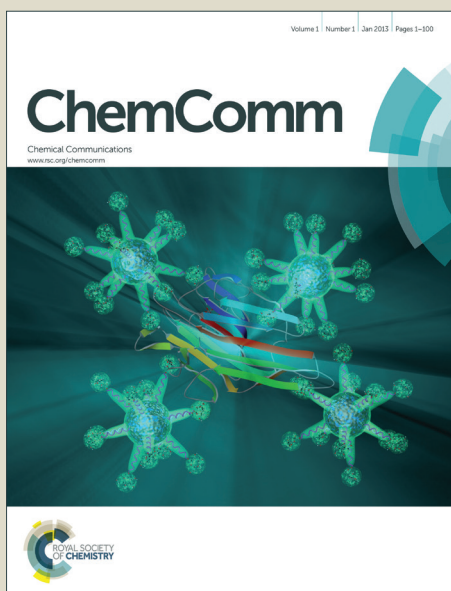


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## COMMUNICATION

# Ultrasensitive impedimetric glycan biosensor with controlled glycan density for detection of lectins and influenza hemagglutinins

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A. Hushegyi, T. Bertok, P. Damborsky, J. Katrlík and J. Tkáč\*

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**Impedimetric glycan biosensor with optimised glycan density was applied for detection of lectins and influenza hemagglutinins down to attomolar concentrations (aM).**

Glycans, complex carbohydrates attached to proteins, lipids or present on the cell surfaces, play an important role in many cellular processes including immune response, tumour metastasis, infection by bacteria/viruses, inflammation, molecular recognition and cell signaling.<sup>1</sup> The importance of involvement of glycans in the cellular processes is recognized and understood at an amazing pace and such knowledge has a great potential for developing novel therapeutic and diagnostic tools for numerous diseases.<sup>2</sup> Glycan biochips based on a fluorescent reading technology developed for highly parallel DNA and protein analysis<sup>3</sup> became a “must have” tool for multiplexed analysis of binding of glycans with proteins or intact cells/viruses.<sup>4</sup>

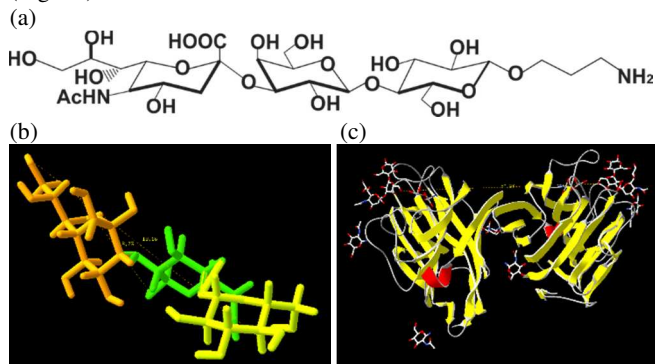
Glycan biochips relying on a fluorescent signal reading have some limitations including a need to fluorescently label either glycan or a glycan-binding molecule with a risk that such modification can alter a biorecognition process between a glycan binding protein and surface confined glycan molecules.<sup>5</sup> Moreover, bleaching of a fluorophore can lead into false negative results or can alter quantitative output from such assays. Traditionally glycans are not printed on the microarray slide with a controlled density, what can influence robustness and reliability of results obtained by application of fluorescent glycan biochips. Additionally, detection limit of fluorescent glycan biochips are not particularly impressive and thus, low abundant analyte molecules cannot be detected.<sup>6</sup> Thus, various alternative and novel assay platforms working in a label-free mode of operation including electrochemical impedance spectroscopy (EIS) and other electrochemical techniques; field-effect transistor (FET)-based sensing; quartz crystal microbalance; surface plasmon resonance; microcantilever arrays; nanopore sensing; quenching of intrinsic fluorescence of single walled carbon nanotubes; etc. integrated with immobilised glycans and lectins (glycan recognising proteins) have been intensively sought to overcome such limitations as reviewed recently.<sup>7</sup> It was recently summarised that nanoscale controlled immobilisation process for glycans and lectins is required to achieve high selectivity and sensitivity assays by these newly developed devices.<sup>6, 8</sup> Moreover, from a diverse range of detection

