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Neu5Acα2,6Gal and Neu5Acα2,3Gal receptor specificities on influenza viruses determined by a waveguide-mode sensor

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ABSTRACT

To characterize the differences in the receptor-binding specificities of human and avian influenza viruses with glycan chains, the authors performed binding analyses using an evanescent field-coupled waveguide-mode biosensor. The experiments were performed on intact viruses and hemagglutinin proteins, using gold-nanoparticle-conjugated Neu5Acα2,6Gal and Neu5Acα2,3Gal glycan chains. Several influenza viruses belonging to subtypes H3N2 (A/Udorn/307/1972, A/Shandong/9/1993, A/Kiev/301/1994, A/Panama/2007/1999, A/Wisconsin/67/2005 and A/Brisbane/10/2007), H1N1 (A/Brisbane/59/2007 and A/California/07/2009) and H5N1 (A/chicken/India/NIV33487/2006) were used. High levels of glycan-based discrimination were observed with the H3N2 strain A/Brisbane/10/2007 due to its specificity with Neu5Acα2,6Gal, but not with Neu5Acα2,3Gal. Possible amino acid residues responsible for the discrimination of human and avian influenza viruses are discussed. These types of sensor-based discriminatory analyses would be very useful for distinguishing between influenza pandemics, especially during the transition and overlapping periods of human and avian influenza viruses with evolutionary changes.

1. Introduction

Influenza, also called "flu", is a viral infection that causes respiratory diseases in mammals. It is divided into three major types: A, B and C. Influenza A is found in a wide variety of bird and mammal species, whereas influenza B is mainly confined to humans. Influenza C is not very common; it infects human, dogs and pigs and may cause both illness and local epidemics. Influenza is a pandemic virus that spreads worldwide with seasonal variations; these seasonal epidemics can infect large proportions of human populations with the emergence of different subtypes (Supplementary Fig. 1). Different subtypes of influenza A according to differences in the membrane proteins hemagglutinin (HA) and neuraminidase (NA) are found in a wide variety of bird and mammal species. These subtypes of influenza A virus are classified according to their antigenicity. Recently, the H1N1 viral strain (A/California/04/2009) implicated in the 2009 flu pandemic among humans was known as "swine flu", since initial testing revealed that all the genes in the virus were similar to influenza viruses occurring in swine. To differentiate the new virus from the old seasonal A (H1N1) viruses circulating in humans before the pandemic (H1N1) 2009, the World Health Organization recently named this pandemic strain A(H1N1)pdm09. Another subtype of influenza A virus, known as "bird flu" A (H5N1), causes illness in birds, humans and many other animal species.

The HA of influenza viruses is an integral membrane glycoprotein with a trimeric receptor-binding pocket on the globular head of each monomer; it contains up to 556 amino acid residues and is the principal determinant of antigenicity. HA accounts for more than 80% of the envelope proteins present in the virus particle and is critical for binding to cellular receptors and viral fusion [1–3]. The binding of HA to sialic acid-containing receptors on the surface of its target host cell allows the virus particle to attach to the cell. Then, the virus penetrates the cell and releases its contents, including its RNA genome, into the cytoplasm; this process is mediated by fusion of the endocytosed virus particle's own membrane and the endosomal membrane of the host. Two major terminated sugar chains, Neu5Acα2,6Gal and Neu5Acα2,3Gal, preferentially bind to HA on human and avian influenza viruses, respectively. However, some closely related viral strains are exceptions that can bind non-preferential sialyl-Gal-terminated structures [4–9]. To maintain the host cell specificity, influenza viruses undergo HA-based host range selection of their receptor binding with respect to receptor sialo-sugar chains on the host; several molecular species of glycans are involved in this process [8].

At present, several anti-HA detection systems using anti-HA probes including anti-influenza aptamers and antibodies are routinely used to characterize these binding events [10–17]. Previously, using an antibody developed against the HA of the virus, the present authors developed a method of detecting the HA of

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viruses that infect humans or birds on the monolithic sensing plates of evanescent field-coupled waveguide-mode (EFC-WM) sensors [3]. The monolithic sensing plate consists of a SiO_2 glass substrate, single crystalline Si layer and thermally grown SiO_2 waveguide. Similarly, using the aforementioned probes, several diagnostic methods have been shown to be capable of detecting and characterizing these viruses [11,13–18]. Since glycans are the major determinants of influenza infection in human cells, the present study followed an approach for binding of influenza viruses with different glycans using an EFC-WM biosensor. This sensor has been used to detect the biomolecular interactions with the sensitivity range of low picomolar concentrations [3,13,19–24]. The principles and design of the waveguide sensor system used were similar to those of a surface plasmon resonance (SPR) system, the only difference being that the mode used for measurement is not a surface mode, but a waveguide mode. In the case of the SPR sensor, the wavelength of an incident light is restricted by the material used, in order to induce the surface plasmon, whereas there is no such restriction in the case of the waveguide sensor. In addition, amorphous SiO_2, one of the most popular materials of the waveguide layer of the waveguide sensor, is physically and chemically more stable than Au or Ag, which are typical materials for SPR. In addition, the advantage of the waveguide sensor over the SPR sensor is that higher sensitivity is easily obtained by perforating the waveguide layer and/or by using dyes or metal nanoparticles as a label. As a common phenomenon, using gold nanoparticles (GNP) with this sensor increases its sensitivity to the molecules [22]. The present study used GNP-conjugated glycans Neu5Acα2,6Gal and Neu5Acα2,3Gal to discriminate between human and avian influenza viruses.

