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Influenza virus surveillance using surface plasmon resonance

Emi Suenaga, Hiroshi Mizuno and Penmetcha K.R. Kumar*
RNA Processing Group; Biomedical Research Institute; National Institute of Advanced Industrial Science and Technology (AIST); Tsukuba City, Ibaraki, Japan

The hemagglutinin (HA) proteins derived from avian influenza viruses bind specifically to the α2-3 sialoglycan (Sia glycan), whereas human-adapted influenza viruses prefer to bind to the α2-6 Sia glycan. A switch of glycan specificity from α2-3 Sia glycan to α2-6 Sia glycan appears to be critical for a virus to become pandemic, therefore, it is important to monitor the influenza virus adaptation to glycan binding. In this article, we described surface plasmon resonance (SPR) methodology for reliable analyses of HA-glycan interactions. The methodology explores the synthetic tetravalent glycans (α2-3 Sia glycan and α2-6 Sia glycans) which facilitates not only the surface capacity of the sensor chip for better SPR signal but also enhance the affinity to the HA resulting an improved sensitivity. To adopt this method routinely for multiple samples of HA or virus, CAP-chip was adopted so that the regeneration of the sensor chip can be achieved. By combining the above developments with BiacoreT100 device, it is possible to program for analyzing multiple samples in continuous fashion under closed environment. Taken together we believe the above methodology is useful in influenza surveillance to monitor the HA adaptations to glycans among influenza viruses.

Keywords: influenza viruses, hemagglutinin, glycan, surface plasmon resonance, surveillance

Abbreviations: SPR, surface plasmon resonance; HA, hemagglutinin; NA, neuraminidase; PCR, polymerase chain reaction; *KD*, equilibrium dissociation constant; SA, streptavidin

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*Correspondence to: Penmetcha K.R. Kumar; Email: pkr-kumar@aist.go.jp

Introduction

Influenza A viruses cause major respiratory tract infections in humans, birds and lower mammals and are responsible for many deaths and economic losses worldwide every year. Influenza virus belongs to the Orthomyxoviridae family and is composed of eight different single-stranded RNA segments surrounded by a lipid membrane that harbors two membrane glycoproteins, hemagglutinin (HA) and neuraminidase (NA). Among these 16 subtypes, only three HA subtypes—H1N1, H2N2 and H3N2—have successfully adapted to humans.[1-3] However, in the past four decades, a growing number of human cases of avian influenza virus infections have been identified, including H5N1, H7N2, H7N7 and H9N2 sub-types.[4-6]

The HA protein of influenza viruses binds to host cell surface complex glycans through a terminal sialic acid (Sia) with α2-3 and α2-6 linkages. This binding is essential for the infection, transmission and virulence of influenza viruses.[7,8] The crystal structures of H1, H2, H3, H5, H7 and H9 HA subtypes and their complexes with α2-3 Sia and/or α2-6 Sia glycans have been reported,[9-16] revealing specific interactions between HA and α2-3 Sia or α2-6 Sia glycans. HAs derived from avian influenza viruses bind specifically to the α2-3 Sia glycan, which is preferentially expressed in the intestinal tracts of waterfowl. By contrast, human-adapted influenza viruses prefer to bind to the α2-6 Sia glycan, which is extensively expressed on the epithelial cells of the human upper respiratory tract.[17] It was suggested that the HA of avian influenza viruses must adapt to bind to the α2-6 Sia glycan for efficient transmission to humans.[18] In pandemic sub-types of avian influenza viruses, the HA switches its glycan specificity from α2-3 Sia to α2-6 Sia by adjusting the glycan-binding pocket through a combination of mutations. Therefore, it is important to monitor the switching of influenza virus binding specificity from α2-3 Sia to α2-6 Sia glycans, especially viruses originating in birds and swine,

to understand clearly their potential for interspecies transmission.

