Supporting Information

Attomolar Detection of Influenza A Virus Hemagglutinin Human H1 and Avian H5 Using Glycan-blotted Field Effect Transistor Biosensor

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Materials

The influenza hemagglutinin (HA) (A/H1N1/New Caledonia/20/1999, A/H5N1/Vietnam/1203/2004, and A/H5N1/Indonesia/05/2005; recombinant, full-length, and maintaining the oligomeric structure of the protein) was purchased from Protein Sciences Co. (Meriden, CT, USA). The glycans, Siaα2,6'Lac (6'-sialyllactose, Neu5Acα2,6GalGlc) and Siaα2,3'Lac (3'-sialyllactose, Neu5Acα2,3GalGlc), were purchased from Carbosynth Limited (Berkshire, UK). Self-assembled monolayer reagent, 3-aminooxypropyltriethoxysilane (AOPTES), was purchased from Medicinal Chemistry Pharmaceuticals, LLC (Sapporo, Japan). Acetic acid was purchased from Wako Pure Chemical Industries, Ltd. The antibody, Anti-H1 (A/New Caledonia/20/1999), was purchased from Immune Technology Corp. Albumin from human serum (HSA) was purchased from Sigma-Aldrich Corporation. The surfactant Tween 20 was purchased from Tokyo Chemical Industry Co., Ltd., while all other chemicals were purchased from Kanto Chemical Co. Inc. The proteins were used without further purification. Phosphate buffered saline (1 × PBS) of pH 7.4 was made in-house using 137 mM NaCl, 8.1 mM $Na_2HPO_4 \bullet 12H_2O$, 2.7 mM KCl, and 1.5 mM KH_2PO_4. Diluted PBS, 0.01 × PBS (pH 7.4), was prepared by diluting 1 × PBS with ultrapure water. The HA was diluted in 1 × PBS. All the experiments handling HA proteins were conducted in a laboratory rated at biosafety level 2 (BSL-2). All used samples were treated with 1% sodium hypochlorite solution after the measurements.

Experimental details

Fabrication of the glycan-immobilized FET The glycan-immobilized FET was prepared by using the aminooxy-terminated SAM, which is reactive with the reducing end of the glycans (Scheme 1). The FETs (n-type FET; gate size: 10 μm (length) × 1000 μm (width)), which were essentially based on the technology we developed,[1] were fabricated by Toppan Printing Co., Ltd. The surface of silicon dioxide as a gate insulating film of the FET was exposed to O_2 plasma (200 W for 1 min) to introduce hydroxyl groups on the surface, followed by coating with SAM of 3-aminooxypropyltriethoxysilane (AOPTES). First, the SAM was formed on the silicon dioxide surface by immersing in 0.1% (v/v) AOPTES in toluene at 60ºC for 15 min in an argon atmosphere. Subsequently, trisaccharides terminating in sialic acid-α2,6-galactose (6'-sialyllactose, hereafter denoted as Siaα2,6'Lac) and in sialic acid-α2,3-galactose (3'-sialyllactose, hereafter denoted as Siaα2,3'Lac), (100 μM in acetic acid, pH 5.3) were allowed to react with the aminooxy moiety of aminooxypropylsilane (AOPS)-modified surface at 60ºC for 90 min. This reaction resulted in the fabrication of the glycan-immobilized FET.

Electric measurements The gate voltage (V_g) - drain current (I_d) relationship of the glycan-immobilized FET was measured and used as the reference. The measurements were made in the dark with a semiconductor parameter analyzer (2612A, Keithley Instruments Inc., USA) at room temperature in 0.01 × PBS (pH 7.4) by sweeping the V_g from -3 V to 0.5 V with a 0.1 V drain voltage. The reference electrode was Hg/Hg_2SO_4. Glycan-immobilized FETs were immersed in HA solutions for 10 min. After the immersion, the residue was washed with 1% Tween 20 containing 1 × PBS. The V_g - I_d characteristic of the HA-reacted FET was measured and compared with the reference. The threshold voltage shift (ΔV_g) was calculated.