techniques applied so far, especially electrochemical based ones including EIS belong to the most sensitive ones.<sup>9</sup> For example glycan biosensors with electrochemical detection (cyclic voltammetry and differential pulse voltammetry) exhibit detection limit down to 0.5 nM<sup>10</sup>, FET-based glycan biosensor offer low nM level<sup>11</sup>, 1 fM<sup>12</sup> or aM<sup>13</sup> detection limits depending on construction formats and EIS-based glycan biosensors were able to detect glycan binding proteins down to 0.12 nM.<sup>14</sup> For comparison glycan biosensors based on surface plasmon resonance or localised surface plasmon resonance can detect analytes down to 0.2 nM<sup>15</sup> and cantilever arrays based glycan devices can detect analytes down to 91 pM<sup>16</sup>.

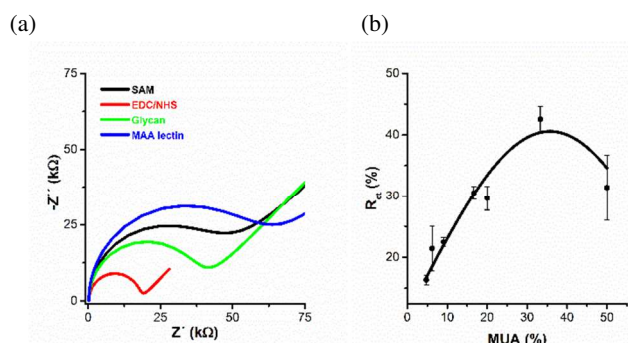
Impedimetric antibody and lectin based biosensors can detect proteins and glycoproteins down to aM level,<sup>9b</sup> but so far impedimetric glycan biosensors could not detect such low levels of analytes.<sup>14</sup> In our previous work we focused on development of ultrasensitive impedimetric lectin biosensors with controlled architecture at nanoscale in glycoprofiling (glycoprotein analysis) of some diseases.<sup>17</sup> In this work we wanted to apply protocols previously developed for immobilisation of lectins for development of ultrasensitive glycan biosensors. We show for the first time that aM detection limits by impedimetric glycan biosensors are achievable. This ultrasensitive analysis was possible by application of an optimised immobilisation process with controlled density of glycans achieved by formation of a mixed self-assembled monolayer to tune density of functional carboxylic groups for analysis of three different analytes i.e. *Maackia amurensis* agglutinin (MAA) and two hemagglutinins H1N1 and H5N1.

Polycrystalline gold electrodes were extensively cleaned to remove organic contaminants and gold oxide layer deposited during electrochemical cleaning as described previously<sup>18</sup> with details provided in ESI. Gold electrodes were patterned by a mixed self-assembled monolayer (SAM), prepared from a mixture of two thiols, what is an effective way how to control density of functional ligands on surfaces (initially developed for immobilisation of lectins).<sup>17b</sup> A functional thiol (11-mercaptoundecanoic acid, MUA) was diluted by 6-mercaptohexanol (MH) by changing amount of MUA in a mixture from 5% to 50%. In the next step a –COOH group of MUA was activated by EDC/NHC mixture to form an active ester.<sup>17b</sup> Then,

amine-terminated glycan (Fig. 1a, 1b) was added for covalent immobilisation of a glycan. Finally, the glycan biosensor was incubated with MAA (Fig. 1c) to find out what glycan density is optimal for binding of MAA. Faradaic EIS investigation described in ESI, showed that charge transfer resistance  $R_{ct}$ , read as a diameter of a semicircle from a Nyquist plot (Fig. 2a), of SAM layer was 42.1 k $\Omega$  and this value decreased to 17.6 k $\Omega$  after EDC/NHS treatment due to effective decrease of a negative charge on the SAM layer. After glycan immobilisation  $R_{ct}$  increased to 36.4 k $\Omega$  and further to value of 55.5 k $\Omega$  after incubation of the glycan biosensor with MAA (770 pM) (Fig. 2a). Optimisation study aimed to find optimal composition of a mixed SAM layer revealed that the glycan biosensor can detect 770 pM MAA with the highest response, when SAM was prepared from a thiol mixture containing 33.3% of MUA (Fig. 2b).



