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Langmuir, **Just Accepted Manuscript** • DOI: 10.1021/acs.langmuir.6b01456 • Publication Date (Web): 16 Jun 2016

ACS Publications

Langmuir is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

Mixed zwitterionic-based self-assembled monolayer interface for impedimetric glycomic analyses of human IgG samples in an array format

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KEYWORDS

Array electrodes, arthritis, betaine, immunoglobulin, impedimetric biosensor, self-assembled monolayer, lectin

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ABSTRACT

Impedimetric lectin biosensor for detection of changes in the glycan structure of antibodies isolated from human serum is here correlated with 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 non-specific interactions. Carboxyl terminated one was applied also for covalent immobilization of lectin *Ricinus communis* agglutinin I (RCA-I). The process of building of a bioreceptive layer was optimized and characterized using a diverse range of techniques. Impedimetric assays were integrated on a chip consisting of 8 gold working electrodes, what is an important step towards achievement of a moderate level of multiplexing for analysis of human serum samples. At the end, the results obtained by impedimetric analysis of IgGs isolated from serum samples were compared to two other standard bioanalytical methods employing lectins i.e. lectin microarrays (MA) and enzyme-linked lectin assays (ELLBA). The impedimetric results agreed very well with DAS28 index (RA disease activity score 28), suggesting impedimetric assays could be used for development of a new diagnostic procedure sensitive to glycosylation changes on human IgG and thus RA progression.

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, as well. They also increase solubility and stability (thermal and proteolytic) of glycoproteins and regulate their function.[3, 4, 5, 6] A single molecule of sialic acid in N-linked complex glycan may change the nature of human immunoglobulin G (IgG, the most common type of serum antibodies) from being an anti-inflammatory to become a pro-inflammatory agent.[7, 8] Despite these facts, the very first therapeutic antibody with precisely engineered glycan part was introduced only in 2012, although about 70% of all therapeutic proteins on 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, 11, 12]. Glycan-lectin binding approach can thus be used in early diagnostics of various diseases, including cancer biomarkers[13, 14, 15], congenital disorders[16] or autoimmune diseases (immunoglobulins with changed glycan structure, targeting their own tissues)[17, 18] or even whole influenza viral particles and free agglutinins[19, 20].

For the "glycocode" deciphering different labelled lectins (glycan recognizing proteins) are often applied as an effective tool in many clinical diagnostic procedures, mostly in a microarray format of analysis, offering an extremely high throughput, simplicity and high reproducibility of analysis with a low sample consumption.[21, 22, 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 biomarker structure in advance, as in 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 to detect various analytes down to 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 evaluation[29], 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 self-assembled monolayer (SAM) with sufficient anti-fouling properties for the real samples analysis, since the main challenge for these interfaces is proper blocking of non-specific interactions.[34] Such molecules provide a high resistance to any non-specific binding and high stability of the prepared surfaces.[35, 36] This effect is caused by a strong binding of water molecules around the SAM layer, as in 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 solubility 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 preparation of such an interface as a result.[17] A SAM layer composed of a single component (a carboxybetaine derivative), offered a dual role i.e. resistivity towards non-specific interactions and availability of a functional group (-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 towards non-specific interaction after –COOH group activation and subsequent lectin immobilization.[18]

Here we wanted to address both problematic issues by designing and preparing a mixed SAM layer 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 a diluting thiol and providing resistivity towards non-specific interactions for the first time. Both thiols are soluble in the same solvent (water), thus an enhancing surface patterning reproducibility to design interfaces with resistivity towards non-specific interactions should be achieved. The other beneficial feature of application of a mixed SAM is a better control over immobilization of lectin, while preserving resistivity of the SAM layer towards non-specific interaction by a sulfobetaine derivative. Poly- and oligo-zwitterionic molecules are not an optimal choice for the modification of an impedimetric biosensor surface because they produce 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.

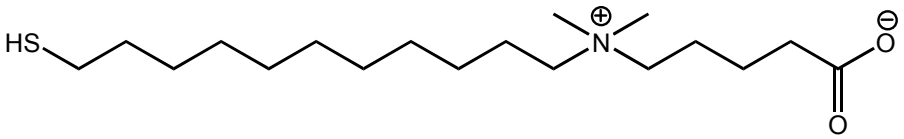
Moreover, based on a previous study[37], in collaboration with an industrial partner (BVT Technologies, Inc., Czech Republic), we managed to develop an impedimetric lectin-based eight-electrode array in order to enhance assays throughput, to shorten electrode cleaning steps and to increase reproducibility of the surface preparation compared to commercially available and standardly used gold disc electrodes. Moderately high assays throughput by the impedimetric analysis in an array format allowed us to measure significantly more samples then previously described.[17, 18] This array was subsequently used for successful electrochemical glycoprofiling of human IgG isolated from human sera, confirming 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 Fc fragment of IgG) IgGs during rheumatoid arthritis (RA) progression.[38] A degalactosylation process made a GlcNAc residue in the N-glycan more accessible to a biorecognition by a mannose-binding 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 a presence or absence of two different RA biomarkers in serum, namely rheumatoid factor antibodies and antibodies against cyclic citrullinated peptides), in order to distinguish possible effect of these biomarkers on a complex glycoprofile of isolated IgGs.[40]

