Supplementary information

Ultrasensitive detection of influenza viruses with a glycan-based impedimetric biosensor

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Experimental section

Chemicals

HS-$(CH_2)_3$-EG_2-OCH_2-COOH (EG=(OCH_2CH_2), OEG-COOH), HS-$(CH_2)_3$-EG_2-OH (OEG), HS-$(CH_2)_8$-EG_2-OH, HS-$(CH_2)_8$-EG_2-OCH_2-COOH and HS-$(CH_2)_8$-EG_3-OH were obtained from Prochimia (Poland). HS-$(CH_2)_{10}$-COOH, HS-$(CH_2)_6$-OH, HS-$(CH_2)_8$-OH, potassium hexacyanoferrate(III), potassium hexacyanoferrate(II) trihydrate, potassium chloride, sodium hydroxide, sulphuric acid, N-hydroxysuccinimide (NHS), *N*-(3-dimethylaminopropyl)-*N′*-

ethylcarbodiimide hydrochloride (EDC), ethanolamine hydrochloride, sodium dodecyl sulfate (SDS), phosphate buffered saline tablet (PBS, one tablet dissolved in 200 ml of deionized water yields 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4, at 25 °C), a lectin isoform I from *Maackia amurensis* (MAA), lectin from *Datura stramonium* (DSL) and lectin isoform I from *Ulex europaeus* (UEA) were purchased from Sigma Aldrich (USA). Influenza A virus H3N2 and H7N7 were acquired from the Department of Chemistry and Biochemistry, Mendel University in Brno. Amino terminated glycan, 3'sialyllactose-β-aminopropyl was purchased from Elicityl Oligotech (France). Ethanol for UV/VIS spectroscopy (ultra-pure) was purchased from Slavus (Slovakia). All buffer components were dissolved in deionized water (DW).

Electrode pre-treatment and SAM preparation

The cleaning of planar polycrystalline gold electrodes (d=1.6 mm, Bioanalytical systems, USA) was made by a previously published protocol (Tkac and Davis 2008) with laboratory potentiostat/galvanostat Autolab PGSTAT 128N (Ecochemie, Netherlands) in a three electrode cell with Ag/AgCl reference and a counter Pt electrode (Bioanalytical systems, USA). The first step was a reductive desorption of previously bound thiols in 0.1 M solution of NaOH, by applying a cyclic potential scanning from - 1,500 mV to -500 mV under N_2 atmosphere with a rate of 1 V s^{-1} for about 50 scans (until a stable scan was obtained). The second step was the mechanical polishing of the electrodes on a polishing pad using 1 μm particles for 5 min and 0.3 μm particles for 5 min (Buehler, USA). After mechanical polishing, electrodes were sonicated two times in DW for 5 min. The third step was immersion of the electrodes into the solution of piranha (H_2O_2:H_2SO_4 – 1:3, **caution**: *handle with a special care since hot piranha is a strongly oxidizing agent*) for 20 min. After piranha treatment, the electrodes were again sonicated in DW for 5 min. The fourth and fifth steps were electrochemical polishing and gold oxide stripping in 0.1 M solution of H_2SO_4. Electrochemical

polishing was made by a cyclic voltammetry run from -200 mV to 1,500 mV at a scan rate of 100 mV s^{-1} up to 25 scans, and gold oxide stripping was realized by 10 cycles starting from +750 mV to +200 mV at a scan rate of 100 mV s^{-1}. The electrodes were then washed with DW and ultra-pure ethanol. After the cleaning process the electrodes were immediately immersed into the 1 mM solution of thiols (OEG-COOH and OEG 1+5, if not specified otherwise). The electrodes were incubated overnight in dark at room temperature.

Preparation of a glycan biosensor

Subsequently, thiol modified electrodes were washed with ultra-pure ethanol and DW. Carboxyl groups present within a mixed SAM layer were activated with 40 µl solution of 200 mM EDC and 50 mM NHS in ratio 1:1 (solution of EDC and NHS were previously prepared in DW and stored at -20°C until use in aliquots) for 15 min. After activation, the electrodes were washed with DW and 100 µM amino terminated glycan was added to the biosensor surface (glycan was previously dissolved in 10 mM PBS pH 7.4 and stored at -20°C in aliquots until use). The glycan immobilization was carried out at room temperature for 1.5 h (**Scheme S1**).

