% of sialic acid, a desialylated form of fetuin), and IgGs from a human serum. We chose a change in the charge transfer resistance as a signal (R_{CT} in $\% \Omega$). For the analysis of real samples, an important step was to determine a proper dilution. This parameter was optimized by the subtraction of the biosensor signal obtained by the incubation of the HSA (a nonspecific binding probe) from the signal obtained by the incubation with IgGs in a series of measurements with different (glyco)protein concentrations employed. A concentration where the sigmoidal curve plateau occurs (the highest signal difference and highest possible dilution to eliminate the nonspecific interactions on the surface) was chosen (Figure S6), in this case a 1000 $\times$ dilution, which is in agreement with our previous studies.^{17,18} Biosensor calibration was used for three glycoprotein standards (and five different concentrations ranging from 0.00001 to 0.1 $\text{mg}\cdot\text{mL}^{-1}$) to evaluate the sensor sensitivity toward galactose-containing glycans. The results (Figure 2) suggested that the RCA-I lectin on the surface maintains its activity and ability to distinguish between different glycoforms. The highest signal was obtained for asialofetuin with a sensitivity of $(53.7 \pm 2.8) \text{ } \% \Omega \cdot \text{decade}^{-1}$ with the galactose moieties exposed, a lower signal with a sensitivity of $(28.1 \pm 1.7) \text{ } \% \Omega \cdot \text{decade}^{-1}$ was obtained for human IgGs, and a signal sensitivity of $(24.1 \pm 4.1) \text{ } \% \Omega \cdot \text{decade}^{-1}$ was read for fetuin with sialic acid as a terminal saccharide, covering the galactose residues in N-glycans (Figure 2).

3.4 Lectin Screening Using ELLBA

Initial experiments with IgGs isolated from selected human serum samples were performed with ELLBA and lectin MAs offering high throughput and low sample consumption to select a lectin showing the highest difference for the analyzed three different sample groups. Five different lectins showing affinity to some part of complex-type N-glycans were chosen for the preliminary study, namely *Griffonia simplicifolia* II (α - or β -GlcNAc specific), *L. tetragonolobus* (α -Fuc specific), *L. esculentum* (GlcNAc2–4 specific), *R. communis* I (β -Gal specific), and *S. nigra* agglutinin (Neu5Ac α 2,6Gal/GalNAc specific). RCA-I lectin was chosen for further experiments because the other lectins showed very little to almost none difference between the groups observed (data not shown). This is in a good correlation with the previously published data, suggesting the loss of sialic acid and even galactose during the RA disease progression. For the data evaluation, box plots in OriginPro software were chosen as a statistical evaluation method (Figure 3A) because in a heterogeneous group, the average and median values are often quite distant and the average value could be a less accurate parameter compared with the median. Using quantiles, the observed sets of individuals were divided into equally sized groups by so-called percentiles. Percentiles in Figure 3A are shown for 1 and 99% (-), 25% (first quartile), 50% (median), and 75% (third

quartile) of the output value. Minimum and maximum values (●), as well as an average value (■), are also shown. From Figure 3B, it can be clearly seen that the median values are different for RA patients compared with those of healthy individuals. Moreover, healthy individuals create a more compact group with low signal variability.

3.5 Lectin Screening Using Protein MAs

As for ELLBA experiments, the same configuration of analysis was investigated (the sample adsorbed on a surface with subsequent incubation with a biotinylated lectin). The results shown in Figure 3C indicate an increase of an output signal with the progression of the RA disease when median values are considered.

3.6 EIS-Based Biosensor Analysis

These preliminary EIS results suggest that the biosensor device could be reliably used to discriminate the different IgG glycoforms in real human samples during RA progression. The samples were prepared according to the above-described protocol and diluted in 0.01 M PB pH 7.4 as previously optimized. The measurements were performed the same way as the other EIS measurements on Au BVT array chips to increase the throughput and also to prepare a biorecognition surface in a more reproducible way compared with standardly used disk electrodes. The reproducibility (calculated as RSD) of the preparation of the RCA-I-modified surface (with a mixed betaine SAM) in the case of BVT Au array chips was calculated to be 19%, which is a better result as opposed to 27% for widely used Au disk electrodes ($d = 1.6$ mm, BASi, USA). The results obtained by the analysis of IgGs isolated from human serum samples are shown in Figure 3A with real distinction in results obtained from healthy individuals and RA patients.