2. MATERIALS AND METHODS

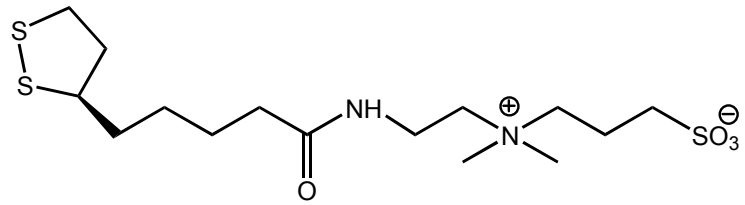
2.1. Chemicals

Potassium hexacyanoferrate(III), potassium hexacyanoferrate(II) trihydrate, phosphate buffer saline (PBS) tablets, KH_2PO_4 and K_2HPO_4 (1 M solutions for preparation of phosphate buffer

(PB), pH 7.4), sulphuric 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 since it is a biological toxin*), fetuin (contains 8.7% of sialic acid), asialofetuin (contains ≤0.5% of sialic acid) and human serum albumin were purchased from Sigma Aldrich (USA). Biotinylated lectins (*Griffonia simplicifolia II, Lotus tetragonolobus*, *Lycopersicon esculentum*, *Ricinus communis I* and *Sambucus nigra* agglutinin), streptavidin-peroxidase (STR-HRP) conjugate and a carbo-free blocking solution for lectin microarray experiments were purchased from Vector Laboratories (USA). A CF647-streptavidin fluorescent label was purchased from Biotium (USA). All solutions were prepared in 0.055 μS ultrapure deionized water (DW) and were subsequently filtered prior to 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 published protocol[17] and a CB derivate (5-((11-mercaptoundecyl)dimethylammonio)pentanoate was synthesized according to a protocol and scheme described in the supporting information file together with ^{1}H NMR and FTIR spectra. Chemical structure of CB and SB is depicted in **Scheme 1**.



CB



SB

Scheme 1. Chemical structure of carboxybetaine (CB, upper) and sulfobetaine (SB, lower).

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, what could negatively affect correlation between the 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 the centrifugation, the serum aliquots were stored at -80 °C until they were subsequently used for the IgG isolation. For the IgG purification, the 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 a same protein concentration and were stored at -80 °C until they were analyzed. C-reactive protein (CRP) was measured using ELISA kit (IBL Hamburg, Germany). Disease activity score 28 (DAS28) was

calculated from number of swollen (SJC) and tender joint counts (TJC) and CRP concentration using formula:

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

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 $\mu g.ml^{-1}$) 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 RCA-I (0.1 $\mu g.ml^{-1}$) was added and incubated for 1 h and afterwards washed out. Then, STR-HRP (0.33 $\mu g.ml^{-1}$) 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 dark). After addition of 3.6 M H_2SO_4, the absorbance at 490 nm was measured using a microplate reader Synergy HT (BioTek). The total volume of liquids present in wells was 100 µl and each sample was measured in triplicate.

2.4. Lectin microarray (MA) measurements

Lectin microarray experiments were performed with PBS as a printing buffer, PBST as a washing buffer and PBST containing a 10x diluted carbo-free blocking solution as a blocking buffer. Shortly, at least three different concentrations of diluted IgG samples (87.5 $\mu g.ml^{-1}$) were spotted using SpotBot3 Microarray Protein edition (Arrayit, USA) on epoxide coated slides Nexterion E (Schott, Germany) using a previously optimized protocol. Spotting temperature was

set to 10 °C and humidity to 60%. Subsequently, the slide was placed in a humidity chamber for 1 h at the room temperature with 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 $\mu g.ml^{-1}$ 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 $\mu g.ml^{-1}$ 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 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 layer formation

Gold disk electrodes on an array chip (BVT Technologies, Czech Republic) were treated by an electrochemical reductive desorption and electrochemical polishing and stripping procedures according to a slightly modified, previously described protocol.[42] Shortly, 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 N_2 atmosphere from -500 mV to -1,500 mV at a scan rate of 100 $mV.s^{-1}$). After that, CV was performed once again in 10 mM H_2SO_4 (5 scans run from -200 mV to +1,500 mV at a scan rate of 100 $mV.s^{-1}$) for the electrochemical polishing and gold oxide stripping (10 scans, run from +750 mV to +200 mV at a scan rate of 100 $mV.s^{-1}$), respectively. All steps were performed using an Electrochemistry Kit and PalmSens3 potentiostat/galvanostat (BVT Technologies, Czech Republic; PalmSens BV, Netherlands) in a three electrode cell system, using auxiliary Pt and reference Ag/AgCl (3M

KCl) electrode (BASi, USA). Immediately after a gold oxide stripping procedure was completed, the electrodes were washed with DW, left to dry in a dustless environment and subsequently used for self-assembled layer (SAM) formation from a 1 mM thiol stock solution prepared freshly prior to use.

Real gold electrode surface area was calculated from 25th scan of cyclic voltammetry assays performed in 0.1 M H_2SO_4 by sweeping potential from -0.2 V to +1.5V at a sweep rate of 100 mV.s^{-1} by integration of a peak attributed to reduction of Au/α gold oxide layer as described previously[42]. Moreover a reductive desorption to strip SAM from the gold electrode by application of a negative voltage could be applied to calculate density of thiols present on the electrode surface[14] by running CV at cathodic potentials (-0.5 V to -1.5 V) in 0.1 M de-aerated NaOH (at least 15 min of nitrogen purging) at sweep rate of 10 mV.s^{-1}, as described previously.[42]