Surface analyses by using atomic force microscopy and X-ray photoelectron spectroscopy

Surface morphologies before and after HA addition onto the FET surface were analyzed using atomic force microscopy (AFM). The topographic images of the gate surface of the FETs were investigated by dynamic mode AFM (SPM-9600, Shimadzu Co.). In the measurement, silicon cantilever (OMCL-AC240TSC2, Olympus Co., spring constant 2 N/m, resonance frequency 70 kHz) was used, and the image size was 1 μm × 1 μm with 512 × 512 pixels. Roughness parameters were obtained from AFM images. X-ray photoelectron spectroscopic (XPS) measurements were performed on a spectrophotometer (PHI-5000 Versa Probe WS, ULVAC-PHI Inc.) using an Al Kα X-ray source. Narrow-scan spectra in the N 1s region of the binding energies ranging from 395 to 410 eV were obtained.

Merit of glycoblotting method using an aminooxy-terminated self-assembled monolayer for glycan immobilization

Glycans are of key importance in life phenomena through specific glycan-protein interactions[2-4], and the technology of immobilizing glycans is expected to be applied in various related fields. Application of the aminooxy-terminated monolayer as an intermediate layer offers two advantages in glycan immobilization, *i.e.*, (1) elimination of the modification step using an additional cross-linker between reactive FET surfaces and (2) facile orientation control of the immobilized glycan. Direct immobilization of glycans at the aldehyde/hemiacetal group in their reducing end using aminooxy functional group is one of the most convenient, effective and simplest protocols.[5] In this approach, only two steps were taken to immobilize the glycans; modification of the SiO_2 surface with the AOPS layer, followed by the coupling of the unmodified glycans to the aminooxy moiety. Unlike common immobilization methods for glycans, the method does not require any other chemicals and preparation for special cross-linking, resulting in elimination of the tedious protocols such as the reaction of cross-linkers under a carefully controlled condition to avoid various side reactions.[6-8] In addition, the orientation of the immobilized glycans appears to be uniform as cell surface because the well-ordered aminooxy-terminated surface is allowed to react selectively with the reducing-end sugar ring of glycan. Thus, the glycan immobilization method based on the glycoblotting in this study with a flexible and hydrophilic linker formed by the opening of the reducing-end sugar ring, leads to the high specificity to capture the target proteins and the suppression of the nonspecific adsorption.

Limitation of the detection of FET biosensor by Debye screening effect

In buffer solutions, an electric double layer is formed near the FET gate with a thickness within the range of the solution Debye length. For the aqueous solution, this Debye length is given by

$$\lambda_{\mathrm{D}} = \sqrt{\frac{\varepsilon_0 \varepsilon_w kT}{2 \times 10^3 N_{\mathrm{A}} e^2 I}}$$

where ε_0 is the permittivity of water at 25˚C, ε_{w} is the vacuum permittivity, k is the Boltzman constant, T is the absolute temperature in Kelvin, N_A is the Avogadro constant, e is the elementary charge, and I is the ionic strength of the buffer solution. The response of the FET biosensor depends on the ionic strength of the measuring solutions. The intrinsic charges of the target proteins within the Debye length could be detectable by the FET, thus the charge-detectable region needs to be effectively used. In the present study, we propose the use of a smaller receptor, glycan, to realize an efficient application of the region than the antibodies, resulting in the highly sensitive detection.

Atomic force microscopy observation of the glycan-immobilized surface caused by the addition of proteins

To examine the specificity of the glycan-immobilized surface to HA, the morphological changes were characterized by atomic force microscopy (AFM) (Figure S1). Changes in surface morphology were observed after immersion of the Siaα2,6'Lac-immobilized surface in a 50 nM (10 μg/mL) solution of human influenza virus H1 HA (A/H1N1/New Caledonia/20/1999) for 10 min with an increase in the root mean squared value of surface roughness from 0.439 nm at 1 μm × 1 μm area for the Siaα2,6'Lac-immobilized surface to 0.726 nm after the immersion. On the other hand, the surface roughness after the immersion of 150 nM (10 μg/mL) solution of human serum albumin (HSA) for 10 min was 0.470 nm, suggesting that nonspecific adsorption of HSA onto the glycan-immobilized surface hardly occurred.