In the past, traditional methods such as antigenic assays after isolating HA and NA from the viruses and serological analyses were commonly used for rapid detection and surveillance. However, these assays are insufficiently sensitive and require large amounts of sample. Newer label-free biosensing technologies are emerging, including optical detection platforms, such as SPR, localized surface plasmon resonance, surface-enhanced Raman scattering, fluorescence and colorimetry and several interference methods, such as microelectromechanical system cantilevers, reflectometric interference spectroscopy, interferometry and ellipsometry. Among these technologies, SPR-based analyses have attracted the most attention for many types of biomolecular interactions. The first paper published on the interaction between whole influenza virus and an antibody based on SPR was reported by Schofield and Dimmock.[19] In that study, they immobilized different antibodies on a CM5 chip, analyzed whole influenza virus interactions and reported the kinetic parameters of their interactions. As an alternative to this strategy, by exploiting the efficient glycan-binding property of HA, Hidari et al.[20] reported whole virus interactions with glycans, which were initially immobilized on the surface of liposomes and subsequently immobilized on the L1 sensor chip. These two studies suggest that SPR-based methodology may also be used to analyze influenza viruses. Further development of SPR hardware and software and parallel analyses of multiple samples may enable the development of a standard approach for influenza surveillance based on the SPR system in the near future.

Toward this goal, we recently reported a protocol (**Fig. 1**) that allows the kinetic analysis of glycan-HA interactions by surface plasmon resonance (SPR),[21] a technique that is commonly used to assess various bio-molecular interactions.[22-24] In these analyses, first, chemically synthesized biotinylated tetravalent glycans was explored to facilitate the efficient recognition of HA. HA is a homotrimer. Based on the structure of the HA-glycan complex, each trimeric HA possesses three glycan-binding sites, which are estimated to be approximately 5 nm apart. The synthetic biotinylated tetravalent glycan has four Sia glycan moieties at the distal end of the biotinylated tetravalent glycan, and our building model predicts that the distance between the Sia glycan moieties is approximately 4 nm. Thus, the biotinylated tetravalent glycan would bind to HA monovalently but would capture other HA trimers using the three remaining unbound Sia glycan moieties. Thus, each biotinylated tetravalent glycan is expected to bind maximum of four HA trimmers, facilitating higher affinity compared with a monovalent glycan. Moreover, the application of mutlivalent glycans in our studies not only mimic the natural recognition of cell surface glycans for HA recognition but also increase the surface capacity of the sensor chip to facilitate the enhance binding of HA. Next, to expand this