2. Experimental

2.1. Reagents and biomolecules

N,*N*′-carbonyldiimidazole (CDI) was from Wako Chemicals (Osaka, Japan). Anti-HA serum for H1N1 (Brisbane/59/2007) and inactive viruses were purchased from Denka Seiken (Tokyo, Japan). The following influenza samples were from Prospec (Rehovot, Israel): intact viruses belonging to H3N2 including A/Shandong/9/1993, A/Kiev/301/1994, A/Panama/2007/1999, A/Wisconsin/67/2005 and A/Brisbane/10/2007; HA proteins of A/H1N1/California/04/2009 and A/H5N1/Vietnam/1203/2004. Recombinant HA from avian influenza virus H5N1 (A/chicken/India/NIV33487/2006) and influenza A H1N1 (A/California/07/2009), rabbit monoclonal antibodies to influenza A virus H5N1 (avian flu) HA and to influenza A virus H1N1 (swine Flu 2009) HA were from Sino Biological Inc., Beijing, China. GNP-conjugated glycans, Neu5Acα2,6Gal-GNP and Neu5Acα2,3Gal-GNP, were from SUDx-Biotec Corporation (Kagoshima, Japan). Horseradish peroxidase-conjugated anti-chicken IgY and anti-rabbit IgG were from Promega, Medison, USA. The Clear Blot membrane-P of ATTO and nitrocellulose membrane of Millipore was purchased. ECL-plus western blotting detection kit was purchased from Amersham (GE Healthcare, USA). The TMB-blotting detection solution was from Thermo Scientific, USA. Gradient polyacrylamide pre-casted gels were purchased from Wako, Japan. Protein markers were from Bio-Rad Laboratories Inc., CA. All samples were stored according to the suppliers' recommendations.

2.2. Preparation of the EFC-WM sensor chips and measurements

The EFC-WM sensor uses a sensing plate with a multilayer structure consisting of a dielectric waveguide, highly refractive index layer and glass substrate [21]. The sensing plate illuminated under the Kretschmann configuration operates as a sensor that is capable of detecting modifications in the dielectric environment near the waveguide surface by measuring changes in reflectivity. The present study used a compact optical system of SPR sensors as a spectral readout system in conjunction with the EFC-WM sensor. The optical setup is shown in Supplementary Fig. 2. In this system, a tungsten halogen lamp was used as a light source. The light from the lamp was guided to a collimator lens; the collimated light was radiated into a prism, where the incident angle was parallel to the bottom face of the prism. Then, the monolithic sensing plate placed on the bottom of a prism was illuminated, and the spectrum of the reflected light was recorded by a spectrophotometer. The prism was made of SiO_2 glass, and the bottom angle of the prism was 38°. A monolithic sensing plate developed previously was applied [21]. This consists of a SiO_2 glass substrate, single crystalline Si layer and thermally grown SiO_2 waveguide. In the present experiment, the thicknesses of the SiO_2 glass waveguide and single crystalline Si layer were 45 and 360 nm, respectively. The sensing plate was etched to show a decrease in reflectance at ~530 nm, which corresponds to the optical absorption of GNP-conjugated glycans. Upon attachment to the waveguide sensing plate, colorless material gives horizontal spectral changes, whereas metal particles show vertical changes of the spectrum. If GNP are attached to the waveguide surface, the dip is deepened by the optical absorption of the GNP [22].

2.3. Discrimination between human and avian influenza viruses on the sensor surface

Before performing the chemical modifications, the chips were treated with alkali solution for 30 min, washed thoroughly with water and dried to attach OH groups to the surface of the silica. Free hydroxide ions were removed by washing several times with phosphate buffered saline (PBS), pH 7.4. For the CDI modification, the alkali-treated sensor chip was soaked in a 0.5 M solution of CDI in dioxane. The reaction was carried out at 37 °C for 2 h, after which the chip was rinsed with acetone, followed by distilled water, and dried. On this surface, anti-HA antibody was immobilized with a higher concentration (500 nM) to facilitate the capture of the antigen to be immobilized with saturation level and blocked by 1 M ethanolamine. The coupling reaction of CDI and antibody reactions was carried out for 3 h; other incubations were performed for 30 min at room temperature. Virus samples (10 or 100 nM) were placed on this antibody-immobilized sensor surface to further enhance signal prediction by attaching GNP-conjugated glycans. Neu5Acα2,6Gal-GNP and Neu5Acα2,3Gal-GNP at 1:100 and 1:50 dilution, respectively, were used. Reactions were performed for 20 min on the shell of waveguide sensor and, after each step, the sensing surface was thoroughly washed three times with 300 μl of PBS.

2.4. Western blot and dot blot analyses

The specificity of antibody or antiserum interactions with viruses were analyzed by standard immune-blotting with appropriate antibodies. In brief, viruses of different ratios (1:5, 1:1, 2:1 and 4:1; virus: buffer) with PBS, pH 7.4, were fractionated by 5–20% gradient gel electrophoresis and transferred to polyvinylidene difluoride membranes. After immobilization of the protein on the membrane, it was blocked with 3% non-fat milk in TBS buffer at room temperature for 1 h and then treated with a 1:1000 dilution of the primary antibody (anti-serum) at 4 °C overnight. After removing the unbound primary antibody by washing, the membrane was incubated for 1 h with horseradish peroxidase–conjugated anti-chicken immunoglobulin Y. The immunocomplexes thus formed were visualized with an enhanced chemiluminescence kit. Similarly, dot-blot analyses were carried out for different

H3N2 strains. Viruses were spotted with 1 μg of each sample on the nitrocellulose membrane. To determine the specific interactions between primary and secondary antibodies, 1:250, 1:500 and 1:1000 dilutions of primary antibody were also spotted. A blocking step was performed with 3% non-fat milk in TBS buffer at room temperature for 1 h and then treated with a 1:1000 dilution of the primary antibody (anti-Udorn rabbit antibody) at 4 °C overnight. After washing, the membrane was incubated for 1 h with horseradish peroxidase–conjugated anti-rabbit immunoglobulin G. The immunocomplexes thus formed were visualized with TMB-blotting detection solution.