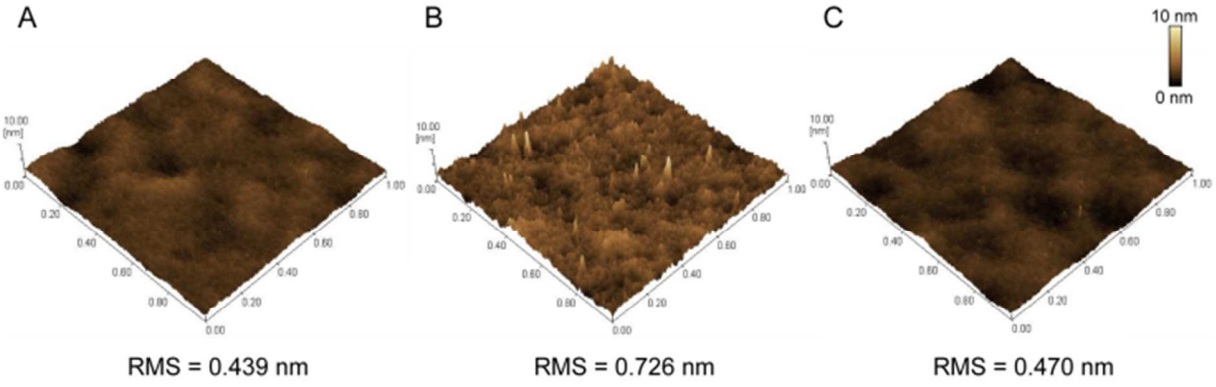

Figure S1 Atomic force microscopy images of surfaces of glycan-immobilized FETs treated with human serum albumin (HSA) and HA. The images show the change of surface morphology before (A) and after the treatment with proteins (B, HA and C, HSA). The scan size was 1 × 1 μm^2. These AFM observations were made in air. Z range = 10 nm.

Optimization of the reaction condition for glycan immobilization

To optimize the reaction condition to produce the glycan-immobilized surface, we examined nonspecific adsorption of HSA on the glycan-immobilized surface made by changing the concentration and the reaction time of the glycan solution by using AFM (Figure S2). The concentrations used were 100 μM and 1 mM, and the reaction time was in the range of 10 min to 90 min. Nonspecific adsorption of HSA was suppressed when the reaction time was increased, suggesting that the increased time promoted the reaction between the glycan and AOPS-modified surface. The concentration of the glycan solution less influenced the suppression of the nonspecific adsorption. The concentration (100 μM) and the reaction time (90 min) were determined to the optimal for the glycan immobilization.

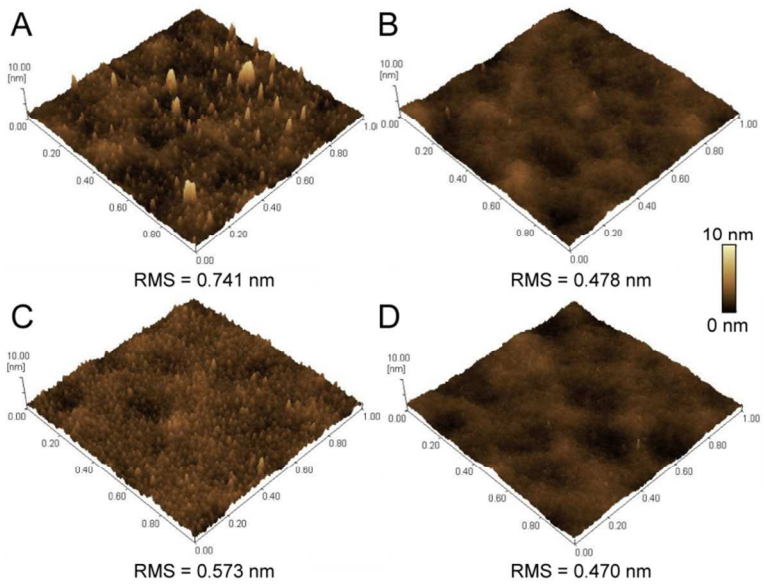

Figure S2 Atomic force microscopy images of glycan-immobilized surfaces produced by using (A, B) 100 μM and (C, D) 1 mM glycan solution. The surfaces were produced by changing the reaction time of (A, C) 10 min and (B, D) 90 min. The images show the change in surface morphology after the immersion of the surfaces in HSA solution for 10 min. Z range = 10 nm.