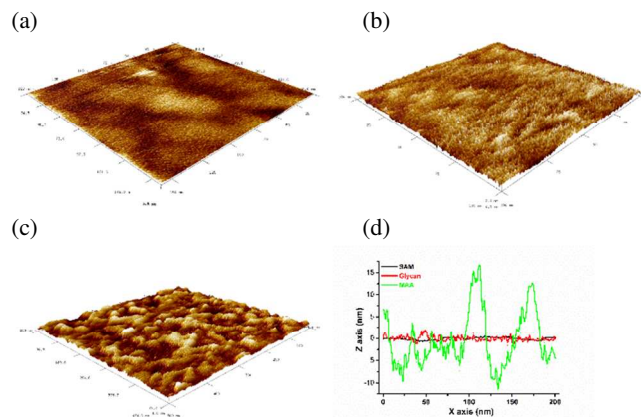
**Fig. 1** (a) Chemical structure of amine glycan derivative of 2,3-sialyllactose with a formula Neu5Ac $\alpha$ 2-3Gal $\beta$ 1-4Glc $\beta$ -O-(CH<sub>2</sub>)<sub>3</sub>-NH<sub>2</sub> applied for construction of glycan biosensor, (b) a 3-D structure of 2,3-sialyllactose and (c) a structure of *Maackia amurensis* agglutinin (lectin) docked with 2,3-sialyllactose (pdb code: 1DBN).



**Fig. 2** (a) Impedimetric characterisation of the build-up process for preparation of the glycan biosensor with formation of a mixed SAM composed of 11-mercaptoundecanoic acid (MUA) and 6-mercaptohexanol (SAM), after activation by a coupling reagents (EDC/NHS), after covalent immobilisation of 2,3-sialyllactose (Glycan) and after interaction with *Maackia amurensis* agglutinin (MAA lectin) and (b) optimisation of MUA ratio within a mixed SAM for preparation of a glycan biosensor evaluated by MAA lectin interaction.

Atomic force microscopy (AFM) analysis of glycan modified surface showed presence of individual features not seen on SAM modified gold surface (Fig. 3) separated by an average distance of  $(2.7 \pm 0.3)$  nm (Fig. S1), which are attributed to individual glycan chains. Glycan patterned surface exhibited mean square roughness  $R_q=0.65$  nm, what is value significantly larger compared to plain

mixed SAM layer with  $R_q=0.39$  nm (Fig. 3). When the glycan surface was finally incubated with MAA  $R_q$  increased to value of 6.45 nm, confirming ability of a glycan bioreceptive layer to bind its analyte MAA (Fig. 3d). Difference of the topology between glycan bound and plain SAM layer is clearer compared to a recently published study<sup>19</sup> and allowed us to calculate glycan density as  $(22.7 \pm 2.7)$  pmol cm<sup>-2</sup>  $((1.4 \pm 0.2) \times 10^{13}$  glycan molecules per cm<sup>2</sup>).



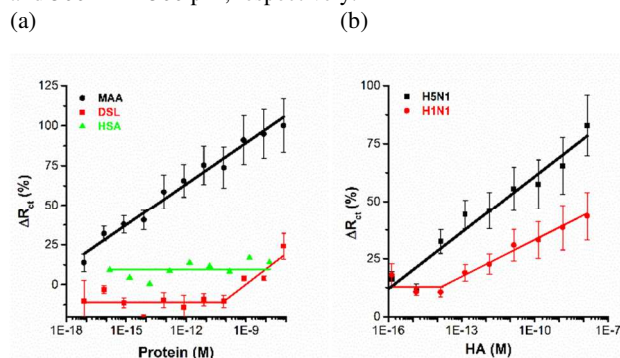
**Fig. 3** Atomic force microscopy (AFM) images of (a) mixed SAM layer composed of 11-mercaptoundecanoic acid and 6-mercaptohexanol, (b) 2,3-sialyllactose covalently immobilised on SAM, (c) incubation of *Maackia amurensis* agglutinin (MAA) with glycan surface and (d) AFM profile showing surface roughness for mixed SAM (SAM), glycan bound surface (Glycan) and after incubation of glycan interface with MAA lectin (MAA).