2.6. Electrochemical impedance spectroscopy (EIS) measurements

On a chip/electrode modified by a mixed SAM by a simple passive incubation at room temperature overnight, the biorecognition element (previously selected RCA-I lectin, based on ELLBA and protein microarray 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 (SB derivative remained intact). Lectin was covalently immobilized on the activated SAM layer by 30 min incubation from a 2 µl stock solution (0.1 mg.ml^{-1} in PB). All electrochemical impedance spectroscopy (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) under PSTrace 4.7.1 software

(PalmSens BV, Netherlands). Data acquired were evaluated by the EIS Spectrum Analyzer software using a Nyquist plot with R(Q[RW]) equivalent circuit employed. Change in a charge transfer resistance (R_{CT}) relative to a reference surface (biosensor surface after the 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 (±SD) calculated in Excel. Human samples were diluted in a sterile and filtered 10 mM PB, pH 7.4 in order to prevent any significant ionic strength changes during experiment. All stock solutions (lectins, standard glycoproteins, and human samples) were freshly prepared prior to analysis.

2.7. Atomic force microscopy (AFM) measurements

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 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 = 50-90 kHz and k = 0.4 N.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, Netherlands) equipment using an EQCM module. The changes of a 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 (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 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 a frequency constant 0.0815 Hz.ng^{-1}.cm^2. The measurements were monitored and evaluated using the Nova 1.10 software and all measurements were run 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, acetone, were purchased from Aldrich as used as received. Ultrapure water was obtained from Millipore system.

^{1}H NMR spectra were recorded on a Varian 400 MR spectrometer instrument at 298 K. Chemical shifts are reported in ppm downfield to solvent standard. The solvent was used as a reference. Working frequency was 400 MHz. Coupling patterns were designated as s − singlet, d − doublet, t − triplet, q− quartet, m − multiplet. Concentration of samples was around 10 mg of sample dissolved in 0.7 mL of solvent.

ATR-FTIR data were recorded 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 tertiary amine. Quarterlization of tertiary amine group with ethyl bromoacetate yielded in carboxybetaine ester. 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 condition.

3. Results and discussion

3.1. SAM optimization to resist non-specific 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 file describing synthesis and characterization of a newly synthesized CB) and subsequently used for SAM preparation. A mixed SAM formation from two zwitterionic betaine derivatives applied for construction of EIS-based biosensors is an elegant method allowing to tune initial charge transfer resistance of the surface with ability to resist non-specific 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. The large difference in pKa of negative groups[43] allows selective distribution and formation of carboxylic ester via NHS/EDC chemistry to carboxylate derivate for linkage with sensing lectin. Moreover, SB molecules with a shorter length serve also as diluent for CB. CB derivate has to have a length (i.e. to be longer than SB) allowing –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 group within CB molecule was chosen. In a previous study[18], a

carboxybetaine derivate with one methylene distance between charged moieties, which is shorter than SB was applied. This derivate was not employed herein since it is anticipated that dense packed mixed SAM would result in difficult access and less reactivity of carboxylate to reagent and lectin during subsequent immobilization step.

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

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

Moreover, the effect of the co-incubation of both thiols on the SAM layer density was investigated using a reductive desorption procedure employing a cyclic voltammetry, as described previously.[17, 42] The effect was similar as in our previous study, where we suggested more dense layers in case of mixed SAMs compared to SAMs consisting of only one thiol (**Fig. 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 case of the SAM layer formed from a solution having CB+SB ratio of 1+3 was 1.14 molecules.nm^{-2}, while the most dense SAM was

formed from the solution composed of CB+SB having 3+1 ratio (1.87 molecules.nm^{-2}), in an agreement with our previous study.[17]

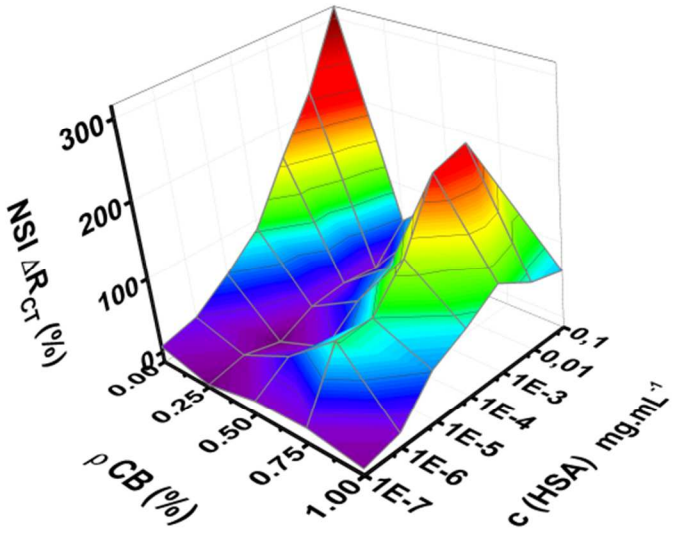


Figure 1. Investigation of a non-specific interaction of human serum albumin (HSA) in a wide concentration window with an interface modified by a mixed SAM layer composed of carboxybetaine and sulfobetaine using EIS. Measurements were performed in 10 mM PB buffer containing 5 mM potassium hexacyanoferrate(III) 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.

