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# Nanostructured electrochemical biosensor for th0065 detection of the weak binding between the dengue virus and the CLEC5A receptor

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**Abstract**

In this paper, we develop an effective method for detecting weak molecular bonding between the dengue virus (DV) and its receptor C-type lectin domain family 5, member A (CLEC5A). The CLEC5A–DV interaction is critical for DV-induced hemorrhagic fever and shock syndrome, so the sensing of CLEC5A–DV binding is crucial to realize a thorough study of the pathogenesis of dengue fever. Through a highly sensitive nanostructured sensing electrode of gold nanoparticles (GNPs) uniformly deposited on a nanohemisphere array, a label-free detection of the ultra weak binding between CLEC5A and the DV can be performed with electrochemical impedance spectroscopy (EIS). Experimental results demonstrate that the proposed approach is a highly promising method for investigating weak molecular interactions such as the ligand–receptor interaction of dengue fever, enterovirus (EV), or the interaction between cancer surface glycoproteins and their receptors.


*Key words:* CLEC5A; Dengue virus; Anodic aluminum oxide; Nanostructured biosensor; Electrochemical impedance spectroscopy

## Background

The dengue virus (DV) is one of the most common mosquito-borne viral diseases in humans with 50-100 million cases being recorded annually. Infection with any of the DV serotypes (serotype 1-4) causes a range of clinical symptoms ranging from dengue fever (DF), which is an undifferentiated febrile illness, to severe clinical symptoms, which are referred to as dengue hemorrhagic fever (DHF). DHF is characterized by hemorrhagic manifestations, thrombocytopenia, and plasma leakage, and has the potential to further develop into dengue shock syndrome (DSS) with a 1%-2.5% mortality rate.[1,2] Because there are no effective anti-DV drugs or vaccines available, the identification of the DV recognition receptor or entry receptors will be very helpful to illustrate the mechanism for DV pathogenesis and to provide specific treatment against DV infection.

Members of C-type lectins (CLRs) on macrophages or dendritic cells have been shown to play critical roles in DV infection.[3–6] Both the mannose receptor (MR) and Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN) receptor have been reported to regulate DV binding and entry,[3-5] while C-type lectin domain family 5, member A (CLEC5A) mediates DV-induced proinflammatory cytokines production and pathogenesis.[6] The Enzyme-linked immunosorbent assay (ELISA)-based innate immunity receptor array[7] has been used to identify DC-SIGN, DC-SIGNR and CLEC5A as DV-specific receptors. Compared to DC-SIGN and DC-SIGNR, the interaction between CLEC5A and DV is very

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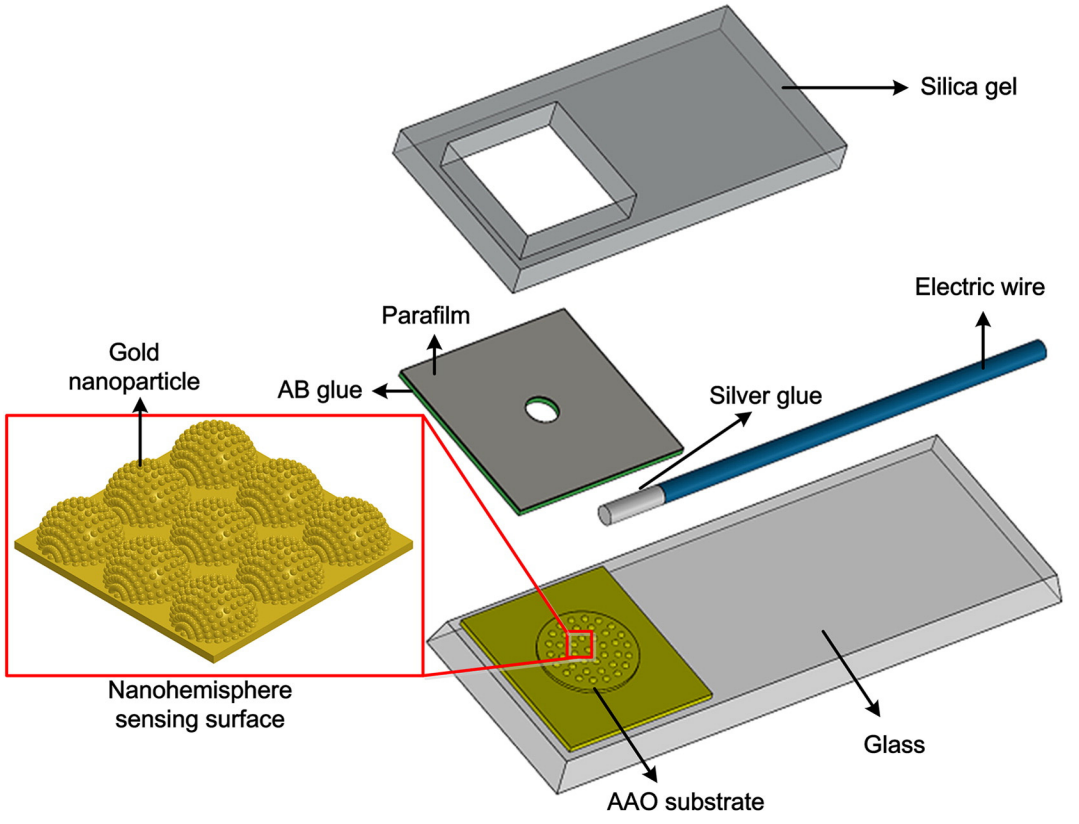