3. Results

Two types of glycan chains were used in this study, Neu5Acα2,6Gal and Neu5Acα2,3Gal, which recognize human and avian influenza viruses, respectively, and were tested on a sensor surface. To mimic the infection mechanism in a host cell (Fig. 1a), an antibody was attached to the CDI-modified surfaces followed by ethanolamine blocking and influenza virus or HA protein attachment (Fig. 1b). The virus or HA-immobilized surface was used to discriminate between human and avian influenza viral specificities to Neu5Acα2,6Gal and Neu5Acα2,3Gal. To attach more viruses or viral proteins, higher concentrations of antibodies were immobilized (500 nM). To enhance the signal with the sensor system, GNP with an average size of 5 nm were conjugated to the end of these glycans (Fig. 1c and d). The effects of non-specific attachments of GNP-conjugated glycans to the antibodies followed by ethanolamine blocking were determined. The results show that Neu5Acα2,6Gal-GNP at 1:100 dilution and Neu5Acα2,3Gal-GNP at 1:50 dilution give <1% reflectivity changes (Supplementary Fig. 3a and b). The experimental values obtained were considered with this background level to determine the binding of glycans on the HA-molecules of the viral membrane. To avoid cleavage of glycans on the surface of the sensor chips by NA antigen, one can adopt two ways, by either incorporating NA-inhibitors to the reaction mix or heat inactivation of viruses. In the present case, the viral samples were incubated at 55 °C as described before [25].

3.1. Intact Brisbane H3N2 virus (A/Brisbane/10/2007)

Several H3N2 strains were screened for glycan-based discrimination. Initially, the ability of Neu5Acα2,6Gal to bind to the intact H3N2 strain A/Brisbane/10/2007 without inactivation was tested in order to monitor the activity of NA. As expected, no change in the decrease in reflectance was observed upon attaching the GNP-conjugated Neu5Acα2,6Gal to the virus particles (Fig. 2a). This is due to the cleavage of sialic acid on the GNP by the enzyme sialidase (i.e., NA) on the virus particle. To confirm this result, the virus particles were heat-inactivated at 55 °C [25] and the same binding events were measured on the sensor chip. This heating process should inactivate sialidase, while maintaining the activity of the HA molecules. Upon attaching the GNP-conjugated Neu5Acα2,6Gal to the Brisbane virus-immobilized sensor chip, a clear change in decrease in reflectance was monitored, indicating the specificity of the Neu5Acα2,6Gal for this strain. Specific binding