Characterization of the glycan-immobilized surface by using XPS

The process of the immobilization of glycan on the surface of SiO_2 substrate was characterized by XPS. The modification of SiO_2 surface with aminooxypropylsilane and the immobilization of glycan on the aminooxy-modified surface were suggested by the increase in intensity of the N 1s peak shown in Figure S3. The appearance of the peak at approximately 399 eV for the surface treated with AOPTES molecules confirms the existence of aminooxy group on the surface, and the increased intensity after the reaction with glycan (Siaα2,6'Lac) is attributed to the acetylamino group in the sialic-acid moiety.

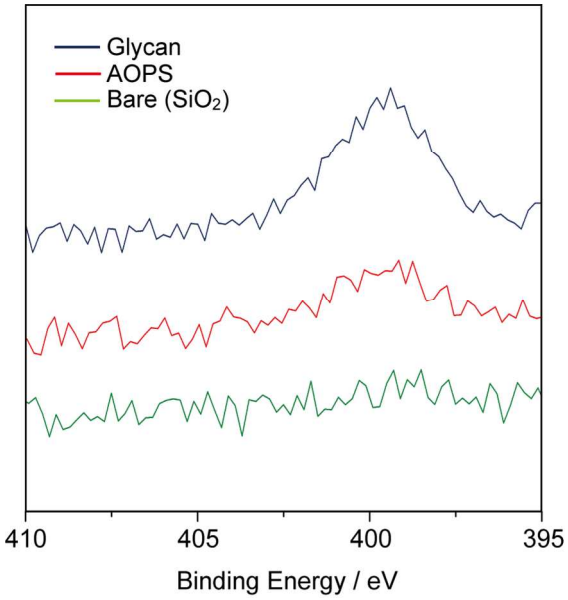

Figure S3 Narrow-scan X-ray photoelectron spectra of N 1s region of (bottom) bare, (middle) AOPS-modified, and (top) glycan-immobilized SiO_2 specimens.

Changes in characteristics of FET biosensor in HA detection

Figure S4 shows the V_g – I_d characteristics before and after the adsorption of HA on glycan-immobilized gate surface. The ΔV_g shifted in positive direction by 71 mV after the addition of 500 pM H5 HA. HA possesses negative charges in PBS (pH 7.4) because the isoelectric point of HA is 6.85. The adsorption of HA molecules converted the surface charge into a negative condition, which caused the ΔV_g in this system.

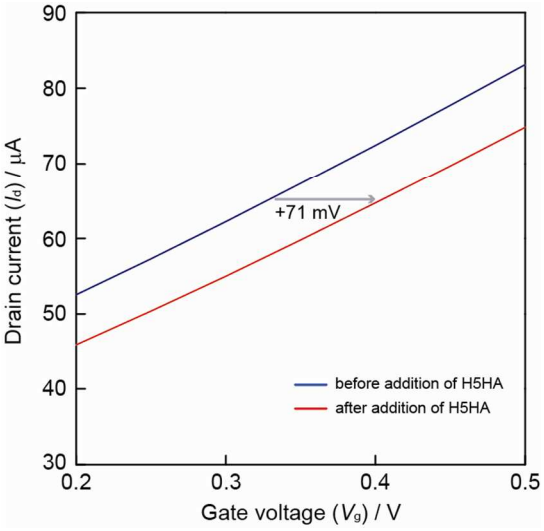

Figure S4 Gate voltage (V_g) – drain current (I_d) characteristics of the Siaα2,3'Lac-immobilized FET before and after adsorption of H5 HA (A/H5N1/Vietnam/1203/2004, 500 pM)..