Figure 1. Schematic of the nanostructured biosensor fabrication process. The nano-hemisphere barrier layer of an AAO membrane was coated with a layer of Au thin film and attached onto a glass slide. An electric wire was connected to the electrode surface with silver glue, after which a parafilm was stuck with a ϕ 6 mm hole onto the AAO membrane with AB glue. The whole chip was sealed with silica gel to prevent leakage, and GNPs were electrochemically deposited onto the surface of each individual nano-hemisphere.

weak, and specific interactions between CLEC5A and DV can only be measured by IIR-EIA in a very narrow window. Thus, a more sensitive and reliable method would be applied to monitor the real-time interaction between CLEC5A and DV to overcome the weak bonds between lectin and its ligand. A biosensor for the effective detection of the weak conjunctions between glycoproteins and their substrates is desirable. EIS, which is sensitive to the conjugation between a receptor and its substrate through the changes of the impedance at the electrode–solution interface, has been widely employed in many biomedical devices.[8,9] EIS based biosensing schemes were employed for the detection of the covalent binding between DV and its antibody.[10,11] The antibody–antigen covalent binding is much stronger than the glycol interaction between DV and its ligand. Furthermore, the enhancement of the EIS sensitivity and detection limit is possible through the modification at the surface of the sensor electrode or exerting some physical influences during the receptor–substrate reactions, for example, using a nanostructured sensor electrode instead of a traditional flat electrode for increasing surface reaction area, decorating the electrode with conductive material (i.e. GNPs or silver nanoparticles, etc) for conductivity improvement,[12,13] and applying a micro-reciprocating motion for enhancing the probe–target conjunctions. Benefiting from recent developments in nanofabrication, EIS has undergone significant advances over the past few years.

Using carbon-nanotube as the substrate of the electrode, a sensitive sensor for the detection of mouse immunoglobulin G (IgG) has been developed.[14] Tsai et al[15] developed a novel nanostructured sensing electrode of golden AAO that detects the dust mite antigen Der p2. The high surface to volume ratio of the golden AAO biosensor enabled more probes to attach onto the electrode. The total enlargement of the effective electrode area between a golden AAO electrode and a flat Au electrode was measured to be more than 4-fold. Hence the improvement of sensor chip sensitivity not only allows the detection of tiny amounts of analytes by immuno-hybridization, but also enables the examination of the single nucleotide polymorphisms (SNPs) at myeloid differentiation-2 (MD2) gene promoter of patients with allergic diseases.[16] Palchetti et al[17] immobilized peptide nucleic acid on gold electrodes to sense the hybridization of complementary DNA by EIS. By electrodepositing GNP on a gold electrode, Ensafi et al[18] fabricated a sensitive DNA sensor of the chronic lymphocytic leukemia associated gene base on EIS. Moreover, the infection of *Salmonella* could be detected by EIS via conjugating a *Salmonella* specific single-stranded DNA probe onto the single-wall carbon nanotube electrode.[19]

Here, we took advantage of the EIS method and the nanostructured biosensor to develop an effective method for verifying the weak conjunction between the glycoprotein at the envelope of DV and CLEC5A. We immobilized CLEC5A and its iso-type proteins separately on the GNP-deposited nano-hemisphere array of the anodic aluminum oxide (AAO) barrier layer surface through a self-assembled monolayer (SAM),