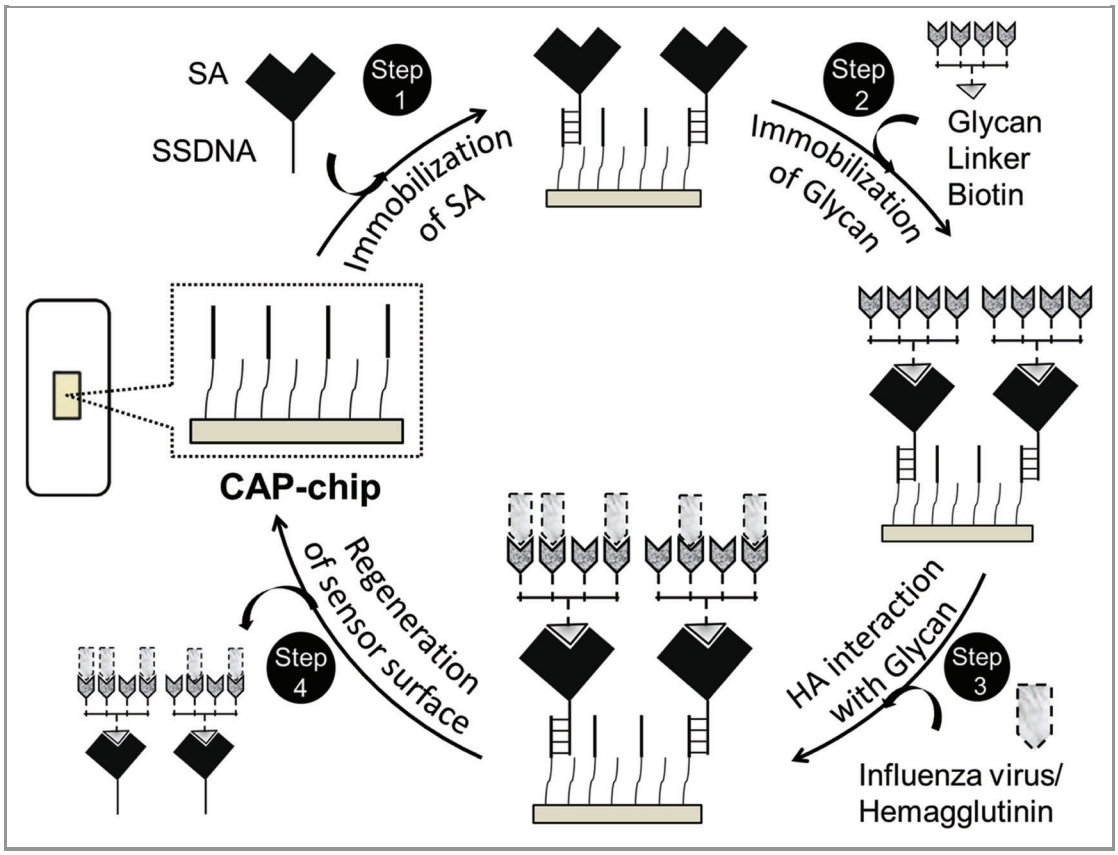

Figure 1. The SPR-based surveillance of influenza virus or HA derived from influenza viruses.

analysis for multiple HA samples derived from influenza viruses or whole influenza viruses we adopted the Biotin-CAP chip. The Biotin-CAP chip surface can be regenerated by incorporating a simple regeneration process (injecting regeneration solution), removing the whole complex (SA-Glycan-HA complex), for the next round of analyses. In terms of sensitivity, the SPR-based analyses known to provide, compared with other reported methods, specificity information with global rate constants [association rate (ka), dissociation rate (kd) and equilibrium dissociation constant (*KD*)], which allows us to monitor how well the HAs of different influenza viruses bind to α2-6 Sia glycans as compared with α2-3 Sia glycans. Importantly, combining the above developments with BiacoreT100 device, it is possible to program analyses for multiple samples continuously in a closed environment. The SPR-based methodology described here has potential applications not only in influenza surveillance to define a pandemic nature of influenza viruses but also useful for screening synthetic glycans and other compounds that may interfere with glycan-HA interactions.

Materials and Methods

Reagents. *Proteins, glycans and buffers.* The soluble form of HA of A/H5N1/Vietnam/1203/2004 (accession number AAW 80717) was obtained from Prospec-Tany TechnoGene Ltd. and was expressed in baculovirus. The purified HA of A/H3N2/Panama/2007/1999 (expressed in a mammalian system) was obtained from Immune Technology. The Biotinylated multivalent glycans; 01-077, [Neu5Acα2-3Gal β1-4GlcNAcβ1-PAA (polyacrylamide)-biotin]; 01-088, [Neu5Acα2-3Galβ1-3GalNAcα1-PAA-biotin]; 01-039a, [Neu5Acα2-6Galβ1-4GlcNAcβ1-PAA-biotin]; and 01-000, [$HOCH_2(HOCH)_4$ CH_2NH-PAA-biotin] were purchased from GlycoTech. All the SPR-based analyses of HA-glycan interactions were performed in HBS-P$^+$ buffer [0.01 M HEPES, 0.15 M NaCl, 0.05% (v/v) Surfactant P20, pH 7.4; GE Healthcare].

Equipment. We used Biacore T100 (GE Healthcare) instrument in evaluating HA and glycan interactions. This system explores the surface plasmon resonance (SPR) phenomena and exploited in the past for analyzing wide range of biomolecular interactions in a label-free environment. In this system the ligand is immobilized on the surface of the sensor chip and passed the cognate analyte (that binds with immobilized ligand) over the immobilized ligand. The binding of the passing analyte to the immobilized ligand generates a response corresponding to bound mass (down to picograms level) that depends on the bulk sample solution concentration down to nano to picomolar levels. Moreover, the interactions are monitored in real time fashion and thus possible to determine binding characteristics including rate constants.

Biotin CAP-chip (GE Healthcare) kit. The sensor chip is the main component in the biacore system which provides suitable condition for generating SPR signal. The glass surface of the sensor chip is coated with thin gold layer (~50 nM thickness) which facilitates the conditions for SPR signal. This signal is the basis for analyzing biomolecular interactions and thus, all biacore sensor chips bear this feature. To provide better environment on the surface of sensor chip, the chips are coated further with different polymers (alkanethiol or carbohydrate polymer). In the case of Biotin CAP-chip, the sensor surface was coated with carbohydrate polymer.