When, an independent method i.e. SPR was applied to calculate glycan density, the SPR response of 440  $\mu$ RIU (Fig. S2), corresponds to a glycan density of 61.8 pmol cm<sup>-2</sup>  $(3.7 \times 10^{13}$  glycan molecules per cm<sup>2</sup>). There are two facts worth mentioning regarding SPR assays: 1) a correlation factor of 1,000  $\mu$ RIU=1 ng mm<sup>-2</sup> provided by the manufacturer for proteins can be different for glycan molecules; and 2) a higher driving force for delivery of EDC/NHS or glycan molecule was applied during SPR experiment run under flow conditions compared to static immobilisation of glycan on an AFM gold chip. Nevertheless, both values of glycan density obtained here fall well within the range  $7.2 \times 10^{12}$  cm<sup>-2</sup> –  $5.2 \times 10^{13}$  cm<sup>-2</sup> obtained in a previous study for diluted mannosyl moieties.<sup>19</sup> Taking into account diameter of 2,3-sialyllactose (Fig. S3), theoretical full glycan monolayer should have density of  $1.4 \times 10^{14}$  cm<sup>-2</sup>, in agreement with experimental value of  $1.2 \times 10^{14}$  cm<sup>-2</sup> obtained for mannose,<sup>19</sup> suggesting that optimal glycan density for lectin binding on the surface in this study is close to 10% of theoretical coverage. Since distance between binding sites in MAA is approx. 5.0 nm (Fig. S4), the highest MAA binding at optimal glycan density (this is in agreement with a previous study, which concluded that the highest lectin binding was achieved on glycan surfaces allowing monovalent interactions.<sup>19</sup> From the structure of hemagglutinin H1N1 (a protein from influenza viruses) (Fig. S5) a protein diameter with a binding site bound to the glycan modified surface of  $\sim 3.5$  nm could be read, a value smaller than for MAA ( $\sim 5.3$  nm, Fig. S4) and thus binding of HA to the glycan modified surface should be less constrained compared to monovalent MAA binding.

The glycan biosensor developed was then calibrated with two different glycan binding proteins – lectins (MAA and *Datura stramonium* lectin - DSL) and hemagglutinins H1N1 and H5N1. Results indicated that the glycan biosensor containing sialyllactose



with  $\alpha$ -2,3-bound sialic acid specifically interact with MAA, which is preferentially binding glycans with terminal  $\alpha$ -2,3-bound sialic acid. The glycan biosensor could detect this lectin in the concentration window 8 aM – 80 nM (i.e. 10 orders of magnitude) (Fig. 4a). DSL binding to  $\beta$ -1,4-linked *N*-acetylglucosamine oligomers, being a negative control, did not exhibit any significant binding until 8 pM and only then the response toward 2,3-sialyllactose modified interface increased (Fig. 4a). Serum albumins are quite often applied as probes to investigate non-specific interactions with bioreceptive layers,<sup>20</sup> this is why an additional control experiment was performed by investigation of binding of human serum albumin (HSA) to the glycan modified surface (HSA with an isoelectric point  $pI=4.4$ <sup>21</sup> comparable to  $pI=4.7$  for MAA lectin<sup>22</sup>) without any significant binding in a concentration window 140 aM – 14 nM (Fig. 4a), suggesting a specific biorecognition of surface confined 2,3-sialyllactose by MAA. H5N1 could be detected in a concentration window 140 aM – 14 nM (i.e. 9 orders of magnitude, if a background value of  $\Delta R_{ct}(\%)$  at a concentration of 1.4 fM is not taking into account), while response towards H1N1 was negligible up to 14 fM and only then linearly increased up to concentration of 14 nM (6 orders of magnitude) (Fig. 4b). The lowest detection limit for determination of lectin with EIS-based glycan biosensor so far is 0.12 nM.<sup>14c</sup> Field-effect transistor (FET)-based device relying on silicon nanowires could detect lectins down to 2 nM<sup>11b</sup> or 1 fM<sup>12</sup>. Other techniques exhibit detection limits for lectins comparable to glycan biochips.<sup>6</sup> So far hemagglutinins have not been determined by EIS-based glycan biosensors, but FET-based analysis was successfully applied. In this particular case H1N1 and H5N1 could be detected in a concentration window 50 aM – 5 nM and 500 zM – 500 pM, respectively.<sup>13</sup>



**Fig. 4** Calibration of the glycan biosensor by (a) two different lectins *Maackia amurensis* agglutinin (MAA), *Datura stramonium* lectin (DSL) and human serum albumin (HSA); and (b) two different influenza hemagglutinins H5N1 (black) and H1N1 (red).