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 an agreement with previous studies.[36, 47, 48, 49, 50] There might be several reasons why SAM layer described herein is not that effective in preventing non-specific protein binding compared to previous studies: thiol coverage is too low (1.87 molecules.nm^{-2}

compared to 5.5-5.7 molecules.nm^{-2} for densely packed single component SAMs composed of chemisorbed aliphatic thiols[42]), non-optimal composition of thiol solutions applied for gold modification since thiol solvent and thiol concentration can significantly affect resistivity towards non-specific interactions[49]; different sensing platform for investigation of nonspecific protein binding using EIS in this study compared to 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 to CB with a shorter separation between charged groups within CB[43]. Despite all this, a proper selection of 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

In order 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 (**Fig. S4a**) compared to 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 nanoscale smooth enough to prepare a well defined SAM layer for RCA-I immobilization (**Fig. S4b**). This was confirmed by comparing both surfaces after RCA-I immobilization as shown in **Fig. S5**, indicating very similar dimension of isolated protein islets found on the surface. Any differences between the two structures are very likely caused by a different orientation of the protein on the biosensor surface, since the orientation during 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 phase) after lectin immobilization was calculated to be 196 $ng.cm^{-2}$, corresponding to 1.64 $pmol.cm^{-2}$. A theoretical surface coverage for this lectin, when a hard sphere model[54] is taking into account was calculated as 4.32 $pmol.cm^{-2}$, suggesting that the lectin layer forms 38% of a full monolayer.

3.3. EIS measurements

All the EIS measurements were performed for evaluation of the surface R_{CT}, calibration of the biosensor and real samples measurements. The sample consumption in all cases was 2 µl (incubation for 20 min). For the 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 IgG from a human serum. We chose a change in the charge transfer resistance as a signal (ΔR_{CT} in %Ω). For analysis of real samples, an important step was to determine a proper dilution. This parameter was optimized by subtraction of the biosensor signal obtained by incubation of the human serum albumin (HSA, a non-specific binding probe) from the signal obtained by incubation with IgG 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 non-specific interactions on the surface) was chosen (**Fig. S6**), in this case a 1,000x dilution, what is in agreement with our previous studies.[17, 18]

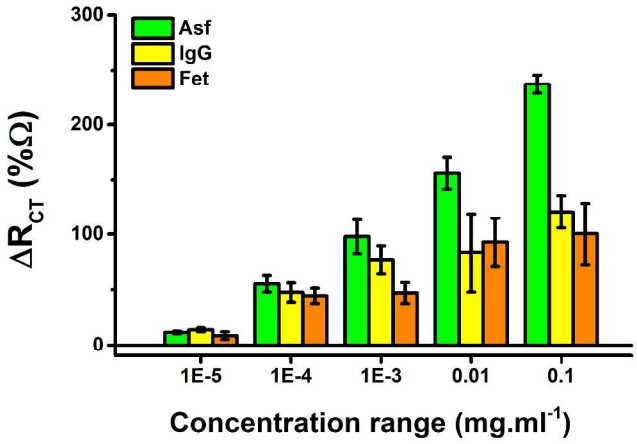


Figure 2. Calibration curve for three glycoprotein standards (fetuin – orange, asialofetuin – green and IgG from human serum – yellow), measured using impedimetric array. For other assay conditions see **Fig. 1**. All measurements were performed at least in triplicates.

Biosensor calibration was used for three glycoprotein standards (and five different concentrations ranging from 0.00001 to 0.1 mg.ml^{-1}) to evaluate the sensor sensitivity towards galactose-containing glycans. The results (**Fig. 2**) suggest 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 ± 2.8) %Ω.decade^{-1} with the galactose moieties exposed, lower signal with a sensitivity of (28.1 ± 1.7) %Ω.decade^{-1} was obtained for human IgG and a signal sensitivity of (24.1 ± 4.1) %Ω.decade^{-1} was read for fetuin with sialic acid as a terminal saccharide, covering the galactose residues in N-glycans (**Fig. 2**).

3.4. Lectin screening using ELLBA

Initial experiments with IgGs isolated from selected human serum samples were performed with ELLBA and lectin microarrays offering high throughput and low sample consumption, in order to select a lectin showing the highest difference for the analyzed three different sample groups. Five different lectins showing affinity to some part of a complex type N-glycans were chosen for the preliminary study, namely *Griffonia simplicifolia II* (α- or β-GlcNAc specific), *Lotus tetragonolobus* (α-Fuc specific), *Lycopersicon esculentum* ($GlcNAc_{2-4}$ specific), *Ricinus communis I* (β-Gal specific) and *Sambucus nigra* agglutinin (Neu5Acα6Gal/GalNAc specific). RCA-I lectin was chosen for further experiments, since 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 (**Fig. 3A**), since in a heterogeneous group the average and median values are often quite distant and the average value could be a less accurate parameter compared to median. Using quantiles, the observed sets of individuals were divided into equally sized groups by so-called percentiles. Percentiles in **Fig. 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 **Fig. 3A**, it can be clearly seen that the median values are different for RA patients compared to healthy individuals. Moreover, healthy individuals create a more compact group with low signal variability.

3.5. Lectin screening using protein microarray (MA)

As for ELLBA experiments, the same configuration of analysis was investigated (sample adsorbed on a surface with subsequent incubation with a biotinylated lectin). The results shown in **Fig. 3B** indicate increase of an output signal with progression of 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 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 to standardly used disc electrodes. The reproducibility (calculated as RSD) of the preparation of RCA-I modified surface (with a mixed betaine SAM) in the case of BVT Au array chips was calculated to be 19%, what is a better result as opposed to 27% for widely used Au disc electrodes (d = 1.6 mm, BASi, USA). The results obtained by the analysis of IgGs isolated from human serum samples are shown in **Fig. 3C** with real distinction in results obtained from healthy individuals and RA patients.