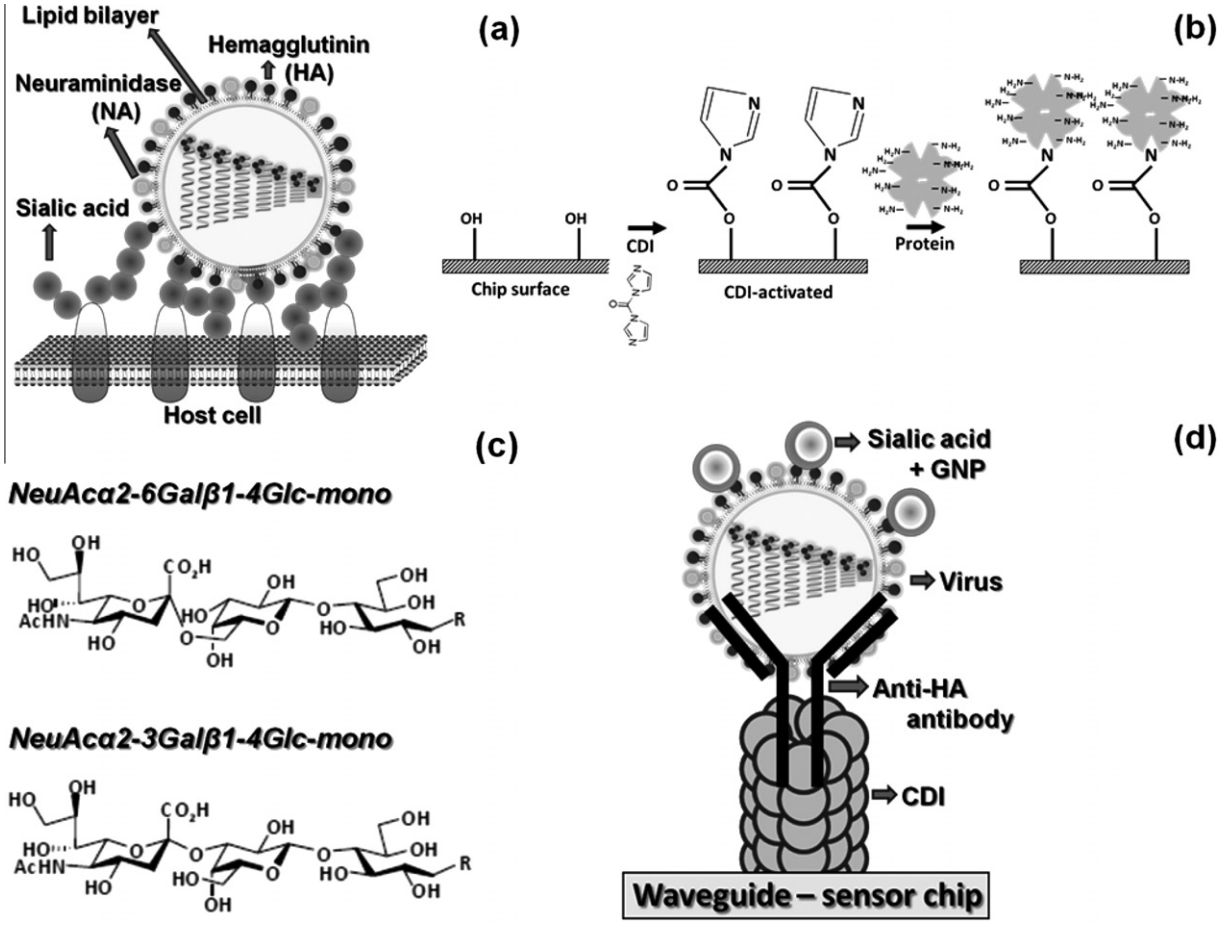

Fig. 1. (a) Representation of influenza virus–host cell interactions through sialic acid (blue spheres). (b) CDI chemical functionalization on the sensor chip used in the EFC-WM sensor. (c) Glycans (Neu5Acα2,6Gal and Neu5Acα2,3Gal) conjugated with GNP. (d) Representation of experimental setup on the EFC-WM sensor chips.

decreased the reflectivity by 25 ± 1%, with the changes appearing from 67 to 42 (Fig. 2b). However, when a similar experiment with the glycan specific for avian influenza (Neu5Acα2,3Gal) was performed, the decrease in reflectance failed in both heated and non-heated viral samples (Fig. 2c and d). These results indicate the effect of heat-inactivation of NA for the specific reaction between Neu5Acα2,6Gal and HA.

3.2. Discrimination between other intact H3N2 strains

Experiments similar to those described above were also performed with the present authors' laboratory strain, A/Udorn/307/1972, which belongs to the same virus subtype H3N2. A signal for the Udorn strain was observed (6.7 ± 0.07%) with the EFC-WM sensor using the human viral-specific glycan Neu5Acα2,6Gal (Fig. 3a). Meanwhile, Neu5Acα2,3Gal, which is specific to avian viruses, failed to bind to the Udorn strain (Fig. 3a; inset). Different incubation times with these glycans were also tested, using the Udorn strain, and similar results were obtained. However, the receptor specificity of the Udorn strain to Neu5Acα2,6Gal was less than that of the Brisbane strain. Similarly, glycan-based discrimination on different H3N2 strains such as A/Shandong/9/1993 (5.7 ± 0.04%), A/Kiev/301/1994 (4.4 ± 0.03%), A/Panama/2007/1999 and A/Wisconsin/67/2005 (2.5 ± 0.03%) were also determined (Fig. 3b–f). With A/Panama/2007/1999 virus, reflectivity changes are observed as 1.7 ± 0.02%. All H3N2 strain glycan-binding analyses were performed using a chip, on which a single anti-Udorn polyclonal antibody was immobilized. Dot-blot analyses were performed with an anti-Udorn antibody against the H3N2 viral strains used in this study, and they had affinities (Fig. 3g). A higher concentration of antibody was used to capture the viruses to be analyzed in order to obtain equal and saturation levels.

With a different approach to substantiating the above studies, purified HA for higher affinity strain (A/Brisbane/10/2007) and strains with relatively lower affinities (A/Wisconsin/67/2005 and A/Panama/2007/1999) to Neu5Acα2,6Gal were obtained. These proteins are migrated with a single protein on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Fig. 4a). As these samples are pure and do not contain sialidase, the sample was placed directly onto the antibody-immobilized sensor surface without heat treatment. Interactions between these HA-immobilized surfaces and glycans were expected to have affinities, as did those in the above-mentioned experiments; however, no changes were noted. This indicates that the binding of the HA molecules with the antibody-coated sensor chip prevents the binding of the GNP-conjugated glycans to the HA molecules. Then, the GNP-conjugated glycans were complexed with the HA molecules in vitro and attached to the antibodies. Excess HA molecules (100 nM) were also used to provide a free surface for antibody binding. Upon binding the complex on the immobilized antibody surface, one could observe the changes in the spectrum as 4.7 ± 0.02 and 1.5 ± 0.01% for the HA of A/Brisbane/10/2007 and A/Wisconsin/67/2005, respectively (Fig. 4b and c). In the case of HA from A/Panama/2007/1999, slight binding was noticed (Fig. 4d). With this experiment, it was clear that the premix of glycan and HA molecules made an ideal situation for immobilizing this complex on the capturing antibody. Values with changes in reflectivity with HA are lower than values obtained from intact viruses; this might be due to more chances of glycan (Neu5Acα2,6-Gal) binding on intact viruses. Among these three tested strains, A/Brisbane/10/2007 showed higher reflectivity changes, as observed in the case of intact virus.

3.3. Intact Brisbane H1N1 virus (A/Brisbane/59/2007)

To further elucidate the interactions between the H1N1 subtypes of human viruses and glycans, experiments were performed with the commercially available virus A/Brisbane/59/2007, and its interactions with an anti-HA serum developed against this virus were analyzed. The as-received virus sample was diluted to 1:100 and 1:600 for specific analyses. As described above, this virus was heat-inactivated to avoid the reaction between NA and the glycans. Analyses using the EFC-WM sensor system to discriminate the H1N1 influenza strain revealed that Neu5Acα2,6Gal