Propagation of influenza viruses in embryonated chicken eggs

Serum pathogen-free (SPF) fertilized chicken eggs were incubated at 37 °C. Virus inoculation was carried out by injection of virus stock into the allantoic cavity of 9-days old eggs using a needle. After 2 days of incubation at 37 °C, the eggs were chilled for 10 h at 4°C. The eggshell above the air sac and the chorioallantoic membrane were opened, and the allantoic fluid containing the virus was harvested. The fluid was cleared from debris by centrifugation at 1,400 rpm for 30 min at 4°C. Supernatant was ultracentrifuged at 40,000 rpm for 2 h at 4°C, resuspended with PBS, aliquoted and transferred to -80 °C for long-term storage.

Hemagglutination assay

Phosphate buffered saline (PBS, 25 µl) was pipetted into wells of 96-well V-bottom plate. 25 µl of PBS containing viruses was added into the first column of the plate. A two-fold dilution series across the 96-well plate by transferring 25 µl was performed. 25 µl of 0.5% red blood cells were added to all wells. The plate was incubated for 30 minutes. The endpoint of agglutination was observed and titer recorded per 25 µl of the sample.

Preparation of proteins and viruses

Lectins were dissolved in 10 mM PBS solution with appropriate pH (MAA at pH 7.4 and DSL at pH 6.8) at concentration of 1 mg ml^{-1} and were stored at -20°C in aliquots. H3N2 and H7N7 viruses were dissolved in 10 mM PBS, pH 7.4 with concentration 256 HAU (hemagglutination units, 1 HAU~$1x10^6$ viruses) in 25 µl and stored at -20°C in aliquots. Intact viruses were inactivated by UV radiation and the glycan biosensor was then incubated with 40 µl of the stock solution of the virus. Human serum albumin (HSA) was stored at -20°C in aliquots with concentration of 1 mg ml^{-1} in 10 mM PBS, pH 7.4.

Electrochemical impedance spectroscopy (EIS)

EIS was measured in an electrolyte containing 5 mM potassium hexacyanoferrate(III), 5 mM potassium hexacyanoferrate(II) and 0.01 M PBS, pH 7.4. The analysis was run at 50 different frequencies (ranging from 0.1 Hz up to 100 kHz) under Nova Software 1.10 (Ecochemie, Netherlands). The results were shown in Nyquist plot, with an equivalent circuit R(Q[RW]). The charge transfer resistance (R_{CT}) parameter was used as the measure for the calibration of the biosensor and for real sample 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.

Quartz crystal microbalance (QCM)

QCM experiments were performed with Autolab PGSTAT 128N (Ecochemie, Netherlands) equipment using an optional EQCM module. The changes of a mass were evaluated using Sauerbrey´s equation:

$$\Delta f = -\frac{2f_0^2}{A\sqrt{\rho_q \mu_q}}.\Delta m \qquad \text{(eqn. 1)},$$

where Δf is the frequency change (Hz), f_0 is the nominal resonant frequency of the crystal (6 MHz), Δm is the change in mass (g cm^{-2}) and μ_q is the shear modulus of a quartz (g cm^{-1} s^{-2}), A is the surface area and ρ_q is density of quartz in g ml^{-1} . For a 6 MHz crystal, the whole equation can be simplified to:

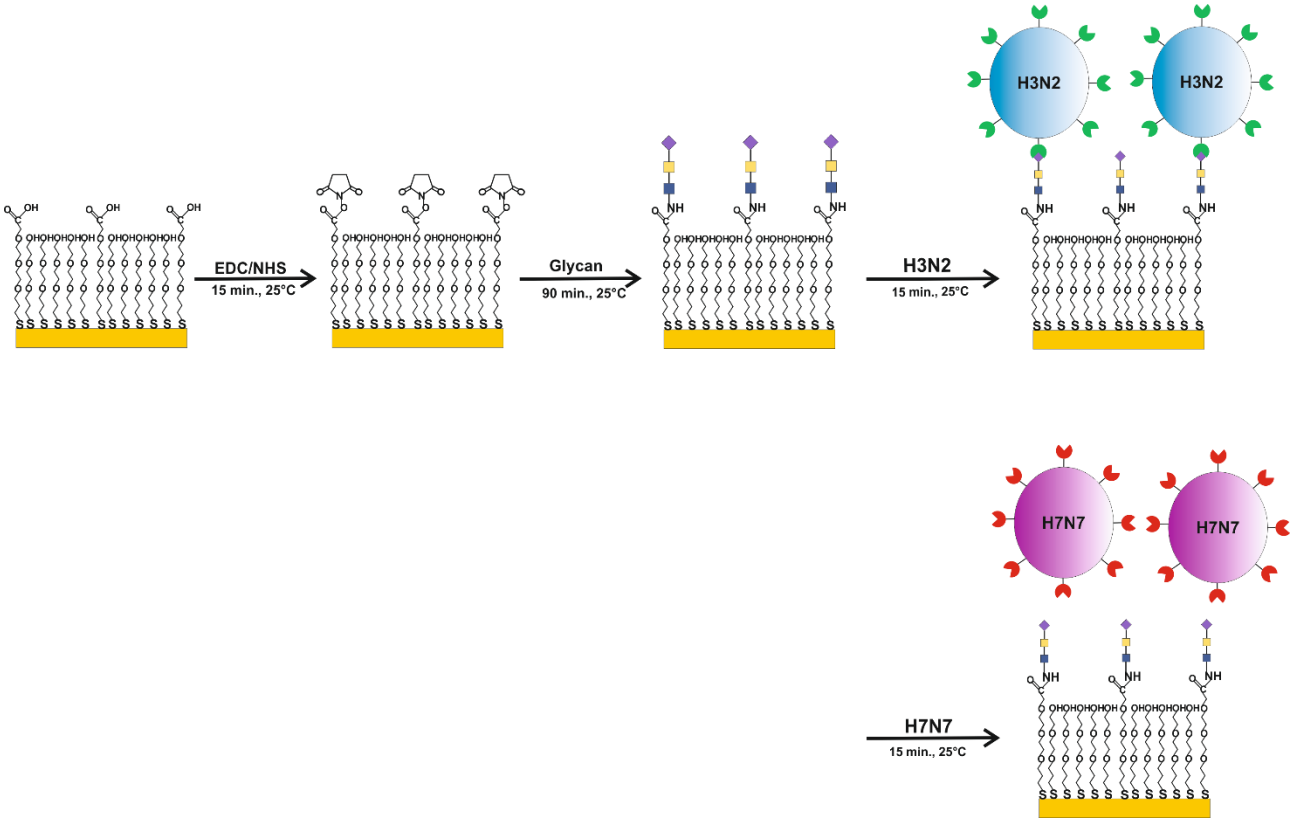
$$\Delta f = -C_f.\Delta m \qquad \text{(eqn. 2)},$$

where C_f is a frequency constant 0.0815 Hz ng^{-1} cm^2. The measurements were monitored and evaluated using the Nova 1.10 software and all measurements were run at room temperature.

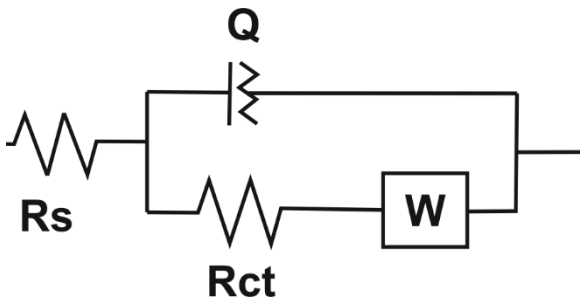
Atomic force microscopy (AFM)