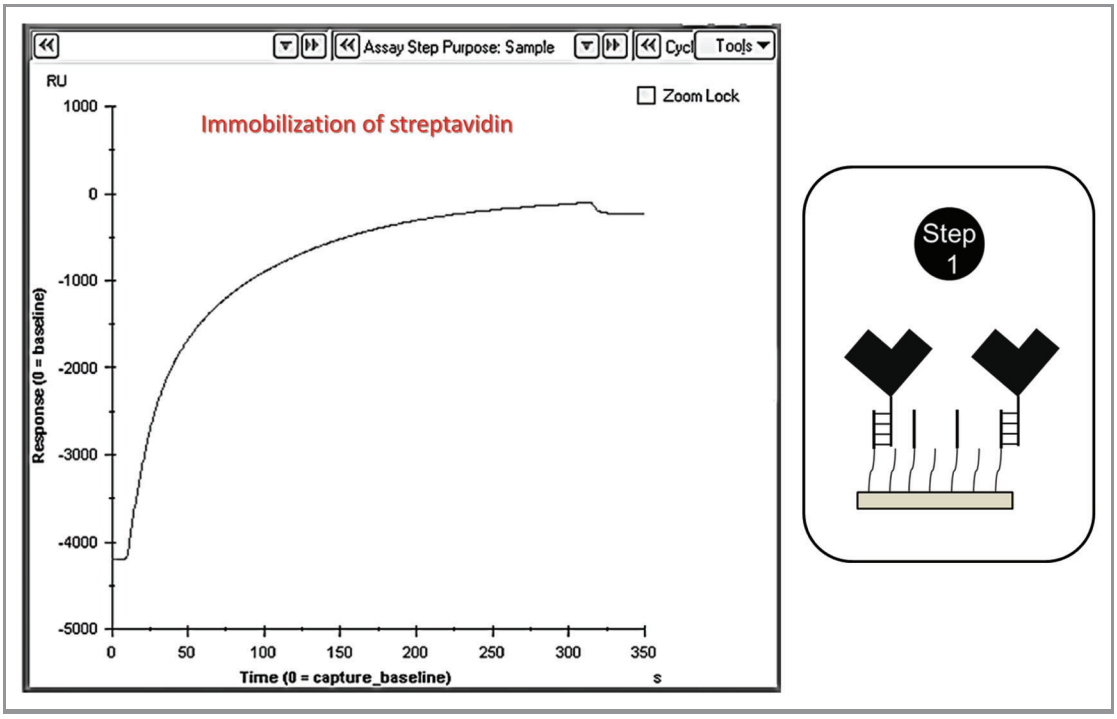

Figure 2. Injecting the biotin CAPture reagent into the flow cells (immobilization of streptavidin).