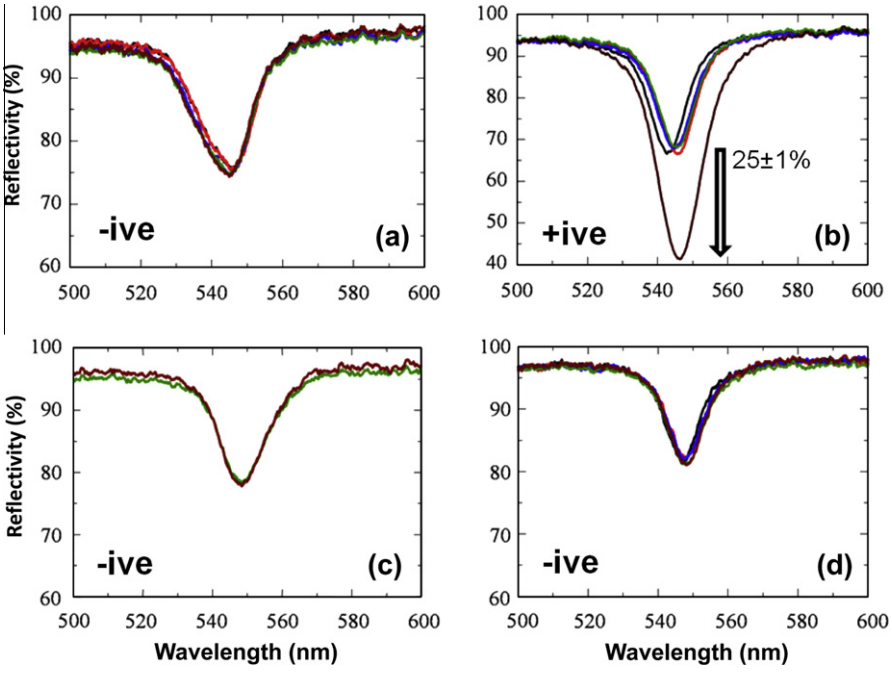

Fig. 2. Measurements on the sensor system with influenza strain H3N2 (A/Brisbane/10/2007) and glycans: (a) intact virus vs. Neu5Acα2,6Gal-GNP; (b) heat-inactivated virus vs. Neu5Acα2,6Gal-GNP; (c) intact virus vs. Neu5Acα2,3Gal-GNP; and (d) heat-inactivated virus vs. Neu5Acα2,3Gal-GNP. The black, red, blue, green and brown lines represent the reflectivity measured after the attachment of CDI, antibody, ethanolamine, virus and GNP-conjugated glycans, respectively.

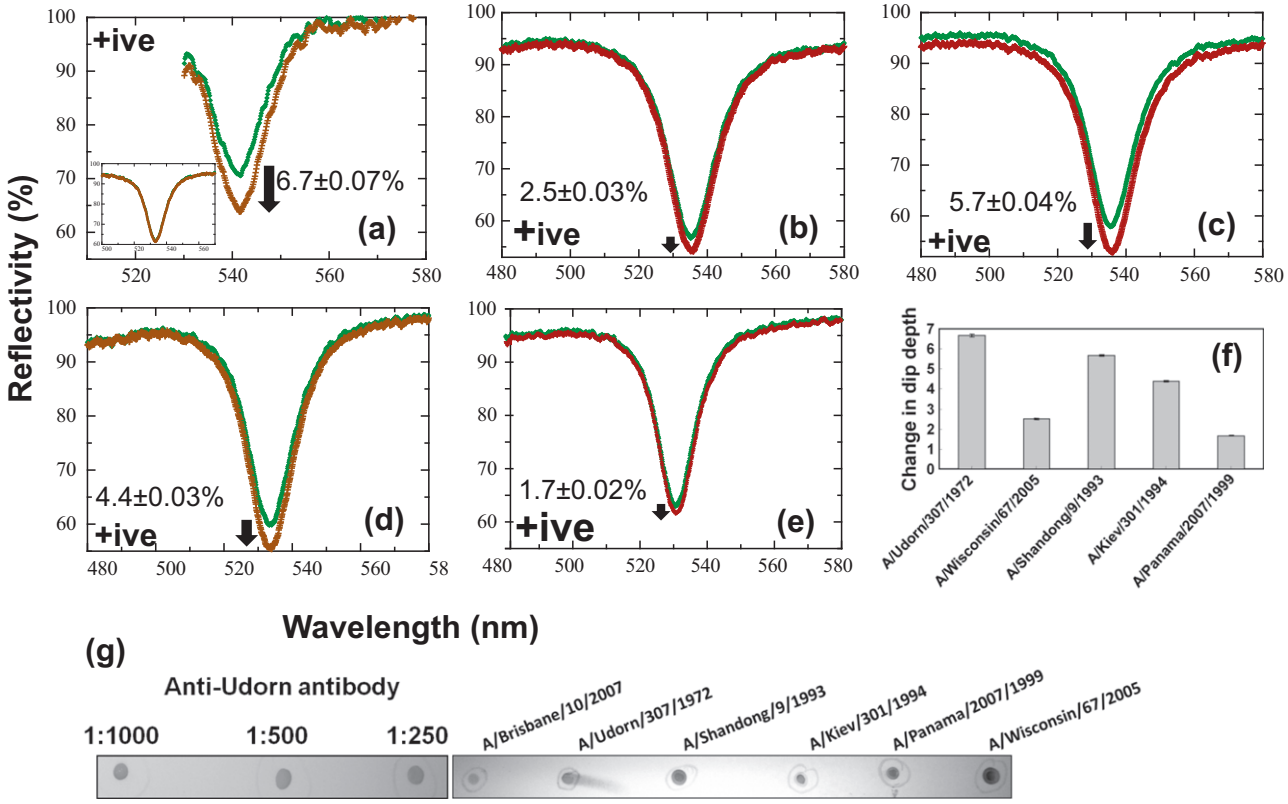

Fig. 3. Measurements on the sensor systems with different influenza H3N2 strains vs. Neu5Acα2,6Gal-GNP: (a) A/Udorn/307/1972; (b) A/Shandong/9/1993; (c) A/Kiev/301/1994; (d) A/Panama/2007/1999; (e) A/Wisconsin/67/2005. The green and brown lines represent the reflectivity measured after the attachment of the viruses and the GNP-conjugated glycan, respectively. (f) Graphical representation of comparative analyses on all tested H3N2 strains with Neu5Acα2,6Gal-GNP. The vertical axis represents the decrease in reflectivity. Averaged values are shown by error bars. (g) Dot-blot analyses of anti-Udorn antibody against H3N2 strains. Primary antibody was used with a dilution of 1:1000, and HRP-labeled secondary anti-rabbit antibody was used with 1:2500 dilution. Specificities between primary and secondary antibodies were tested by spotting 1:1000, 1:500 and 1:250 dilutions of primary antibody. The viral samples were spotted with 1 μg spot^{-1}. Interactions of A/Udorn/307/1972 and Neu5Acα2,3Gal-GNP are shown as inset (a).