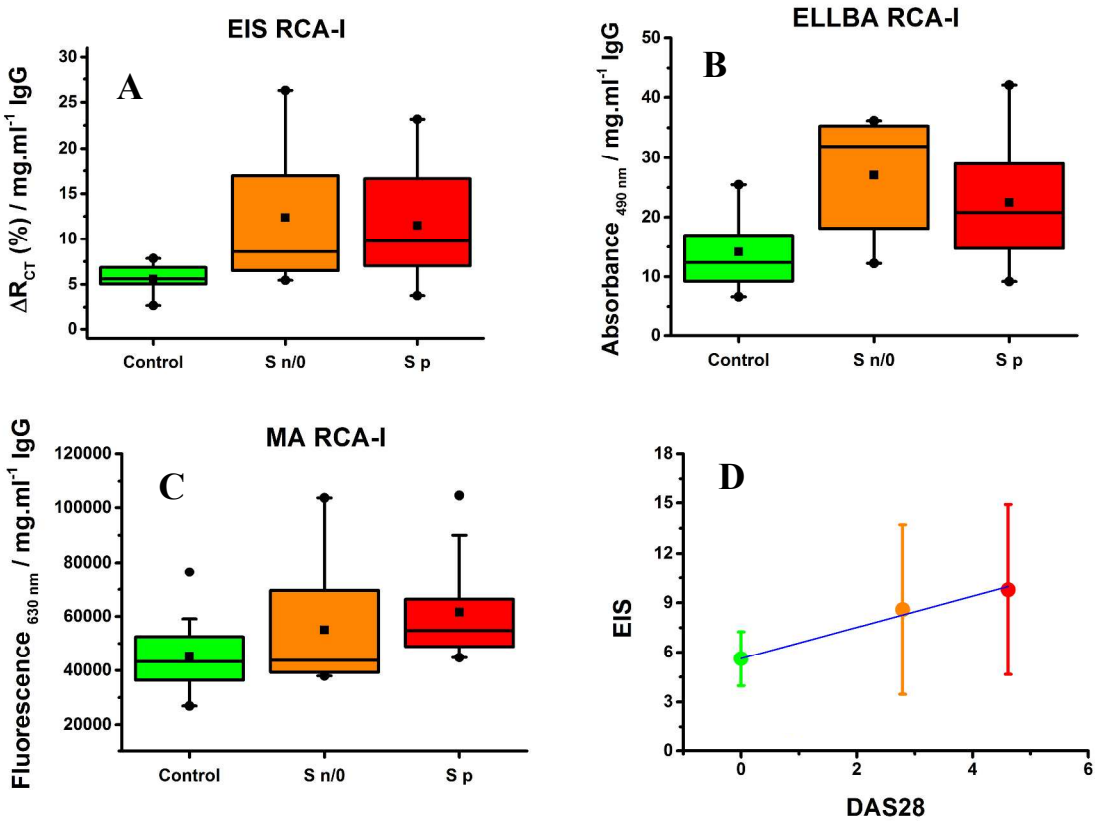


Figure 3. Box plots obtained from (**A**) ELLBA experiment, (**B**) MA experiment and (**C**) EIS experiment using RCA-I (β-Gal specific lectin), showing differences for three groups observed in this study. Green box – healthy individuals (10 samples), orange box – seronegative RA patients (7 samples), red box – seropositive RA patients (11 samples). Individual percentiles (1%, 25%, 50% - median, 75% and 99%) denoted ▬, minimum and maximum value denoted ●, and an average values is indicated as ■. (**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.

3.7. Comparison of the results obtained by all three analytical methods

Analytical performance of all three methods was performed recently[33] showing that the most sensitive is the EIS method (working concentration range 10 aM–1 pM), followed by ELLBA method (390 fM–12 pM) and MA (10 nM– 1 mM). The main two drawbacks of EIS compared to ELLA and MA were identified reproducibility of the EIS biosensor construction and low throughput of analysis. This was addressed in this study using array of gold electrodes. Surprisingly, the results obtained by ELLBA and MA don´t seem to correlate well with 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 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 is simply less sensitive compared to impedimetric analyses.

The most interesting result with possible diagnostic potential is a perfect correlation of results obtained by EIS with DAS28 index showing a correlation coefficient of 0.990 (**Fig. 3D**). This means that EIS-based method could be used for development of a new diagnostic procedure sensitive to glycosylation changes on human IgG and thus RA progression.

4. CONCLUSIONS

The study was focused on development of a biointerface composed of a mixed SAM layer formed by incubation of gold electrodes with two zwitterionic thiolated derivatives with sulfo- and carboxy- terminal functional group. Difference in pKa between these two derivatives

facilitates distribution and selective binding of lectin to carboxylate one while both derivatives possess highly resistive interface towards non-specific protein binding. The lectin biosensor in an array format using a chip with 8 gold electrodes was then employed for impedimetric detection of glycan present on the IgG molecules isolated from the human sera of healthy individuals and patients with RA. The impedimetric results agreed very well with DAS28 index, which is a measure of actual disease activity based on tender and swollen joint counts, and CRP concentrations suggesting impedimetric assays could be used for development of a new diagnostic procedure sensitive to glycosylation changes on human IgG 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 sensitive discriminator between patients with high and low disease activity.

The authors declare no competing financial interest.

ACKNOWLEDGEMENTS

Financial support received from the Slovak Scientific Grant Agency VEGA 2/0162/14 and from the Slovak Research and Development Agency APVV 14-0753 is acknowledged. The research leading to these results 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 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. 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. Authors would like to thank Miroslav Vlček and Richard Imrich for fruitful discussions.