AFM measurements were done on a Bioscope Catalyst instrument and Olympus IX71 microscope in conjunction with NanoScope 8.15 software at a scan rate 0.5 line s^{-1} with the tip set to 200 pN (Scan Asyst, Bruker, USA) used. Measurements were carried out with peak force tapping mode in air. Bare gold SPR sensor chip (XanTec Bioanalytics GmbH, Germany) was cleaned three times with piranha (H_2O_2:H_2SO_4 – 1:3, **caution**: *handle with a special care since hot piranha is a strongly oxidizing agent*) for 2-3 s, after that with DW and finally with ultra-pure ethanol. The SPR chip was

immediately modified with thiols and glycans as it was described previously for gold electrodes. SCANASYST-AIR silicon tip on nitride lever sharpened for a tip radius of 2 nm (Bruker, USA, with f_0=50-90 kHz and k=0.4 N m^{-1}) was used for AFM visualization.



Scheme S1. Schematic representation for development of a glycan biosensor by formation of a mixed SAM, activation of –COOH group by EDC/NHS with covalent immobilization of amine-terminated glycan. The final part shows specific interaction of the glycan biosensor with analyte (H3N2 influenza viruses), while influenza viruses H7N7 are not recognized by the glycan biosensor.



Scheme S2. An equivalent circuit applied for data evaluation from EIS experiments.

Table S1. List of parameters obtained from fitting of data presented in **Fig. 3**.

	R_s (Ω)	R_{CT} (kΩ)	W $(\mu\Omega)^{-1}$	Q - Y_0 $(n\Omega)^{-1}$	n
SAM + glycan	164	46.4	18.9	515	0.911
H3N2 10 μl^{-1}	163	57.6	16.9	508	0.912
H3N2 1000 μl^{-1}	161	94.1	12.1	498	0.915
H3N2 100 000 μl^{-1}	159	134	7.14	473	0.922

Constant phase element (CPE) represented by Q symbol; n – empirical constant. CPE behaves as an ideal capacitor, and on the contrary, when n = 0, it becomes a pure resistor (Pihikova et al. 2016).

