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Label-free detection of glycoproteins by the lectin biosensor down to attomolar level using gold nanoparticles

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ABSTRACT

We present here an ultrasensitive electrochemical biosensor based on a lectin biorecognition capable to detect concentrations of glycoproteins down to attomolar (aM) level by investigation of changes in the charge transfer resistance (R_{ct}) using electrochemical impedance spectroscopy (EIS). On polycrystalline gold modified by an aminoalkanethiol linker layer, gold nanoparticles were attached. A *Sambucus nigra* agglutinin was covalently immobilised on a mixed self-assembled monolayer formed on gold nanoparticles and finally, the biosensor surface was blocked by poly(vinyl alcohol). The lectin biosensor was applied for detection of sialic acid containing glycoproteins fetuin and asialofetuin. Building of a biosensing interface was carefully characterised by a broad range of techniques such as electrochemistry, EIS, atomic force microscopy, scanning electron microscopy and surface plasmon resonance with the best performance of the biosensor achieved by application of HS–$(CH_2)_{11}$–NH_2 linker and gold nanoparticles with a diameter of 20 nm. The lectin biosensor responded to an addition of fetuin (8.7% of sialic acid) with sensitivity of $(338 \pm 11)\ \Omega\ \text{decade}^{-1}$ and to asialofetuin ($\leq 0.5\%$ of sialic acid) with sensitivity of $(109 \pm 10)\ \Omega\ \text{decade}^{-1}$ with a blank experiment with oxidised asialofetuin (without recognisable sialic acid) revealing sensitivity of detection of $(79 \pm 13)\ \Omega\ \text{decade}^{-1}$. These results suggest the lectin biosensor responded to changes in the glycan amount in a quantitative way with a successful validation by a lectin microarray. Such a biosensor device has a great potential to be employed in early biomedical diagnostics of diseases such as arthritis or cancer, which are connected to aberrant glycosylation of protein biomarkers in biological fluids.

1. Introduction

Glycomics is becoming more and more influential member of an "omics" family since glycosylation is the most frequent posttranslational modification of proteins and glycans (saccharides attached to proteins) are actively involved in many physiological and pathological processes [1]. Glycans are better equipped to be an information coding tool compared to DNA and peptides simply because glycans are information rich molecules, i.e. theoretical number of all possible hexamers (consisting of 6 building units) for glycans (1.44×10^{15}) is few orders of magnitude larger compared to peptides (6.4×10^{6}) or DNA (4096) [2]. The size of the cellular glycome is estimated to be in excess of 100,000–500,000 glycan modified biomolecules with a number of unique glycans to be 3000–7000 [3] and this variability can explain human complexity in light of a paradoxically small genome. A pace of advances in the field of glycomics is reduced due to an enormous complexity of glycans on one side with similar physico-chemical properties of glycans on the other side [4], but new high throughput methods have a potential to speed up the process of glycan analysis [5]. The main analytical tools of focus in glycomics include wide range of chromatographic techniques, mass spectrometry, capillary electrophoresis and especially lectin microarray technique.

A microarray technique relying on lectins, which are natural glycan recognising proteins, has a clear advantage over other modern analytical tools applied in glycomics, i.e. glycans do not need to be released from a biomolecule and thus *in-situ* glycan

Abbreviations: AFM, Atomic force microscopy; ASF, Asialofetuin; AuNPs, Gold nanoparticles; DW, Deionised water; EIS, Electrochemical impedance spectroscopy; FET, Fetuin; MH, 6-Mercaptohexanol; MUA, 11-Mercaptoundecanoic acid; OxASF, Oxidised asialofetuin; PVA, Poly(vinyl alcohol); SAM, Self-assembled monolayer; SNA I, *Sambucus nigra* agglutinin; SPR, Surface plasmon resonance

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analysis is possible. Lectin microarrays have been very effective in revealing an active role of glycans in many processes and at present are considered as a standard analytical tool in glycomics [5,6]. However, a typical lectin microarray experiment involves a fluorescent dye being coupled either to lectin or to the glycan/sample for generation of an analytical signal. This requirement for having a label can cause unwanted variability in labelling and biorecognition [6] and thus other formats of analysis working in a label-free mode of detection should be utilised.

Electrochemistry is a very powerful analytical platform with an array of different detection principles and some of them allow to work without any label in a label-free mode of operation [7,8]. Electrochemical impedance spectroscopy (EIS) is quite frequently applied in construction of label-free biosensors due to very sensitive analysis and a simpler set-up compared to field-effect sensing [7]. EIS is based on an electric perturbation of a thin layer on the conductive surface by a small alternating current amplitude and can provide interfacial characteristics such as impedance, resistance and capacitance utilisable in sensing by employment of an equivalent circuit for data evaluation [9]. EIS results are typically transformed into a Nyquist plot, which can provide information about charge transfer resistance R_{ct} in a direct way. After a biorecognition event, R_{ct} increases due to presence of additional layer and thus a subtle change in R_{ct} can be used for detection [10]. Subsequently, EIS allows complex biorecognition events to be probed in a simple, sensitive and label-free manner and EIS is being increasingly popular for development of electrochemical lectin-based biosensors for glycan determination.