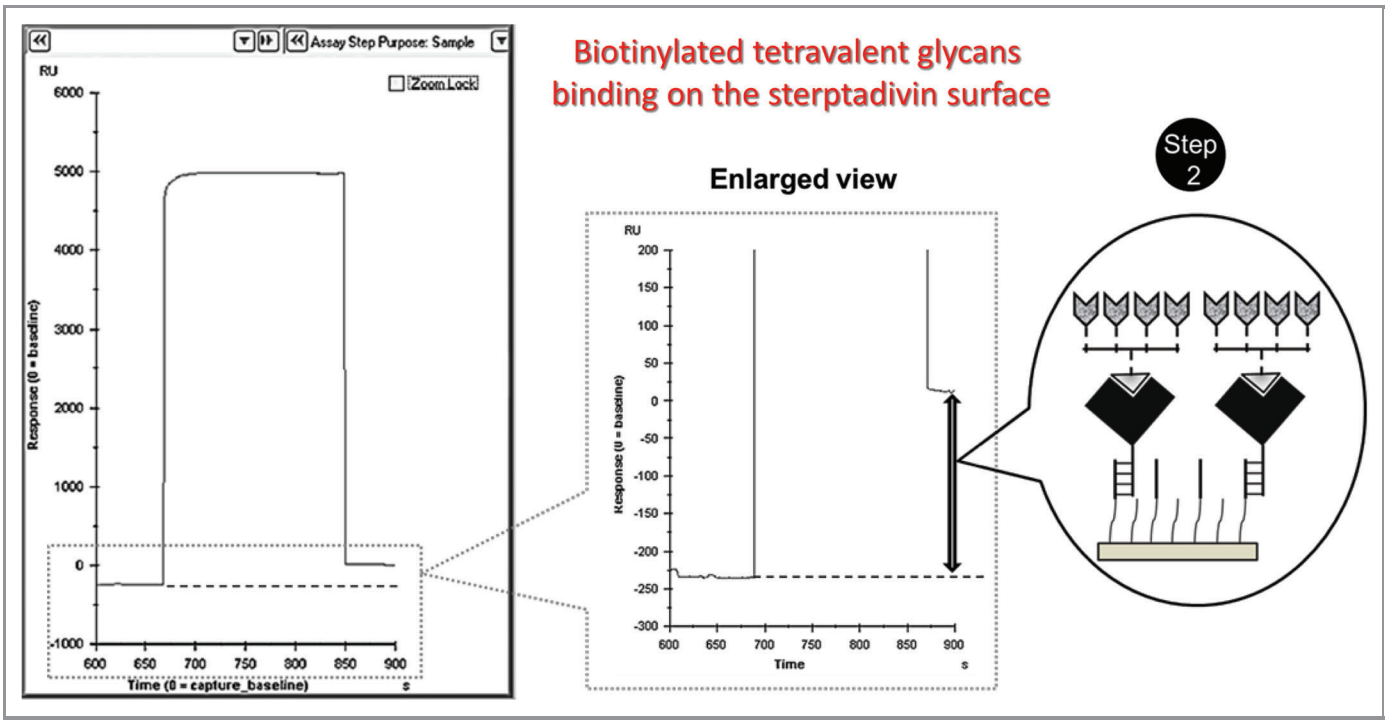

Figure 3. Immobilization of biotinylated tetravalent glycan on the streptavidin surface.