ASSOCIATED CONTENT

Supporting Information shows synthesis of carboxybetaine (CB, **Scheme S1**), synthesis of all compounds needed for preparation of CB together with ^{1}H NMR and FTIR spectra. Moreover, a figure showing density of thiols on the gold electrode surface (**Fig. S3**), AFM images of BVT Au surface (**Fig. S4**), height profile of lectin on Au surfaces (**Fig. S5**) and evaluation of a proper dilution of isolated IgGs is provided, as well (**Fig. S6**). This material is available free of charge via the Internet at http://pubs.acs.org."

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Table of Contents Graphic and Synopsis

Impedimetric glycomic analysis of human IgG by the lectin biosensor in an array format.

REFERENCES

1. Cummings, Richard D.; Pierce, J. M. The Challenge and Promise of Glycomics. *Chem. Biol.* **2014,** *21* (1), 1-15.
2. Dalziel, M.; Crispin, M.; Scanlan, C. N.; Zitzmann, N.; Dwek, R. A. Emerging principles for the therapeutic exploitation of glycosylation. *Science* **2014,** *343* (6166), 1235681.
3. Macauley, M. S.; Crocker, P. R.; Paulson, J. C. Siglec-mediated regulation of immune cell function in disease. *Nat. Rev. Immunol.* **2014,** *14* (10), 653-666.
4. Paleček, E.; Tkáč, J.; Bartošík, M.; Bertók, T.; Ostatná, V.; Paleček, J. Electrochemistry of Nonconjugated Proteins and Glycoproteins. Toward Sensors for Biomedicine and Glycomics. *Chem. Rev.* **2015,** *115* (5), 2045-2108.
5. Stencel-Baerenwald, J. E.; Reiss, K.; Reiter, D. M.; Stehle, T.; Dermody, T. S. The sweet spot: defining virus-sialic acid interactions. *Nat. Rev. Microbiol.* **2014,** *12* (11), 739-749.
6. Lepenies, B.; Seeberger, P. H. Simply better glycoproteins. *Nat. Biotechnol.* **2014,** *32* (5), 443-445.
7. Anthony, R. M.; Nimmerjahn, F.; Ashline, D. J.; Reinhold, V. N.; Paulson, J. C.; Ravetch, J. V. Recapitulation of IVIG Anti-Inflammatory Activity with a Recombinant IgG Fc. *Science* **2008,** *320* (5874), 373-376.
8. Bochner, B. S.; Zimmermann, N. Role of siglecs and related glycan-binding proteins in immune responses and immunoregulation. *J. Allergy Clin. Immunol.* **2015,** *135* (3), 598-608.
9. Beck, A.; Reichert, J. M. Marketing approval of mogamulizumab. *mAbs* **2012,** *4* (4), 419-425.
10. Dall'Olio, F.; Vanhooren, V.; Chen, C. C.; Slagboom, P. E.; Wuhrer, M.; Franceschi, C. N-glycomic biomarkers of biological aging and longevity: A link with inflammaging. *Ageing Res. Rev.* **2013,** *12* (2), 685-698.
11. Adamczyk, B.; Tharmalingam, T.; Rudd, P. M. Glycans as cancer biomarkers. *BBA-Gen. Subjects* **2012,** *1820* (9), 1347-1353.