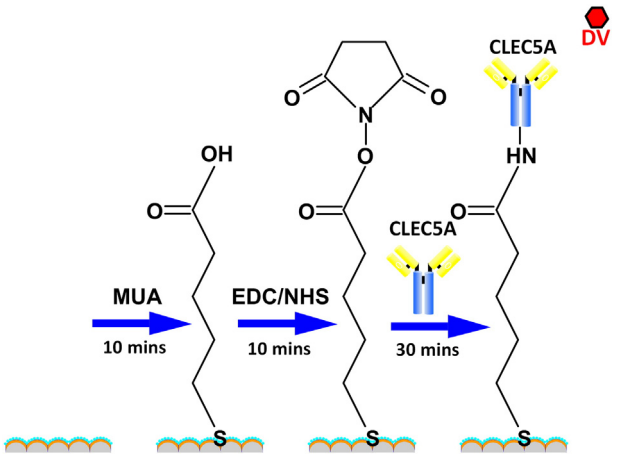


Figure 2. The SAM process used to immobilize probes onto the sensor electrode. The SAM process begins by incubating the chip with MUA, followed by treating the MUA-immobilized surface with EDC/NHS. Then, the NHS groups could be replaced by the N-terminal of the sensing probes.

followed by incubating the DV at several probes. The bonding between CLEC5A and DV can be represented by the impedance difference before and after the immobilization of DV on a CLEC5A coated electrode.

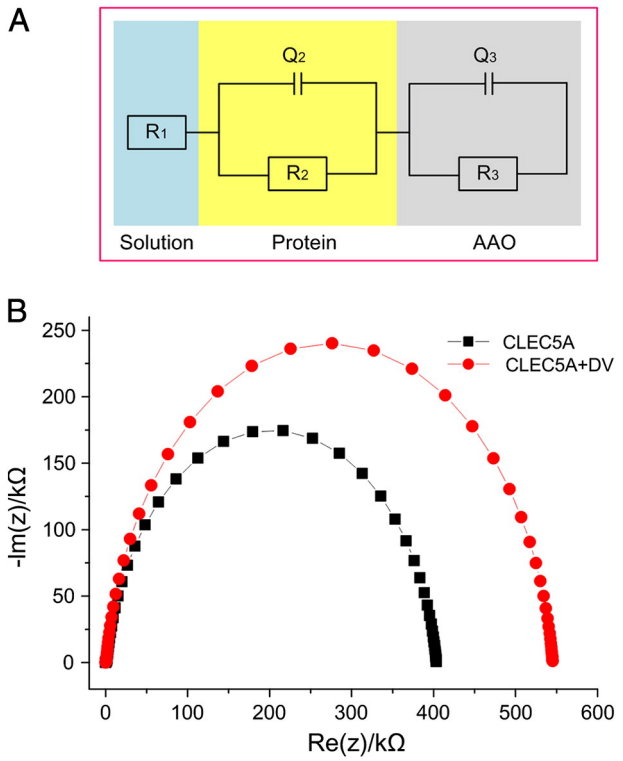


Figure 3. The equivalent circuit model for EIS and Nyquist plots for the CLEC5A–DV conjugation measurements. **(A)** The equivalent circuit model, which can be divided into a series of three electro elements that represent the resistance of the electrolyte ($R_1$), the capacitance ($Q_2$) and resistance of the probes ($R_2$), and the capacitance ($Q_3$) and resistance ($R_3$) of the sensor substrate; **(B)** The representative Nyquist plot that demonstrates the changes in the impedance before and after CLEC5A interacts with DV.

## Methods

### *Fabrication of nanostructured biosensor*

The composition of the nanostructured sensor chip is shown in Figure 1. To increase the surface reacting area, the barrier-layer surface of an AAO membrane was used as the substrate of the sensor chip. The AAO membrane was prepared by the conventional anodization process, which was described in our previous study.[15] After anodic oxidization, the non-oxidized aluminum beneath the barrier layer was removed by $CuCl_2 \cdot HCl$ solution, which was prepared by dissolving 13.45 g of $CuCl_2$ powder in 100 ml of 35-wt% hydrochloric acid solution. The nano-hemisphere structure of the barrier layer was further modified in a 30-wt% phosphoric acid for 30 min. A 10 nm gold thin film was sputtered on the surface of the barrier-layer through a DC sputter as the electrode for further electrochemical deposition of GNPs. The consistency of the working area was assured by gluing a parafilm with a $\phi = 6$ mm hole on the substrate. An SP-150 potentiostat (Bio-Logic, USA) was used to conduct the electrochemical deposition of GNPs. The gold thin film covered sample was placed at the working electrode (WE), with the gold thin film serving as the electrode. The counter electrode was constituted of a Pt film, while the reference electrode (RE) was Ag/AgCl. GNPs were uniformly deposited on the nano-hemisphere surface via using 0.5 mM $HAuCl_4$ as working electrolyte and applying a DC −0.7 V electric potential for 3 min. The whole chip was sealed with silica gel to prevent any EIS working buffer from leaking into the sensor chip.