Comparison of the response between glycan-immobilized and antibody-immobilized FET for the detection of HA molecules

In view of the size of antibodies ranging from 4 to 12 nm in general, glycans have the merit of effective use of the interfacial region for the detection. To investigate the cause of difference in the sensitivity between glycan- and antibody-immobilized FET's we calculated the adsorbed density of the HA molecules from the measured magnitudes of ΔV_g. The relation between the surface charge density, σ_0, of adsorbed HA molecules on the sensor surface and the magnitude of ΔV_g is given by:[9]

$$\sigma_0 = \sqrt{8000 I N_A \varepsilon_0 \varepsilon_w kT} \sinh \frac{e\Delta V_g}{2kT}$$

The change in the surface charge density corresponds to that in the number of HA molecules adsorbed on the surface. The number of HA molecules is obtained by dividing the surface charge density by a valid charge number of a single HA molecule. The valid charge number, q_{valid}, under Debye screening effect is given by:

$$q_{valid} = \sum_{i=1}^{n} q_i \, e^{-\kappa x_i}$$

where q_i and x_i are the charge number and the distance from the surface of i-th charged species of amino acids. The valid charge numbers of the single HA molecule calculated by considering the Debye screening effect are calculated to be equal to -7.9e on the glycan-immobilized surface (1 nm-above the recognition site from the surface) and -5.3e on the antibody-immobilized surface (4 nm-the above recognition site from the surface), respectively, while the total charge number, discounting the effect of the Debye screening, is -22.9e. The difference in the valid charge numbers of the single HA molecule was suggested to result in that in the magnitudes of response of FET. In addition, the glycoside cluster effect of the glycan-blotted SAM can greatly contribute

to enhance the HA-glycan interaction; the K_D value for HA-sialylated glycolipid monolayer is reported to be 10^{-10}–10^{-11} M,[10] while corresponding K_D value for monovalent glycan is 10^{-3} M,[11] and for antibody (monovalent Fab) is 10^{-4}–10^{-7} M.[12] In this study, the size of immobilized molecule is reflected in its occupation area on the gate surface, from which the number of potential sites for target binding could be higher in case for glycans than for antibodies. Thus, the immobilized glycans capture more HA oligomers by achieving the cluster effect than the immobilized antibodies which are bound to the HA molecules in the monovalent form, suggesting the enhancement of sensitivity.

Dependence of sensitivity of glycan-immobilized and antibody-immobilized FETs at different ionic strengths

We investigated the influence of PBS concentration on device sensitivity of FETs functionalized with glycan. The concentration of 5 nM HA (1μg/mL) was selected for this experiment, because it offers sufficiently high response to be discussed at various ionic strengths (0.01 × PBS, 0.1 × PBS and 1 × PBS). The glycan-immobilized FET shows clear change in the response even at the highest ionic strength, while the response of antibody-immobilized FET in 0.01 × PBS is essentially identical to that of glycan-immobilized FET in 1 × PBS (Figure S5). These results suggest that the use of size-reduced receptor allows sensitive detection of FET biosensor, and the glycan-immobilized FET has a potential to detect proteins even at the high ionic strengths.

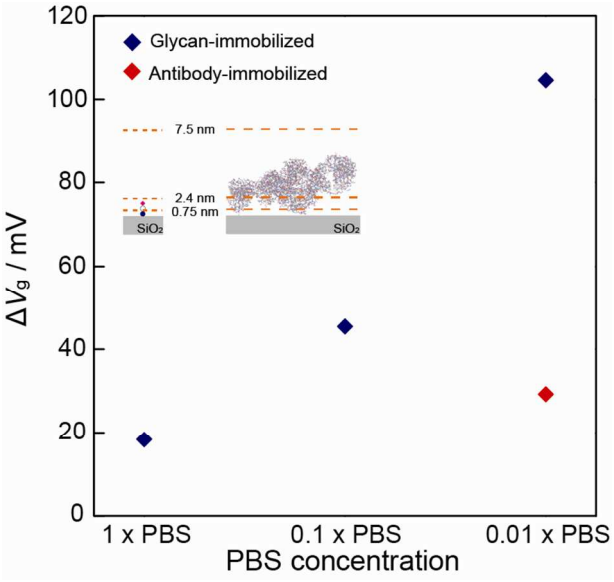

Figure S5 Relation between PBS concentration and the amount of the threshold voltage shifts caused by HA adsorption for glycan-immobilized FET and antibody-immobilized FET. The Debye length in 1 × PBS, 0.1 × PBS and 0.01 × PBS corresponds to 0.75 nm, 2.4 nm, and 7.5 nm, respectively.

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