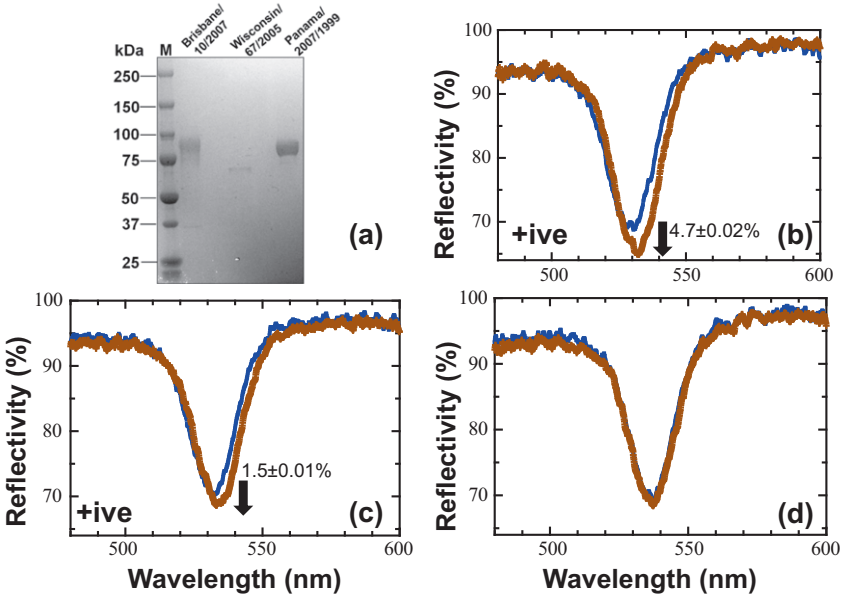

Fig. 4. Measurements on the sensor system with HA of influenza strains from H3N2 against Neu5Acα2,6Gal-GNP. (a) Purity of HA from H3N2 strains on SDS–PAGE; M = marker proteins; (b) A/Brisbane/10/2007; (c) A/Wisconsin/67/2005; and (d) A/Panama/2007/1999. The blue and brown lines represent the reflectivity measured after the attachment of ethanolamine and the premix of HA and GNP-conjugated glycans, respectively. The direction of shift in the resonance is indicated by an arrow.

clearly and specifically bound to this strain under different dilution conditions. The increments with these two dilutions were observed as changes in the decrease in reflectance at 2% and 4.5%, with the higher (1:600) and lower (1:100) dilutions, respectively (Fig. 5a

and c). In the absence of the virus, the background value exhibited a decrease in reflectivity equal to 1%. Neu5Acα2,3Gal, however, did not exhibit specific changes in the spectrum on the EFC-WM sensor in the presence of the virus at various dilutions (Fig. 5b and c). The comparative values obtained from the EFC-WM sensor are represented graphically in Fig. 5c. The specificity of anti-serum and virus interactions was confirmed by western blotting analysis (Fig. 5d).

3.4. HA from influenza A H1N1 (A/California/07/2009)

Experiments were also carried out using recently emerged H1N1, and the secreted recombinant HA of A/California/07/2009 composed of 521 amino acids was purchased. As mentioned above, the sample was immobilized directly onto the antibody-immobilized sensor surface without heating. The complexed GNP-conjugated glycans and the HA molecules under in vitro conditions were placed on the surface with antibodies. During the binding reaction between glycan and HA, Neu5Acα2,6Gal could bind with HA, which could be observed by the decrease in reflectivity (7 ± 0.04%), whereas Neu5Acα2,3Gal was not binding (Fig. 6a and b). With this experiment, the receptor specificity between Neu5Acα2,6Gal and Neu5Acα2,3Gal on HA could be distinguished using the EFC-WM sensor.

Similarly, the glycan-based discrimination with influenza strain (A/Brisbane/04/1999) reported using SPR, which works similarly to the waveguide-mode sensing system [26], was evaluated. HA of this strain was obtained from the same source and tested against Neu5Acα2,6Gal and Neu5Acα2,3Gal using a waveguide-mode sensor. The data clearly indicate that Neu5Acα2,6Gal binds to HA of the tested strain with reflection changes of 2.6 ± 0.02%, whereas other glycan Neu5Acα2,3Gal could not bind (Supplementary Fig. 5a and b).

3.5. HA from avian influenza H5N1 viruses

All the aforementioned experiments were performed using human influenza virus strains, showing a clear virtual binding with the glycan Neu5Acα2,6Gal. To evaluate the direct binding of the glycan Neu5Acα2,3Gal, the secreted recombinant HA was obtained from influenza A H5N1, A/chicken/India/NIV33487/2006, which is composed of 524 amino acids. As in the above experiment with purified HA, a mix was made in vitro of HA and Neu5Acα2,3Gal or Neu5Acα2,6Gal, before loading it on the antibody-activated sensor surface. The HA molecules of the tested avian species exhibited affinity for Neu5Acα2,3Gal (5.5 ± 0.05%), but not for Neu5Acα2,6-Gal (Fig. 6c and d).