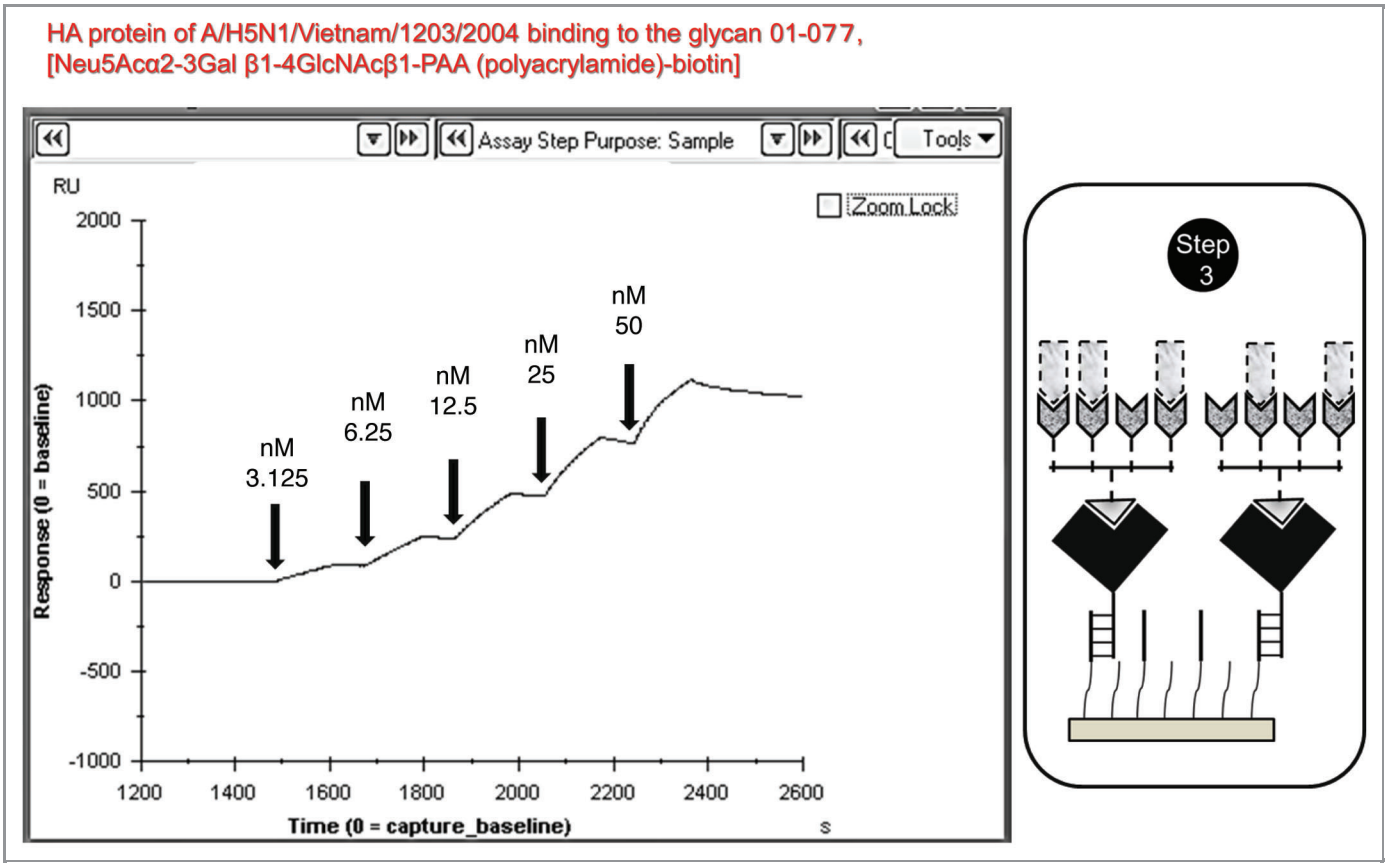

Figure 4. Biotinylated tetravalent glycan interaction with increasing concentrations of HA (single-cycle-kinetics mode).

Reagent setup. The procedure for the SPR-based surveillance of influenza virus or HA derived from the A/H5N1/Vietnam/1203/2004 virus is summarized in **Figure 1** and consists of four steps. The purified HA proteins were stored at 4°C and the diluted HA samples were used within 24 h of preparation. All the multivalent glycans stocks (33.3 µM) were stored at −80°C in 0.3 M sodium phosphate buffer pH 7.4 as small aliquots. For each analysis, the tetravalent glycans and control compounds were diluted freshly in 0.3 M sodium phosphate buffer, pH 7.4, to prepare 500 nM stocks. The diluted glycans stocks can be stored at 4°C up to three days. All buffers (10× stock solutions) were stores at room temperature and diluted freshly to 1× buffers followed by filtration (through 0.45 µM Millipore filters). The Biotin CAP-chip and supplied reagents with the kit are stored at 4°C and diluted freshly before use. Regeneration solution was prepared by mixing 3 parts of regeneration stock solution 1 (8 M guanidine-HCl) with 1 part regeneration solution stock 2 (1 M NaOH) provided by the supplier. The resulting stock should be used within 2 d when stored at 4°C. Upon completion of the analyses, the Biotin CAP-chip should be stored at 4°C.

Step 1. Immobilization of SA on the CAP chip using biotin CAPture reagent (provided by the supplier, do not dilute the reagent, GE Healthcare) at a flow rate of 2 µl/min for 5 min for all the flow cells (1–4) to facilitate the binding of biotinylated glycans (**Fig. 2**).

Step 2. Immobilization of synthetic α2-3 or α2-6 glycans on the surface through SA-biotin interactions.

(1) Initially, the control 01-000 compound (500 nM stock) was injected into flow cell 1 and allowed to bind to the SA surface for approximately 3 min at a flow rate of 10 µl/min. This compound lacks the glycan but has all of the backbone structure (biotin and PAA chain). This compound is useful as a control to eliminate non-specific interactions of HA with the backbone chain in the absence of glycan (**Fig. 3**).

(2) Next, α2-3 glycan (01-077, 500 nM stock) was injected into flow cell 2 and allowed to bind to the SA surface at the same flow rate used above. This glycan is used for the analysis of influenza viruses that frequently infect birds.

(3) Another type of α2-3 glycan (01-088, muscine type 500 nM stock) was injected into flow cell 3 and allowed to bind to the SA surface at the same flow rate. This glycan is also used for the analysis of influenza viruses that frequently infect birds.