It is quite interesting that an interface modified by  $\alpha$ -2,3-linked sialic acid in sialyllactose bind in our study besides H5N1 also H1N1, which should recognise  $\alpha$ -2,6-linked sialic acid.<sup>23</sup> Hemagglutinins from avian influenza viruses (i.e. H5N1) preferentially bind to  $\alpha$ -2,3-linked sialic acid terminated glycans, while hemagglutinins from human influenza viruses (i.e. H1N1) preferentially bind to  $\alpha$ -2,6-linked sialic acid terminated glycans, but such interactions are more complex than previously thought.<sup>24</sup> Other attributes such as glycan topology (i.e. cone-like vs. umbrella-like structure), the effect of neighbouring glycan and proper spacing of minimal glycan epitopes can play significant role in viral recognition of its host cellular glycans *via* hemagglutinins.<sup>23b, 25</sup> Interrogation of glycan biochips with hemagglutinins isolated from different influenza viruses revealed that some human H1N1 hemagglutinins can bind to  $\alpha$ -2,3-linked sialic acid terminated glycans and avian

H5N1 hemagglutinins can bind to  $\alpha$ -2,6-linked sialic acid terminated glycans.<sup>26</sup> Another recent study showed that H1N1 hemagglutinin isolated from viral strain New Caledonia 20/99 (used in this study) not only binds effectively to various glycans with  $\alpha$ -2,3-linked sialic acid termination, but more interestingly enhancement of binding was observed, when galactose next to terminal sialic acid was modified by a negatively charged sulfo group.<sup>27</sup> The most recent study showed that binding preference of intact H1N1 influenza viruses (A/Puerto Rico/8/34) to 2,3-sialyllactose or 2,6-sialyllactose modified glycan microarrays exhibit binding specificity depending on a glycan density i.e. at low printed glycan density (from 0.1 mM glycan solution) the H1N1 influenza virus exhibit preferential binding to 2,6-sialyllactose, while at higher printed glycan density (1-10 mM of glycan in solution), the same virus preferentially bind to 2,3-sialyllactose.<sup>28</sup> This study is in agreement with conclusion of another study that alternative moieties including sulfonated ones may be important in recognition of host glycan receptors by influenza viruses.<sup>29</sup> Thus, more systematic investigation of glycan density, topology and presence of various charged species within glycan interfaces on binding of viral agglutinins is needed for determination of various different viral strains. Impedimetric glycan biosensors based on gold surfaces patterned by thiols terminated in numerous functionalities in a flexible way can shed light on interaction between glycan epitopes and viral proteins.<sup>14b</sup> Moreover, impedimetric glycan biosensors could be useful for analysis of activities of glycan processing enzymes, not yet performed.<sup>30</sup>

We have developed the most sensitive impedimetric glycan biosensor for analysis of lectins with detection limit 7 orders of magnitude lower compared to the best described glycan biosensor. The glycan biosensor described here is the first impedimetric glycan biosensor for analysis of hemagglutinins with detection limits down to attomolar level with sensitivity similar to the best glycan biosensor based on FET described so far.<sup>13</sup> Moreover, the study provides ultrasharp visualisation of glycans immobilised on surfaces by AFM.

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Department of Glycobiotechnology, Institute of Chemistry, Slovak Academy of Sciences, Dúbravská cesta 9, 845 38 Bratislava, Slovakia. \* Corresponding author. Jan Tkac, E-mail: [Jan.Tkac@savba.sk](mailto:Jan.Tkac@savba.sk), Tel.: +421 2 5941 0263.

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Electronic Supplementary Information (ESI) available: full description of experimental conditions, a detailed topological profile of glycan patterned surface (Fig. S2), SPR sensorgram (Fig. S2), structures of 2,3-sialyllactose glycan (Fig. S3), *Maackia amurensis* agglutinin docked with 2,3-sialyllactose (Fig. S4) and H1N1 hemagglutinin docked with 2,3-sialyllactose (Fig. S5). See DOI: 10.1039/c000000x/

Ultrasensitive impedimetric analysis of lectins and viral agglutinins was achieved by the glycan biosensor built up on a mixed SAM (left) with a controlled glycan density (right)