### *Preparation of recombined sensor probe*

The IgG-like sensing probes with human IgG1 Fc region and lectin ligands (CLEC5A, DC-SIGN, or Dectin-2) at its Fab region, were constructed as described in a previous study,[6] and were overexpressed in a FreeStyle 293 Expression System (Invitrogen). In summary, $3 \times 10^7$ 293-F cells were transfected with a mixture of 40 μl of 293fectin™ and 30 μg of probe constructs. At days 3 and 5 after transfection, the culture supernatants were collected. The recombinant sensor probes were further purified from the supernatants by protein A beads (GE Healthcare).

### *Preparation of virus stock*

The propagation of the dengue virus (DV2/PL046) was performed in C6/36 cells. The viral titers were measured by plaque-forming-assays with BHK-21cells. The EV71 (BrCr strain; ATCC VR784) was propagated according to Shih's method[20] with Vero cell at 37 °C.

### *Immobilization of sensing probe*

The SAM process was applied to immobilize the sensing probes onto the sensor surface. First, we treated the electrode surface with 20 μL, 10 mM 11-mercaptoundecanoic acid (11-MUA) solution for 10 min, followed by interaction with a 20 μL mixed solution of 50 mM *N*-hydroxysuccinimide (NHS) and 100 mM 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC). Then, we incubated it with the probes for 30 min (15 μL, the concentration of the implemented probes was 0.02 μg/μL for hIgG1and 0.012 μg/μL

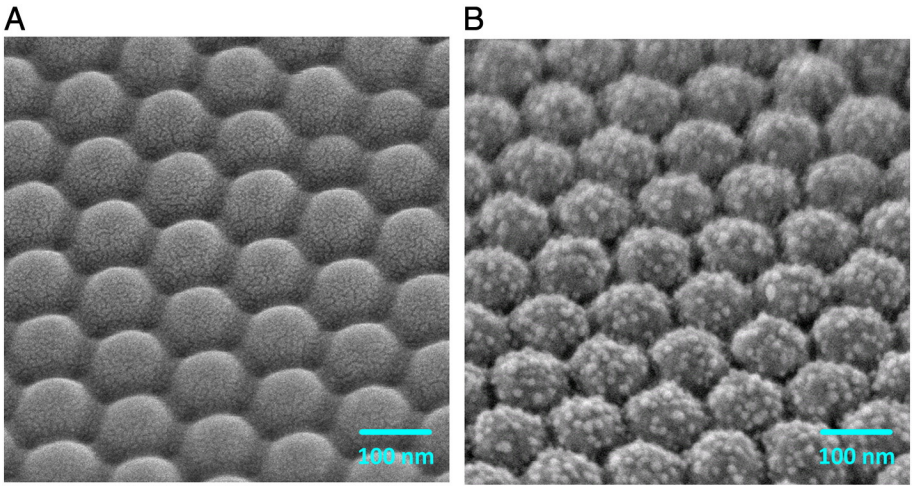


Figure 4. SEM images of the barrier layer before and after the deposition of GNPs. **(A)** A nano-hemispheric barrier layer surface with a nano-hemisphere of 200 nm after anodic oxidiation; **(B)** GNPs (less than ϕ 10 nm) were uniformly deposited on the surface of each nano-hemisphere.

for the other probes). The sensor chips were then blocked with the culture medium for 45 min, after which the sensor chip was incubated with DV (DV titer is $9.5 \times 10^7$ plaque forming units/mL; pfu/mL) or enterovirus (EV) for another 30 min (Figure 2).