12. Svarovsky, S. A.; Joshi, L. Cancer glycan biomarkers and their detection–past, present and future. *Anal. Methods* **2014,** *6* (12), 3918-3936.
13. Liu, J.; Wang, J.; Wang, T.; Li, D.; Xi, F.; Wang, J.; Wang, E. Three-dimensional electrochemical immunosensor for sensitive detection of carcinoembryonic antigen based on monolithic and macroporous graphene foam. *Biosens. Bioelectron.* **2015,** *65*, 281-286.
14. Pihikova, D.; Belicky, S.; Kasak, P.; Bertok, T.; Tkac, J. Sensitive detection and glycoprofiling of a prostate specific antigen using impedimetric assays. *Analyst* **2016,** *141* (3), 1044-51.
15. Pinho, S. S.; Reis, C. A. Glycosylation in cancer: mechanisms and clinical implications. *Nat. Rev. Cancer* **2015,** *15* (9), 540-555.
16. Scherpenzeel, M.; Willems, E.; Lefeber, D. J. Clinical diagnostics and therapy monitoring in the congenital disorders of glycosylation. *Glycoconjugate J.* **2016**, 1-14.
17. Bertok, T.; Klukova, L.; Sediva, A.; Kasák, P.; Semak, V.; Micusik, M.; Omastova, M.; Chovanová, L.; Vlček, M.; Imrich, R.; Vikartovska, A.; Tkac, J. Ultrasensitive Impedimetric Lectin Biosensors with Efficient Antifouling Properties Applied in Glycoprofiling of Human Serum Samples. *Anal. Chem.* **2013,** *85* (15), 7324-7332.
18. Bertok, T.; Šedivá, A.; Filip, J.; Ilcikova, M.; Kasak, P.; Velic, D.; Jane, E.; Mravcová, M.; Rovenský, J.; Kunzo, P.; Lobotka, P.; Šmatko, V.; Vikartovská, A.; Tkac, J. Carboxybetaine Modified Interface for Electrochemical Glycoprofiling of Antibodies Isolated from Human Serum. *Langmuir* **2015,** *31* (25), 7148-7157.
19. Hushegyi, A.; Bertok, T.; Damborsky, P.; Katrlik, J.; Tkac, J. An ultrasensitive impedimetric glycan biosensor with controlled glycan density for detection of lectins and influenza hemagglutinins. *Chem. Commun.* **2015,** *51* (35), 7474-7477.
20. Hushegyi, A.; Pihíková, D.; Bertok, T.; Adam, V.; Kizek, R.; Tkac, J. Ultrasensitive detection of influenza viruses with a glycan-based impedimetric biosensor. *Biosens. Bioelectron.* **2016,** *79*, 644-649.
21. Hushegyi, A.; Tkac, J. Are glycan biosensors an alternative to glycan microarrays? *Anal. Methods* **2014,** *6* (17), 6610-6620.
22. Katrlík, J.; Švitel, J.; Gemeiner, P.; Kožár, T.; Tkac, J. Glycan and lectin microarrays for glycomics and medicinal applications. *Med. Res. Rev.* **2010,** *30* (2), 394-418.
23. Chandra, H.; Reddy, P. J.; Srivastava, S. Protein microarrays and novel detection platforms. *Expert Rev. Proteomics* **2011,** *8* (1), 61-79.
24. Belicky, S.; Tkac, J. Can glycoprofiling be helpful in detecting prostate cancer? *Chem. Pap.* **2015,** *69* (1), 90-111.
25. Bertók, T.; Katrlík, J.; Gemeiner, P.; Tkac, J. Electrochemical lectin based biosensors as a label-free tool in glycomics. *Microchim. Acta* **2013,** *180* (1-2), 1-13.
26. Loaiza, O. A.; Lamas-Ardisana, P. J.; Jubete, E.; Ochoteco, E.; Loinaz, I.; Cabañero, G.; García, I.; Penadés, S. Nanostructured Disposable Impedimetric Sensors as Tools for Specific Biomolecular Interactions: Sensitive Recognition of Concanavalin A. *Anal. Chem.* **2011,** *83* (8), 2987-2995.
27. Xu, Y.; Xie, X.; Duan, Y.; Wang, L.; Cheng, Z.; Cheng, J. A review of impedance measurements of whole cells. *Biosens. Bioelectron.* **2016,** *77*, 824-836.
28. Cao, H.; Yang, D.-P.; Ye, D.; Zhang, X.; Fang, X.; Zhang, S.; Liu, B.; Kong, J. Protein-inorganic hybrid nanoflowers as ultrasensitive electrochemical cytosensing Interfaces for evaluation of cell surface sialic acid. *Biosens. Bioelectron.* **2015,** *68*, 329-335.

29. Santos, A.; Carvalho, F. C.; Roque-Barreira, M.-C.; Bueno, P. R. Impedance-derived electrochemical capacitance spectroscopy for the evaluation of lectin–glycoprotein binding affinity. *Biosens. Bioelectron.* **2014,** *62* (0), 102-105.
30. Patil, A. V.; Bedatty Fernandes, F. C.; Bueno, P. R.; Davis, J. J. Immittance Electroanalysis in Diagnostics. *Anal. Chem.* **2015,** *87* (2), 944-950.
31. Janegitz, B. C.; Cancino, J.; Zucolotto, V. Disposable Biosensors for Clinical Diagnosis. *J. Nanosci. Nanotechnol.* **2014,** *14* (1), 378-389.
32. Komarova, E.; Reber, K.; Aldissi, M.; Bogomolova, A. New multispecific array as a tool for electrochemical impedance spectroscopy-based biosensing. *Biosens. Bioelectron.* **2010,** *25* (6), 1389-1394.
33. Klukova, L.; Bertok, T.; Petrikova, M.; Sediva, A.; Mislovicova, D.; Katrlik, J.; Vikartovska, A.; Filip, J.; Kasak, P.; Andicsová-Eckstein, A.; Mosnáček, J.; Lukáč, J.; Rovenský, J.; Imrich, R.; Tkac, J. Glycoprofiling as a novel tool in serological assays of systemic sclerosis: A comparative study with three bioanalytical methods. *Anal. Chim. Acta* **2015,** *853* (0), 555-562.
34. Vaisocherová, H.; Zhang, Z.; Yang, W.; Cao, Z.; Cheng, G.; Taylor, A. D.; Piliarik, M.; Homola, J.; Jiang, S. Functionalizable surface platform with reduced nonspecific protein adsorption from full blood plasma—Material selection and protein immobilization optimization. *Biosens. Bioelectron.* **2009,** *24* (7), 1924-1930.
35. Yang, W.; Ella-Menye, J.-R.; Liu, S.; Bai, T.; Wang, D.; Yu, Q.; Li, Y.; Jiang, S. Cross-Linked Carboxybetaine SAMs Enable Nanoparticles with Remarkable Stability in Complex Media. *Langmuir* **2014,** *30* (9), 2522-2529.
36. Sun, F.; Ella-Menye, J.-R.; Galvan, D. D.; Bai, T.; Hung, H.-C.; Chou, Y.-N.; Zhang, P.; Jiang, S.; Yu, Q. Stealth Surface Modification of Surface-Enhanced Raman Scattering Substrates for Sensitive and Accurate Detection in Protein Solutions. *ACS Nano* **2015,** *9* (3), 2668-2676.
37. Dock, E.; Christenson, A.; Sapelnikova, S.; Krejci, J.; Emnéus, J.; Ruzgas, T. A steady-state and flow-through cell for screen-printed eight-electrode arrays. *Anal. Chim. Acta* **2005,** *531* (2), 165-172.
38. Pasek, M.; Duk, M.; Podbielska, M.; Sokolik, R.; Szechiński, J.; Lisowska, E.; Krotkiewski, H. Galactosylation of IgG from rheumatoid arthritis (RA) patients – changes during therapy. *Glycoconjugate J.* **2006,** *23* (7), 463-471.
39. Goulabchand, R.; Vincent, T.; Batteux, F.; Eliaou, J.-f.; Guilpain, P. Impact of autoantibody glycosylation in autoimmune diseases. *Autoimmun. Rev.* **2014,** *13* (7), 742-750.
40. Sahatciu-Meka, V.; Izairi, R.; Rexhepi, S.; Manxhuka-Kerliu, S. Comparison of seronegative and seropositive rheumatoid arthritis with regard to some clinical characteristics. *Reumatizam* **2009,** *56* (1), 8-16.
41. Aletaha, D.; Neogi, T.; Silman, A. J.; Funovits, J.; Felson, D. T.; Bingham, C. O.; Birnbaum, N. S.; Burmester, G. R.; Bykerk, V. P.; Cohen, M. D.; Combe, B.; Costenbader, K. H.; Dougados, M.; Emery, P.; Ferraccioli, G.; Hazes, J. M. W.; Hobbs, K.; Huizinga, T. W. J.; Kavanaugh, A.; Kay, J.; Kvien, T. K.; Laing, T.; Mease, P.; Ménard, H. A.; Moreland, L. W.; Naden, R. L.; Pincus, T.; Smolen, J. S.; Stanislawska-Biernat, E.; Symmons, D.; Tak, P. P.; Upchurch, K. S.; Vencovský, J.; Wolfe, F.; Hawker, G. 2010 Rheumatoid arthritis classification criteria: An American College of Rheumatology/European League Against Rheumatism collaborative initiative. *Arthritis Rheum.* **2010,** *62* (9), 2569-2581.
42. Tkac, J.; Davis, J. J. An optimised electrode pre-treatment for SAM formation on polycrystalline gold. *J. Electroanal. Chem.* **2008,** *621* (1), 117-120.