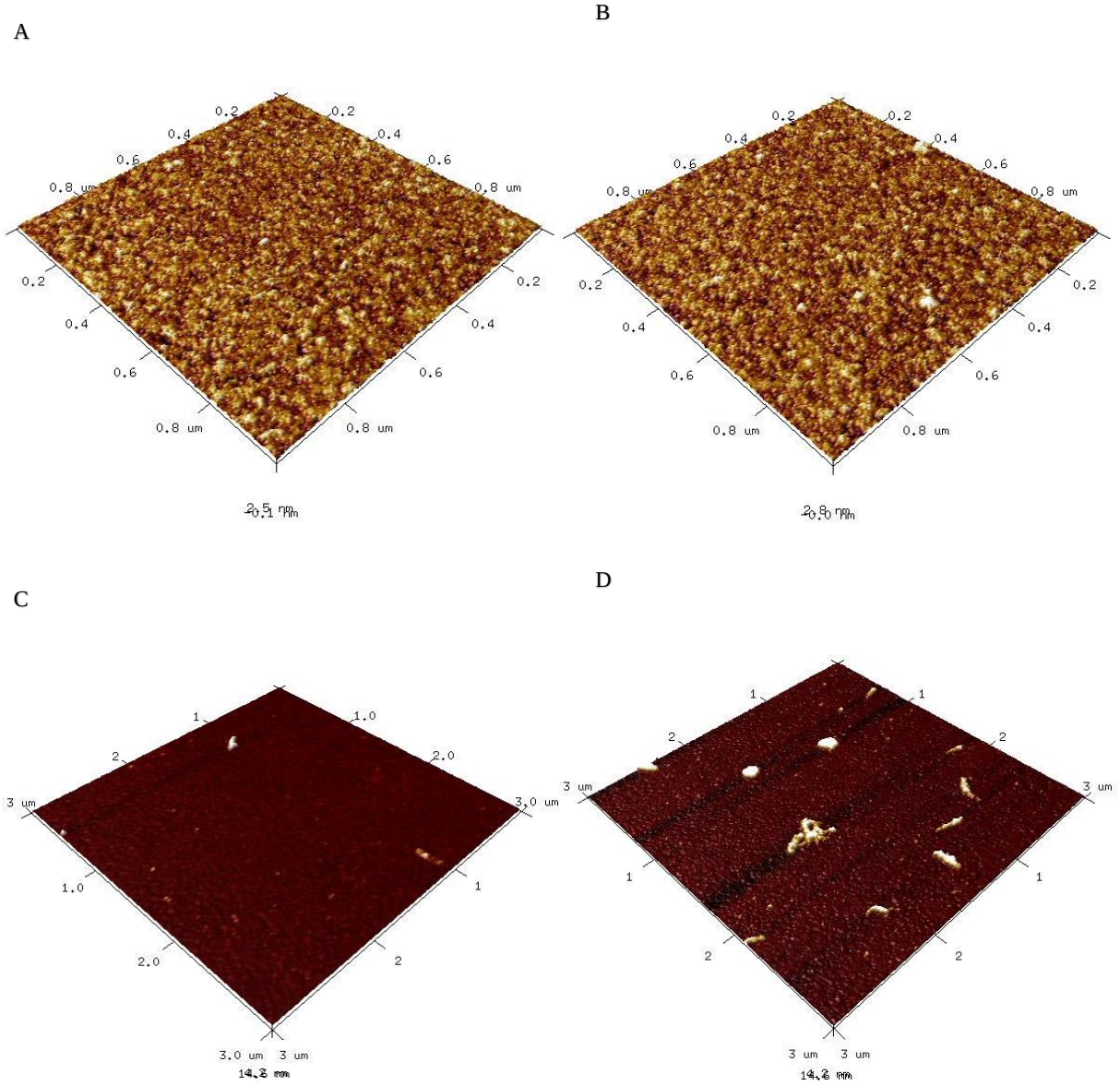


Figure S1. AFM images of various surfaces. A) Au surface 1 x 1 μm (z=5.1 nm), B) Au + SAM + glycan 1 x 1 μm (z=5.6 nm), C) glycan biosensor + H7N7 virus 3 x 3 μm (z=21 nm) and D) glycan biosensor + H3N2 virus 3 x 3 μm (z=21 nm).

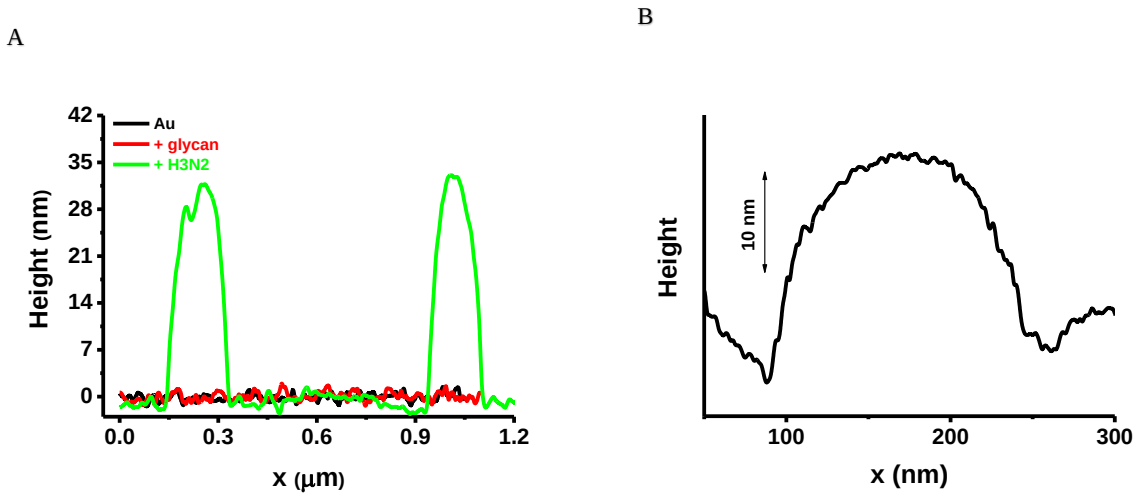


Figure S2. A) AFM profile of bare gold, glycan modified gold surface and the surface after interaction with influenza H3N2 viral particles and B) AFM profile with a focus on one influenza H3N2 viral particle.