HA of other reported [26] H5N1 strain (A/Vietnam/1203/2004) was also tested against glycans Neu5Acα2,3Gal or Neu5Acα2,6Gal. As in the case of A/chicken/India/NIV33487/2006, the virtual binding of Neu5Acα2,3Gal to HA of A/Vietnam/1203/2004 with the reflection changes of 3.0 ± 0.01% was noticed, and HA did not bind to Neu5Acα2,6Gal (Supplementary Fig. 5c and d). Based on all the above experiments, it was possible to clearly distinguish between human and avian influenza viruses or HA using Neu5Acα2,6Gal and Neu5Acα2,3Gal (Table 1).

4. Discussion

Influenza pandemics have occurred several times in recent decades. The antigen HA on the virus is the dominant target of the host antibody response [27]. However, minor mutations in the amino acid sequences on these surface antigens occur frequently, which enable the viruses to evade the host immune system (Supplementary Fig. 1). Molecular discrimination is an important event in the field of biology. In the past, several probes have been generated to distinguish between closely related species [11,12,28–30]. The waveguide sensing system shown here for discrimination of receptor specificity has several advantages, including specificity, selectivity, portability and the sensing plate with higher stability against chemical and physical alterations, and is also comparable with other sensing systems that are in vogue. The sensitivity of this sensor can be increased using GNP as a label [22]; the present authors therefore conjugated GNP to these glycans. As expected, it was possible to clearly distinguish between human and avian influenza viruses using Neu5Acα2,6Gal and Neu5Acα2,3Gal,

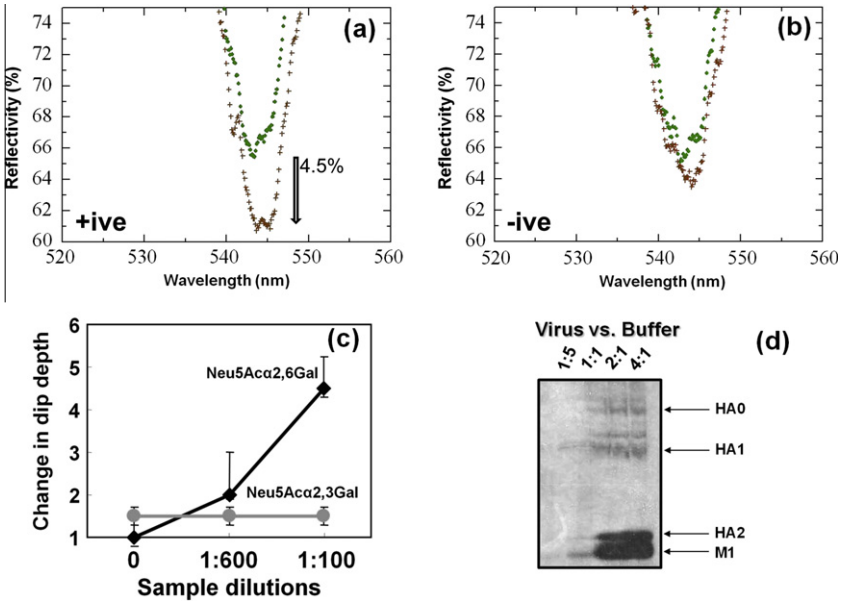

Fig. 5. Measurements on the sensor system with influenza strain H1N1 A/Brisbane/59/2007 against glycans at different dilutions: (a) heat-inactivated virus vs. Neu5Acα2,6Gal-GNP; (b) heat-inactivated virus vs. Neu5Acα2,3Gal-GNP. The green and brown lines represent the reflectivity measured after the attachment of the viruses and the GNP-conjugated glycan, respectively. (c) Graphical representations of binding reactions with different dilutions. The vertical axis represents the decrease in reflectivity. (d) Specific virus–antibody reaction shown by immunoblotting. Anti-serum was used with a dilution of 1:1000, and HRP-labeled secondary anti-chicken antibody was used with 1:2500 dilution. Detections were made using an ECL-plus western blotting system.

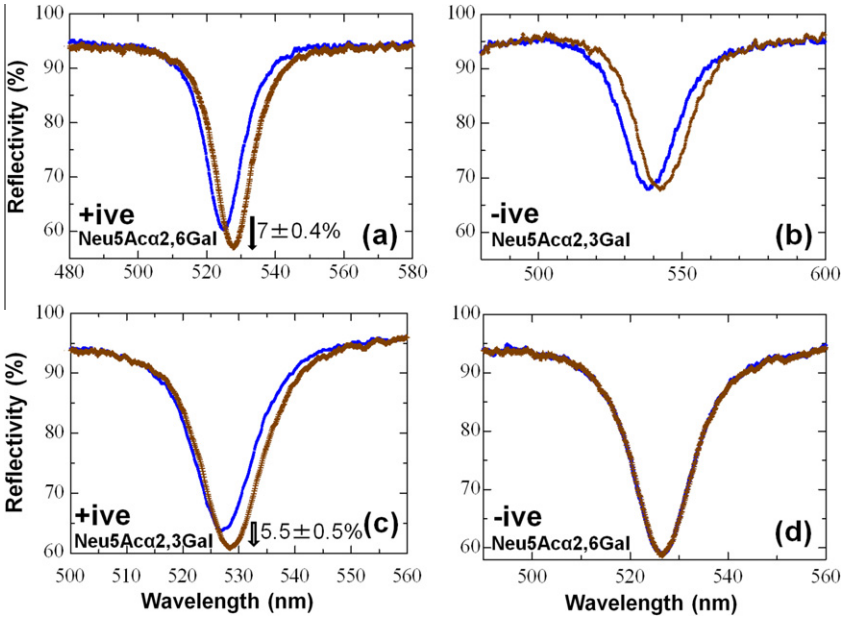

Fig. 6. Measurements on the sensor system with the HA of influenza strains H1N1 and H5N1: (a) A/California/07/2009 vs. Neu5Acα2,6Gal-GNP; (b) A/California/07/2009 vs. Neu5Acα2,3Gal-GNP; (c) A/chicken/India/NIV33487/2006 vs. Neu5Acα2,3Gal-GNP; (d) A/chicken/India/NIV33487/2006 vs. Neu5Acα2,6Gal-GNP. The blue and brown lines represent the reflectivity measured after the attachment of ethanolamine and the premix of HA and GNP-conjugated glycans, respectively. The direction of shift in the resonance is indicated by an arrow.