43. Shao, Q.; Jiang, S. Molecular Understanding and Design of Zwitterionic Materials. *Adv. Mater.* **2015,** *27* (1), 15-26.
44. Davis, J. J.; Tkac, J.; Humphreys, R.; Buxton, A. T.; Lee, T. A.; Ko Ferrigno, P. Peptide Aptamers in Label-Free Protein Detection: 2. Chemical Optimization and Detection of Distinct Protein Isoforms. *Anal. Chem.* **2009,** *81* (9), 3314-3320.
45. Shen, C.-H.; Lin, J.-C. Improving the Surface Biocompatibility with the Use of Mixed Zwitterionic Self-Assembled Monolayers Prepared by a Proper Solvent. *Langmuir* **2011,** *27* (11), 7091-7098.
46. Ooi, Y.; Hobara, D.; Yamamoto, M.; Kakiuchi, T. Ideal nonideality in adsorption of 2-aminoethanethiol and 2-mercaptoethane sulfonic acid to form electrostatically stabilized binary self-assembled monolayers on Au(111). *Langmuir* **2005,** *21* (24), 11185-9.
47. Ostuni, E.; Chapman, R. G.; Liang, M. N.; Meluleni, G.; Pier, G.; Ingber, D. E.; Whitesides, G. M. Self-assembled monolayers that resist the adsorption of proteins and the adhesion of bacterial and mammalian cells. *Langmuir* **2001,** *17* (20), 6336-6343.
48. Holmlin, R. E.; Chen, X.; Chapman, R. G.; Takayama, S.; Whitesides, G. M. Zwitterionic SAMs that resist nonspecific adsorption of protein from aqueous buffer. *Langmuir* **2001,** *17* (9), 2841-2850.
49. Shen, C.-H.; Lin, J.-C. Solvent and concentration effects on the surface characteristics and platelet compatibility of zwitterionic sulfobetaine-terminated self-assembled monolayers. *Colloids Surf., B* **2013,** *101*, 376-383.
50. Cheng, G.; Zhang, Z.; Chen, S.; Bryers, J. D.; Jiang, S. Inhibition of bacterial adhesion and biofilm formation on zwitterionic surfaces. *Biomaterials* **2007,** *28* (29), 4192-4199.
51. Prime, K. L.; Whitesides, G. M. Self-assembled organic monolayers: model systems for studying adsorption of proteins at surfaces. *Science* **1991,** *252* (5009), 1164-1167.
52. Prime, K. L.; Whitesides, G. M. Adsorption of proteins onto surfaces containing end-attached oligo(ethylene oxide): a model system using self-assembled monolayers. *J. Am. Chem. Soc.* **1993,** *115* (23), 10714-10721.
53. Zhang, Z.; Zhang, M.; Chen, S.; Horbett, T. A.; Ratner, B. D.; Jiang, S. Blood compatibility of surfaces with superlow protein adsorption. *Biomaterials* **2008,** *29* (32), 4285-4291.
54. Lahiri, J.; Isaacs, L.; Tien, J.; Whitesides, G. M. A Strategy for the Generation of Surfaces Presenting Ligands for Studies of Binding Based on an Active Ester as a Common Reactive Intermediate: A Surface Plasmon Resonance Study. *Anal. Chem.* **1999,** *71* (4), 777-790.
55. Gerlach, J. Q.; Kilcoyne, M.; Joshi, L. Microarray evaluation of the effects of lectin and glycoprotein orientation and data filtering on glycoform discrimination. *Anal. Methods* **2014,** *6* (2), 440-449.