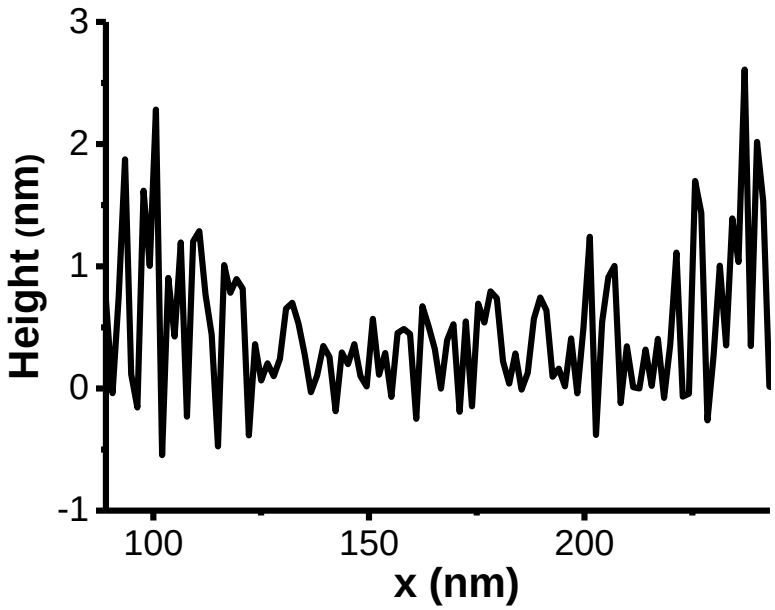


Figure S3. Profile of features appearing on the surface of a single influenza H3N2 viral particle seen in **Fig. S2B** obtaining after subtraction from a curved surface applying spline baseline.

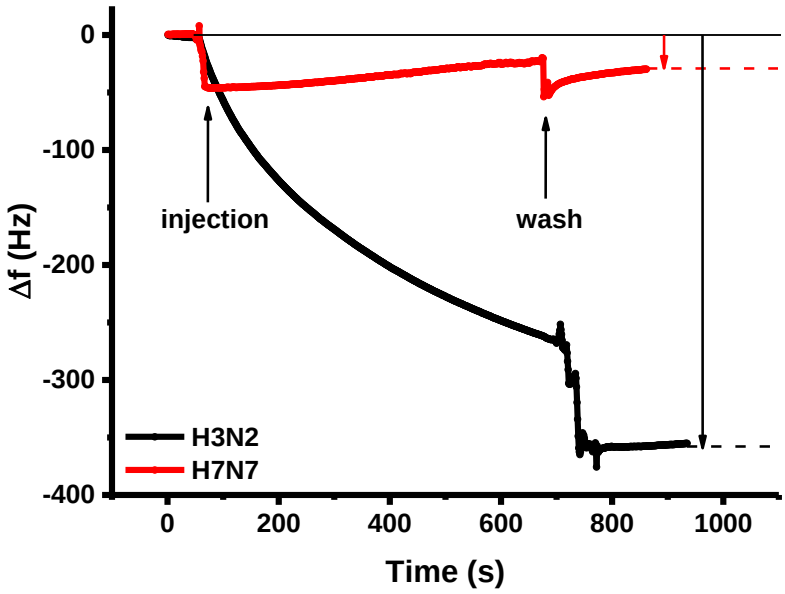


Figure S4. Validation of the specificity of influenza H3N2 viral particles binding over influenza H7N7 viral particles to the glycan biosensor surface using quartz crystal microbalance. Influenza H3N2 and H7N7 viral particles were injected with a concentration of 100,000 viruses μl^{-1}.

References

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