3.7 Comparison of the Results Obtained by All Three Analytical Methods

The analytical performance of all three methods was evaluated recently,³³ showing that the most sensitive is the EIS method (working concentration range of 10 aM–1 pM), followed by the ELLBA method (390 fM–12 pM) and MA (10 nM–1 mM). The main two drawbacks of EIS compared with ELLA and MA were identified, the reproducibility of the EIS biosensor construction and low throughput of analysis. This was addressed in this study using an array of gold electrodes. Surprisingly, the results obtained by ELLBA and MA do not seem to correlate well with those of the impedimetric biosensor ($R^2 = 0.401$ and 0.552 , respectively) when observing the median values. Joshi's group suggested that lectin-based analyses may very often be format-dependent with different results obtained using different assay configurations.⁵⁵ In the case of our ELLBA and MA experiments, the sample was immobilized on a surface and subsequently incubated with lectin and a label. The primary interaction of an IgG sample with lectin on a biosensor surface was in a reverse configuration, possibly affecting the analysis results. The other possible explanation may be that MA and ELLBA are simply less sensitive compared with impedimetric analyses.

The most interesting result with a possible diagnostic potential is a perfect correlation of results obtained by EIS with a DAS28 index, showing a correlation coefficient of 0.990 (Figure 3D). This means that the EIS-based method could be used for the development of a

new diagnostic procedure sensitive to glycosylation changes in human IgGs and thus RA progression.

4 Conclusions

The study was focused on the development of a biointerface composed of a mixed SAM formed by the incubation of gold electrodes with two zwitterionic thiolated derivatives with sulfo- and carboxy-terminal functional groups. The difference in pK_a between these two derivatives facilitates the distribution and selective binding of lectin to the carboxylate derivative, whereas both derivatives possess a highly resistive interface toward nonspecific protein binding. The lectin biosensor in an array format using a chip with eight gold electrodes was then employed for the impedimetric detection of glycans present on the IgG molecules isolated from the human sera of healthy individuals and patients with RA. The impedimetric results agreed very well with the DAS28 index, which is a measure of actual disease activity based on TJC, SJC, and CRP concentrations, suggesting that impedimetric assays could be used for the development of a new diagnostic procedure sensitive to glycosylation changes in human IgGs and thus RA progression. The DAS28 is a combined index that has been developed to measure the disease activity in patients with RA. The score is also a strong predictor of physical disability and radiological progression, and the index is a sensitive discriminator between patients with high and low disease activities.

Supporting Information

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Investigation of a non-specific interaction of human serum albumin (HSA) in a wide concentration window with an interface modified by a mixed SAM layer composed of carboxybetaine and sulfobetaine using EIS. Measurements were performed in 10 mM PB buffer containing 5 mM potassium hexacyanoferrate(III) and 5 mM potassium hexacyanoferrate(II). The analysis was run at a potential of +200 mV at 50 different frequencies (ranging from 0.05 Hz to 50 kHz) at room temperature. CB = carboxybetaine derivative, HSA = human serum albumin, NSI = non-specific interactions expressed as a relative R_{CT} change.

Figure 2.

Calibration curve for three glycoprotein standards (fetuin – orange, asialofetuin – green and IgG from human serum – yellow), measured using impedimetric array. For other assay conditions see Fig. 1. All measurements were performed at least in triplicates.

Figure 3.

Box plots obtained from (A) ELLBA experiment, (B) MA experiment and (C) EIS experiment using RCA-I (β -Gal specific lectin), showing differences for three groups observed in this study. Green box – healthy individuals (10 samples), orange box – seronegative RA patients (7 samples), red box – seropositive RA patients (11 samples). Individual percentiles (1%, 25%, 50% - median, 75% and 99%) denoted $\blacksquare$, minimum and maximum value denoted $\bullet$, and an average values is indicated as $\blacksquare$. (D) Comparison of median values obtained by analysis of IgG isolated from three distinct groups of serum samples with $R^2=0.990$ (green – 10 samples from healthy individuals, orange – 7 samples from seronegative RA patients, red – 11 samples from seropositive RA patients) using EIS with DAS28 index.

Scheme 1.

Chemical structure of carboxybetaine (CB, upper) and sulfobetaine (SB, lower).