*EIS detection and quantification*

The changes in the charge transfer resistance before and after the incubation of the sensor chip with DV or EV were measured by a SP-150 potentiostat (Bio-Logic, USA). All measurements were performed in a buffer solution (PBS) with a mixing electrolyte of 5 mM $Fe(CN)_6^{4-}$ and 5 mM $Fe(CN)_6^{3-}$. The counter electrode, reference electrode, and working electrode were Pt film, Ag/AgCl, and the nanostructured sensor, respectively. Each experiment result was fitted into the selected equivalent circuit model, as shown in Figure 3, *A* to calculate the charge transfer resistance. The charge transfer resistance changes before and after the incubation with the target virus was demonstrated in Figure 3, *B*.

*Statistical analysis*

The differences in the charge transfer resistances between the CLEC5A experimental group and the other control groups were expressed as mean ± SEM. A Student's *t* test from the Prism software package (GraphPad) was used to analyze the statistical significance of the differences, and a two-tailed *P* value of $< 0.05$ was considered to be significant.

## Results

*Characterization of nanostructured sensing surface*

The nanostructure of our sensor surface was analyzed using a scanning electron microscope (SEM) (Figure 4). Through our fabrication process, a modified AAO barrier layer of uniformly distributed nano-hemispheres with a diameter about 200 nm was formed (Figure 4, *A*). GNPs with an average diameter of less than 10 nm were uniformly and compactly deposited on the orderly hemispheric electrode array (Figure 4, *B*). The uniformly scattering electric flux that is perpendicular to the nanohemispheric electrode attracts the positive charges carrying Au ions in the electrolyte that separately adheres to the electrode surface.

*Detecting the weak bonding between CLEC5A and DV*

As specified in the materials and methods section, HIgG1, DC-SIGN, and Dectin-1 were utilized to verify the specific bonding between CLEC5A and DV. The DC-SIGN (dendritic-cell-specific intercellular adhesion molecule-3-grabbing non-integrin; known as CLEC4L) receptor, which has strong interaction with glycans on the envelope protein of DV, was used as a positive control to demonstrate the normal ligand–receptor interaction. Because the engineered probes used in this study have an IgG-like structure, the dectin-2 receptor (also known as CLEC6A), which recognizes α-mannans in the cell wall of fungi, was used as the iso-type control to eliminate any influence of the IgG-like structure. The human IgG1 was chosen as the negative control for displaying the background signals of the experiments.

The experimental results are shown in Figure 5. We observe that $\Delta R_2$, which was calculated by subtracting the charge transfer resistance of the bare probe electrode from the charge transfer resistance of DV immobilized electrode, for the CLEC5A–DV group ($n = 7$) is significantly higher than the corresponding value in the iso-type control group (Dectin-2 + DV, $n = 5$) and negative control groups (HIgG1 + DV, $n = 7$; HIgG1 + EV, $n = 3$) ($p < 0.05$). This fact implies that the weak interaction between CLEC5A and DV, which is barely detected by ELISA, can be clearly and definitely detected with our device. In addition, a large increment of the charge transfer resistance in the positive control group (DC-SIGN + DV, $n = 6$) suggests that the affinity between DC-SIGN and DV is higher than that between CLEC5A and DV. In this experiment, we obtained the same result as Chen's previous work,[6] which was performed by ELISA, and confirmed the interaction between CLEC5A and DV with our EIS sensor chip.

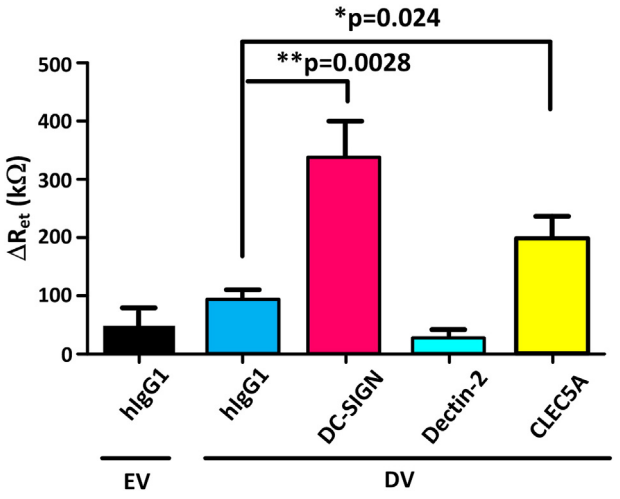


Figure 5. EIS analyzed CLEC5A–DV interaction. The interaction between CLEC5A and DV was measured in terms of the charge transfer resistance difference ($\Delta R_{et}$) through EIS. A significant increment ($p = 0.024$) in the charge transfer resistance was found between experimental (CLEC5A and DV; n = 7) and negative control (hIgG1 and DV; n = 7) groups. A more specific difference ($p = 0.0028$) of the charge transfer resistance was found between the positive control (DC-SIGN and DV; n = 6) and negative control (hIgG1 and DV; n = 7) groups. There is no specific difference ($p > 0.05$) between the negative control (hIgG1 and DV; n = 7) and iso-type control (Dectin-2 and DV; n = 5) groups.