EIS is very often combined with formation of self-assembled monolayers (SAMs) allowing to precisely tune interfacial properties such as capacitance and resistance of the interface and/or to control density of ligands at nanoscale subsequently applied in an immobilisation process [11]. Moreover, once SAM is formed it can be employed as a linker to deposit gold nanoparticles on the surface to enhance loading of biorecognition elements and their accessibility, as well [12]. Gold nanoparticles (AuNPs) are of a great attention both from a fundamental [13] and an application-driven perspective [14]. More specifically, AuNPs due to large surface area, intense visible light scattering/absorption, large electron density and catalytic properties have been employed as cargos able to penetrate cell membrane [15], efficient catalysts [16], image contrast agents [17] and most extensively as parts in biosensors or in bioanalytical devices [18] with electrochemical detection platform as the most dominant one [19].

Electrochemical detection of various analytes provides a highly sensitive tool in comparison with other detection platforms in the area of biosensing with detection limits down to attomolar level (aM) for DNA [20], proteins [21], or even low-molecular weight analytes such as pesticides [22]. In this study, we focused on development of a highly sensitive biosensor device by immobilisation of a lectin SNA I recognising 2,6-linked sialic acid on an AuNP layer with integration of such an interface with a label-free format of assays based on EIS. Sialic acids have a prominent role in many pathological processes such as chronic inflammation, HIV, influenza infection, malaria and cancer [1,23] and thus *in-situ* detection of sialic acid on biomolecules is of special interest in glycomics. This is the first paper showing detection limit for glycoprotein determination down to 1 aM level and when we used the same calculations as in a previous paper [24], we were able to detect 40 yoctomoles (i.e. 1.10^{-18} mol $l^{-1} \times 40.10^{-6}$ l) of a glycoprotein, i.e. lower amount than previously claimed for a protein (200 yoctomoles) [24]. Although the biosensor was designed to detect sialic acid in this study, the same immobilisation protocol can be applied for immobilisation of any other lectin, what is an essential feature for integration of this patterning protocol into an array/biochip format of analysis.

2. Materials and methods

2.1. Chemicals

11-Mercaptoundecanoic acid (MUA), 6-mercaptohexanol (MH), potassium hexacyanoferrate(III), potassium hexacyanoferrate(II) trihydrate, potassium chloride, *N*-hydroxysuccinimide (NHS), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), poly(vinyl alcohol) (PVA, Mowiol® 4-88), sodium periodate, ethylene glycol, cysteamine, acetonitrile, fetuin (FET, 8.7% of sialic acid), asialofetuin (ASF, $\leq$0.5% of sialic acid), cysteamine hydrochloride and gold nanoparticles (5 nm, 10 nm and 20 nm) were purchased from Sigma Aldrich (USA). Phosphate buffer saline tablets (PBST) for SPR buffers were from Merck (Slovakia). Aminoalkanethiol linkers 6-aminohexanethiol and 11-aminoundecanethiol hydrochloride were purchased from Dojindo (Germany). SNA I lectin from *Sambucus nigra* was purchased from Gentaur (Belgium). Biotinylated SNA I lectin was purchased from Vector Laboratories (USA), and CF555-streptavidin fluorescent label was purchased from Biotium (USA). Ethanol for UV/VIS spectroscopy (ultra pure) was purchased from Slavus (Slovakia). Zeba™ spin desalting columns (40k MWCO) for protein purification were purchased from Thermo Scientific (UK). All buffer components were dissolved in deionised water (DW).

2.2. Electrode cleaning and SAM formation

Planar polycrystalline gold electrodes with a diameter of 1.6 mm (Bioanalytical systems, USA) were cleaned as previously described [25]. First, a reductive desorption of previously bound thiols was employed with a potentiostat Autolab PGSTAT 128N (Ecochemie, Netherlands) in a cell with Ag/AgCl reference and a counter Pt electrode (both from Bioanalytical systems, USA) by applying a cyclic potential scanning from −1500 mV to −500 mV in 100 mM NaOH under N_2 atmosphere with a sweep rate of 1 V s^{-1} until a stable cyclic voltammogram was obtained. Then a mechanical polishing of electrodes for 10 min on a polishing pad using 1.0 μm and then 0.3 μm particles (Buehler, USA) for a total polishing time of 20 min was performed, followed by two sonications in DW for 3 min. In the last step the electrodes were left in hot piranha (a mixture of concentrated H_2SO_4 and concentrated H_2O_2 in 3+1 ratio, *handle with a special care*) for 20 min and sonicated in DW for 3 min. Just before the electrode patterning by SAM, CV was employed for an electrochemical polishing of the electrodes (from −200 mV to 1500 mV at a scan rate of 100 mV s^{-1} until a stable CV was obtained—up to 25 scans) and for gold oxide stripping (10 cycles starting from +750 mV to +200 mV at a scan rate of 100 mV s^{-1}) from the electrodes in 100 mM H_2SO_4.

Electrochemical polishing procedure run for two cycles was applied for estimation of a surface coverage of gold nanoparticles attached to polycrystalline gold covered by a thiol linker by integration of a gold reductive peak as proposed previously [25]. A value of electrochemical gold electrode surface area with gold nanoparticles attached was subtracted from a value of electrochemical gold electrode surface area without having gold nanoparticles and this value was then recalculated as coverage of AuNPs.

The electrodes were washed by DW and absolute EtOH, left to dry in dustless environment and subsequently immersed in 1 mM solutions of different aminoalkanethiols for 24 h (to provide a high-density monolayer). After incubation, the electrodes were