(4) Another type of α2-6 glycan (01-039a, 500 nM stock) was injected into flow cell 4 and allowed to bind to the SA surface at the same flow rate. This glycan is used for the analysis of influenza viruses that frequently infect humans and other mammals.

Step 3. Injection of either the virus or HA into the flow cells (1–4) as analytes (step 4) at a flow rate of 30 µl/min after the tetravalent glycan surface is generated on the sensor surface. For each sample, cycles of analyte injection for 120 sec and dissociation for 300 sec and analyte-free

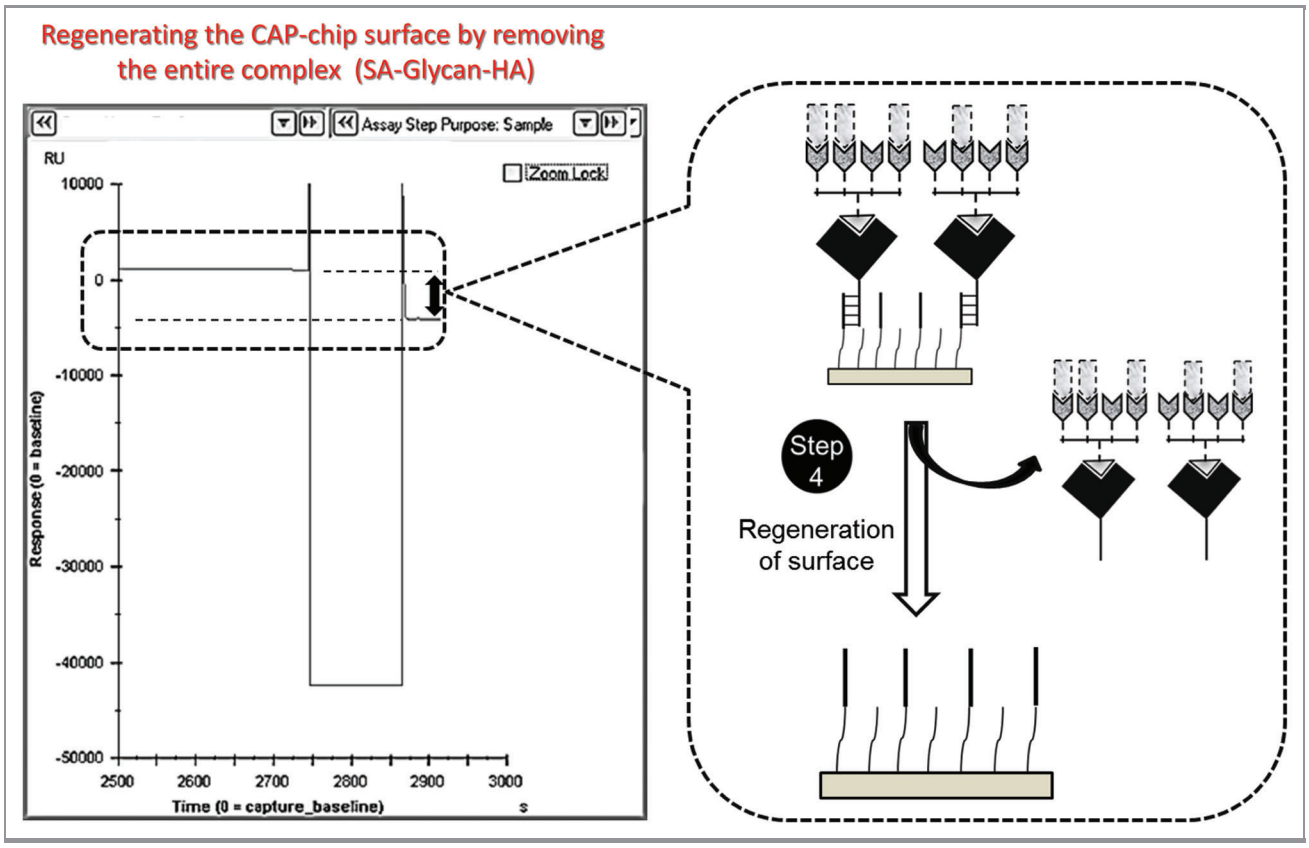

Figure 5. Regeneration of the sensor surface by stripping the entire complex.