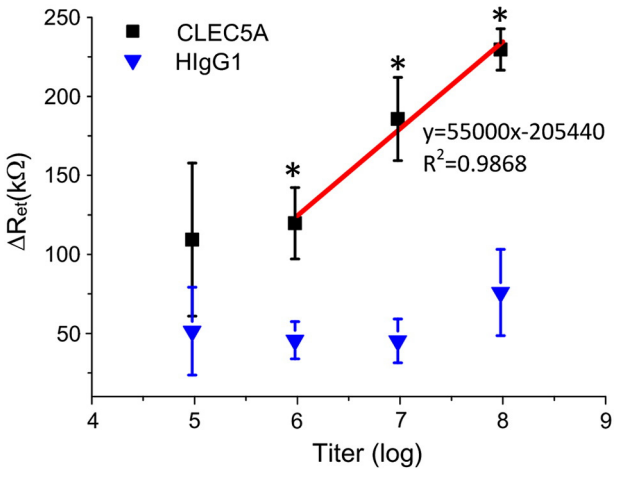


Figure 6. Detecting CLEC5A–DV interaction in different viral titers. Serial dilutions of the virus concentration were conducted to investigate the detecting limit of the proposed sensing device on CLEC5A–DV interactions. The average charge transfer resistance difference (n = 3) was labeled with log viral concentrations. The significant difference of charge transfer resistance between CLEC5A–DV and HIgG1-DV with same viral concentration was found from viral titer of $9.5 \times 10^7$ to $9.5 \times 10^5$ pfu/mL (asterisk, $p < 0.05$). A trendline between CLEC5A–DV charge transfer resistance and viral concentration was found ($R^2 = 0.9868$) between viral titers of $10^8$ and $10^6$ pfu/mL.

### *Investigating the CLEC5A–DV interactions under different viral concentration*

A serial dilution of DV beginning with a virus titer of $9.5 \times 10^7$ to $9.5 \times 10^4$ pfu/mL was used to discover the limitations of our device in sensing the weak interaction between CLEC5A and DV. Compared to negative control (HIgG1 group), a significant increase of charge transfer resistances was found within virus titer $9.5 \times 10^7$ to $9.5 \times 10^5$ ($p < 0.05$), indicating that the limitation of the sensor for detecting weak CLEC5A–DV interaction might be at viral titer of $9.5 \times 10^5$. A trendline ($R^2 = 0.9868$) was found between virus titer $10^8$ and $10^6$ (Figure 6), which enables the estimation of virion titer based on the variations of the charge transfer resistance within the range of 169 kΩ to 224 kΩ. However, for a virus titer of about $10^4$, the noise became large and no significant difference was found between CLEC5A and HIgG1 group. The change of charge transfer resistance at various viral concentrations not only provides a good indicator for virus titer measurement, but also illustrates the high sensitivity of our sensing device. This trendline may be used to sense the slight changes in the number of immobilized viruses by measuring the changes in the charge transfer resistance.

## Discussion

The common approach used to realize high-throughput screening in protein–protein interaction is by using the ELISA or surface plasmon resonance (SPR) technique. ELISA has the advantages of low cost and simple operation; however, its relatively low sensitivity limits the detection of weak molecular-bonding such as the CLEC5A–DV interactions. The SPR technique is a high-sensitivity method used to screen protein–protein interactions, which has the added advantage of being label-free. However, its operating procedure is somewhat complicated, expensive, and time consuming when compared to that of ELISA. Although high sensitivity in SPR enables it to diagnose DV infections,[21] detect the rabies virus,[22] and sense the hepatitis B virus,[23] all of them were based on the specific recognition between the virus sample and its affinity antibodies, and not the weak interaction between the virus and its corresponding ligand as we proposed. Compared to these two commonly used approaches, EIS is relatively sensitive compared to ELISA, and is more simple than SPR, and has been widely applied for detecting small amounts of molecules, analyzing the pinhole defection of the self-assembled octadecanethiol monolayer,[24] distinguishing the SNPs in DNA samples, and serving as an effective tool for sorting cells according to their different impedances.[25] However, to our knowledge, this is the first time that a simple biosensing device is being used as an efficacious tool specified for the detection of weak molecular interactions. Even though the interaction between CLEC5A and DV was determined by Chen et al[6] through ELISA, difficulties remain in understanding its clear mechanism and replicating the experiment due to the low affinity between CLEC5A and DV. With the successful detection of the weak interaction between CLEC5A and DV, our method provides a promising approach for the screening of other kinds of weak molecular bonding, especially for those that occur within glycans and proteins, such as the interactions between P-selectin glycoprotein ligand-1 and Enterovirus 71 (EV71),[26] or the tumor metastasis related protein–glycol conjunction.