Table 1
Receptor binding potentials of HA with Neu5Acα2,6Gal and Neu5Acα2,3Gal.*

Subtype	Strains	Virus form	Neu5Acα2,6Gal	Neu5Acα2,3Gal
H3N2	Brisbane/10/2007	Intact	+(25 ± 1%)	–
H3N2	Udorn/307/1972	Intact	+(6.7 ± 0.07%)	–
H3N2	Wisconsin/67/2005	Intact	+(2.5 ± 0.03%)	–
H3N2	Shandong/9/1993	Intact	+(5.7 ± 0.04%)	–
H3N2	Kiev/301/1994	Intact	+(4.4 ± 0.03%)	–
H3N2	Panama/2007/1999	Intact	+(1.7 + 0.02%)	–
H1N1	Brisbane/59/2007	Intact	+(4.5 + 0.7%)	–
H1N1	California/07/2009	HA	+(7 ± 0.4%)	–
H5N1	Chicken/India/NIV33487/2006	HA	–	+(5.5 ± 0.5%)
H5N1	Vietnam/1203/2004	HA	–	+(3.0 ± 0.01%)

* All strains used belong to influenza A type. Reflectivity changes are indicated in parentheses.

respectively (Table 1). The highest receptor activity was detected between the H3N2 strain A/Brisbane/10/2007 and Neu5Acα2,6Gal; the same strain did not interact with Neu5Acα2,3Gal. Interactions were also observed between Neu5Acα2,3Gal and HA from A/chicken/India/NIV33487/2006 or A/Vietnam/1203/2004. As the mark of clear evidence, two reported strains were evaluated, which were tested by the SPR method for glycan-based discrimination [26]. Since the waveguide sensor system works on a principle similar to SPR, these reported strains [A/Brisbane/04/1999 (H1N1) and A/Vietnam/1203/2004 (H5N1)] were considered, and HA obtained from these strains could bind to Neu5Acα2,6Gal and Neu5Acα2,3-Gal, respectively. Analyses performed with these HA molecules against antibody and glycans showed clearly that the premix of glycan and HA molecules favors immobilizing this complex on the capturing antibody.

Sequence alignments of all strains used in the present experiments were also performed and the potential regions analyzed (Supplementary Fig. 4a). In the H3N2 strains, the strongest interaction with Neu5Acα2,6Gal was observed when the assay was performed with A/Brisbane/10/2007 (Ser 205, Ile 226 and Ser 228); however, this interaction was drastically reduced with A/Shandong/9/1993 and A/Wisconsin/67/2005. In these two cases, the additional changes were predicted based on the sequence homology analyses for the decrease in binding of HA to Neu5Acα2,6Gal. Mutations at V223I and G186V in A/Wisconsin/67/2005 and changes at G186S, N189S, F193S, L194V, R222W and N225G in A/Shandong/9/1993 compared with A/Brisbane/10/2007 may be the reason for the decreased affinity to Neu5Acα2,6Gal. Mutations at the amino acid 226 in A/Kiev/301/1994 and A/Panama/2007/1999 resulted in reduced binding of these strains to Neu5Acα2,6-Gal compared with binding of A/Brisbane/10/2007 to Neu5Acα2,6-Gal. These types of mutation-based discriminations and affinities have been discussed in previous studies [8,9,31]. H1N1 strains showed different amino acid residues at the major positions (205, 226 and 228), as well as less specificity to Neu5Acα2,6Gal compared with the specificity of the H3N2 strain A/Brisbane/10/2007, and no affinity to Neu5Acα2,3Gal. The amino acid sequence alignments between human (H1N1) and avian (H5N1) HA show similarities at the potential regions (226 and 228) (Supplementary Fig. 4a). It is therefore likely that the amino acids from other regions are also involved in the glycan-based discrimination of human and avian influenza viruses. The possible amino acid residues involved in the discriminating receptors for HA molecules are indicated on the three-dimensional crystal structures (*Supplementary* Fig. 4b and c). The differences in binding depend on the position of the mutation and how it distinguishes the receptors. If mutation is at critical binding region, it will show higher receptor specificities.

5. Conclusions

To characterize the differences in the receptor-binding specificities of human and avian influenza viruses, GNP-conjugated glycans Neu5Acα2,6Gal and Neu5Acα2,3Gal were used in this study, and these discrimination analyses were performed using an EFC-WM biosensor. Among the different strains tested, A/Brisbane/10/2007 belonging to H3N2 showed higher levels of discrimination by its specificity to Neu5Acα2,6Gal, but not with Neu5Acα2,3Gal. Based on the specific interactions of tested strains with Neu5Acα2,6Gal or Neu5Acα2,3Gal, the probable potential amino acid residues responsible for glycan binding were discussed. Overall, the data from the present study indicate that glycan-based discriminatory analyses using waveguide sensor systems are very useful for distinguishing between influenza pandemics during the transition and overlapping periods of human and avian influenza viruses.

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Appendix A. Figures with essential colour discrimination

Certain figures in this article, particularly Figs. 2–6, are difficult to interpret in black and white. The full colour images can be found in the on-line version, at http://dx.doi.org/10.1016/j.actbio.2012.09.027.

Appendix B. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.actbio.2012.09.027.

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