Table 1. Potential problems and solution during the analyses of HA-glycan interaction using SPR-based analyses

Step	Observation	Probable reason	Solution
Step 1	No increase of response	Biotin capture reagent is inactivated or CAP chip is damaged	Prepare new capture reagent and change the CAP chip
Step 2	No increase of response	Glycan binding failed	Re-inject the freshly prepared glycans
Step 3	No increase of response	HA failed form the trimeric structure	Use higher HA conc. (up to 1 μM)
Step 4	The response should decrease	Regeneration is not complete	Repeat the regeneration step

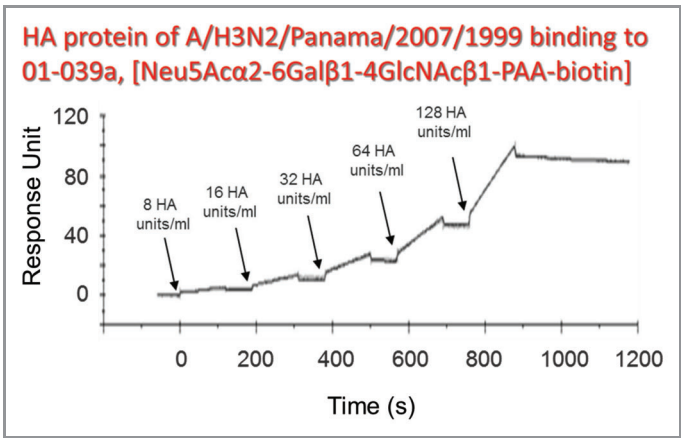

Figure 6. SPR-based analyses of HA-glycan (01-039a) interactions on the CAP chip (HA derived from H3N2/Panama) using single-cycle kinetics.

buffer injection were performed for 10 min. In general, this procedure is sufficient to obtain the response unit (RU). If analyses of the same virus or HA at increased concentrations are planned, continuous injection of samples is possible without surface regeneration (**Fig. 4**).

Step 4. Regeneration of the surface for the next sample of viruses or HA (**Fig. 5**). Using this procedure, approximately two samples can be analyzed per hour, and the entire process can be programmed without interrupting the analyses. A caution for some errors during this analysis also included for ready reference (**Table 1**).

Sensitivity of the SPR-based analyses. To evaluate the sensitivity of the SPR methodology suggested above, we performed analyses using different concentrations of purified HA derived from the H3N2/Panama virus (**Fig. 6**). Initially, the HA units in the purified HA were evaluated with an agglutination assay, and samples with different HA units/ml were prepared. The diluted samples were injected into the flow cell containing the glycan (01-039a), and continuous injection was performed with increasing concentrations of HA units (8–128 HA units/ml). The response unit was increased with increased concentrations of HA units, and a clear response was observed with 16 HA units/ml. However, the agglutination assay showed that 320 HA units/ml was required to obtain clear agglutination (data not shown).

Recent studies[25-28] of H5N1 influenza viruses suggest that HA can evolve enhanced α2-6 glycan recognition by readjusting the glycan-binding region from α2-3 glycans. The HA variants of influenza viruses that attained enhanced binding to the α2-6 glycan may have significant effects on viral attachment to the human respiratory epithelium. This step seems to be important for adapting to humans, in addition to other supporting mutations in other influenza proteins. Thus, these studies suggest that it is important to evaluate the glycan-binding properties of HA derived from H5N1, H7N7 and H9N2 viruses. We believe that the protocol described above will aid in the evaluation of HA evolution among influenza viruses. Taken together, SPR-based analyses using either multivalent glycans or high-affinity ligands, such as anti-HA aptamers,[29] may provide an efficient and rapid method for influenza surveillance, especially in a pandemic scenario.

The SPR system used in the above studies (BiacoreT100) is programmable and can analyze a number of samples automatically and continuously, and the CAP chip surface can be regenerated at least 90 times. More importantly, the SPR-based analysis is a closed system (samples are not exposed to the environment) and requires minimal time and small sample quantities for analysis, which is important when analyzing pandemic influenza viruses. The above procedure may be readily adjusted for portable SPR devices or commercially available systems to expand the application of SPR-based point-of-care influenza surveillance.

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