Compared to our previous study,[6] which is the only work that has reported the interaction between CLEC5A and DV through

ELISA with a virus titer of $5 \times 10^6$ pfu/mL plaque-forming units and probes with a concentration of 1 μg/well, the number of probes used in this study is about one-fifth (0.18 μg/chip) of that of the ELISA. However, our device can still detect CLEC5A–DV interaction at viral concentration of $9.5 \times 10^5$ pfu/mL, indicating that the sensitivity has been enhanced by about 5-fold when compared to ELISA method. This high sensitivity implies that even though most of the conjugated virus is washed off during the experiment process due to the weak interactions between glycoproteins, our method can still provide a sufficiently strong signal for distinguishing the difference between experimental and control groups. The proposed approach not only simplifies the experiment process, but also increases the reproducibility of experiments. Furthermore, the use of the culture medium as the blocking material also facilitates the applications of our device to blood plasma samples due to the similar component within the culture medium and blood plasma.

Some improvements may be done to further enhance our device's performance. To reduce the background signal observed during the experiments, we modified our blocking process to minimize the influence of environmental factors. According to our preliminary results, a severe background noise overwhelmed the signals of the probe–analyte interactions when bovine serum albumin (BSA) was used as the blocking material. However, the background signals reduced dramatically when the culture medium was used as the blocking material. This was probably because the virus particles had been preserved in the culture medium, and the traditional BSA-based blocking process may not be adequate to reduce the noise caused by molecules in the culture medium to interact with CLEC5A or the sensor surface due to the high sensitivity of our device. Hence, a suitable blocking material is critical for retaining the sensitivity of our biosensors. Additionally, the high morphology consistence of each chip substrate can minimize the variations observed in different experiments. Although the well-developed anodization process of AAO membranes is recognized as being relatively consistent, tiny differences between different AAO membranes may still exist. In this study, the charge transfer difference between the targets immobilized the electrode, and the electrode-coated probes were used to reduce the morphology variation effect.

The glycol conjunction between the ligand–receptor was involved in several biological mechanisms, such as cell–cell recognition and communication, signal transduction, immune response, virus infection, and cancer metastasis. Most of the interactions within glycoproteins and their corresponding receptors were hardly detected, and may therefore discourage thorough investigations on the epitopes of the ligand–receptor interactions. In 2012, Gubasekara used nuclear magnetic resonance (NMR) spectroscopy to investigate a tumor metastasis-related protein–glycol conjunction between the cytoplasmic domain of MUC1 and Src-SH3.[27] It was observed that the conjunctions did not occur in the usual binding pocket, which may result in the weak interactions between MUC1 and Src-SH3. Even though the bioinformatics technique that uses an exponentially enhanced protein database enables the prediction of possible binding sites for ligand–glycan interaction, solid evidences to confirm the computer stimulated results are still required. With regards to the DV, there has been speculation that the serotype 2 envelope proteins will interact with CLEC5A through a glycan chip by bioinformatics computer simulation.[28] However, to date there is no direct evidence to confirm this simulated result.

The protein–glycan interaction also plays an essential role in metastasis, including the loss of cell–cell conjunction for tumor intravasation and the attachment of the cancer cell onto the vessel endothelial cells for extravasation,[29] both of which may be accelerated by the truncation or mutation of the glycan structure. The abnormal modification of the glycan on proteins may lead to a weaker interaction between ligands and their receptors. Our highly sensitive biosensor can provide a good solution for investigating those weak glycol interactions, or can be used as a tool for screening the actual binding site of the protein–glycol conjugation.

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