

Detection of Intact Influenza Viruses using Biotinylated Biantennary S-Sialosides

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Influenza, a highly pathogenic virus that belongs to the orthomyxoviridae family, accounts for over 150 million infections and 200000 hospitalizations every year in the U.S. alone.¹ Antivirals are a welcome tool in the fight against this contagious disease.² However, they must be administered within 48 h of infection for optimal efficacy.³ Early and accurate diagnosis of the specific strain in both a clinical and point-of-care setting would significantly facilitate a targeted and rapid response by healthcare providers. Commercial antibody-based rapid diagnostic kits offer results in 15 min., but suffer from sensitivity, selectivity, and variability issues,^{4–6} which could potentially be attributed to antibody degradation upon long-term storage at ambient temperature.⁷ Stability of recognition elements for point of care diagnostics is often underappreciated. It is a major problem in the case of influenza, as recent H5N1 outbreaks have originated in remote areas of the world, where refrigeration for reagents is not always available. Clearly, there is a pressing need to develop synthetic recognition molecules that exhibit antibody-like selectivity and sensitivity, are robust, inexpensive, amenable to scale up, and require no refrigeration for application in influenza diagnostics and therapeutics.

All strains of influenza type A virus bind to terminal sialic acid derivatives. Within type A viruses, there are several serotypes such as H1N1 and H3N2, etc. which are distinguished on the basis of the antibody response elicited in the host. Each serotype is further subdivided into several strains accounting for the enormous antigenic diversity. Binding between a particular strain and the host cell is highly dependent on the interaction of viral glycoproteins, hemagglutinin (HA) and neuraminidase (NA), with specific structural features of the glycan.⁸ Glycan microarrays (developed by the Consortium for Functional Glycomics), that utilize over sixty unique *O*-sialosides, have shown that structure (sulfation, fucosylation, etc.) of the internal sugars plays a critical role in defining HA binding events.^{9,10} While these studies have been extremely valuable in providing insight into HA specificity, NA and intact virus specificities have not been determined as NA cleaves *O*-sialosides rapidly.¹¹ Therefore, detection of the intact virus in the presence of the innate enzymatic activity requires stable glycoside analogues. Here, we report the modular synthesis of viral NA resistant *S*-sialosides and demonstrate their ability to differentiate between two selected influenza strains belonging to H1N1 and H3N2 serotypes. These results show glycoconjugate-based viral capture and a potential path for the discriminatory detection of intact

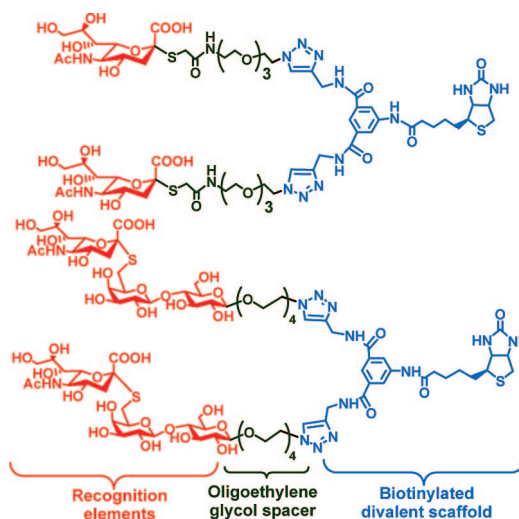


Figure 1. Structures of the two glycoconjugates, **BG-1** (top) and **BG-2** (bottom).

influenza viruses in the presence of the innate enzymatic activity of viral NAs.

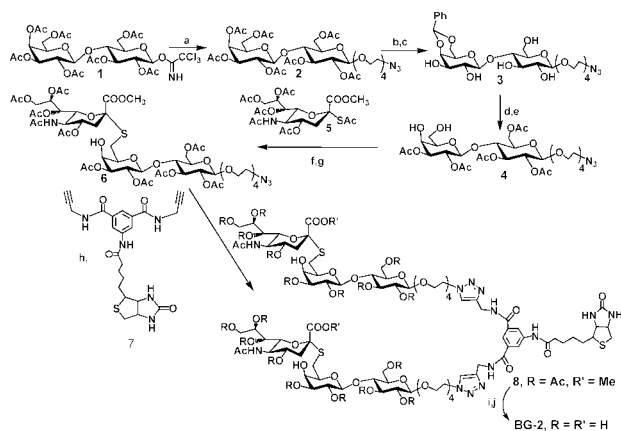
Since the position of sialic acid, orientation, density, tether length, and ancillary groups influence binding efficiency and selectivity, we designed a modular, flexible synthetic strategy that accommodates these variables to yield stable glycoconjugates. (Figure 1) The three important components of the designer ligands are (i) A biotinylated divalent scaffold. Biotin was used because it can attach to any streptavidin matrix. The biotin further affords additional multivalency as avidin binds up to four biotinylated molecules. Indeed, the reporter element was prepared by incubation of the biotinylated ligand with a fluorescently labeled streptavidin molecule to yield a streptavidin–(biotin-saccharide)₄ conjugate. (ii) A robust recognition element. We synthesized *S*-sialosides as these have been shown to be more resistant than *O*-glycosides.^{11,12} (iii) A critical variable spacer that connects and separates the biotin from the recognition motifs. Recent reports have suggested that the topology of the sugars is very critical for differentiation between influenza strains.¹³ We chose oligoethylene glycols to reduce nonspecific binding, to impart a degree of flexibility, and to facilitate development of glycoconjugates with distance optimized between adjacent recognition sites.

We used this modular strategy to synthesize biotinylated biantennary glycoconjugates, **BG-1** and **BG-2** that differ in the presentation of the sialic acid. **BG-1** was designed to exhibit no discrimination, while we expected the sialic acid attached to the 6'

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Scheme 1. Synthesis of the Biantennary α -2,6-S-Sialoside^a

^a Reagents and conditions: (a) $\text{H}(\text{OCH}_2\text{CH}_2)_4\text{N}_3$, TMSOTf, DCM, 0 °C, 1.5 h, 74%; (b) NaOMe, MeOH, 12 h; (c) $\text{PhCH}(\text{OMe})_2$, *p*-TSA, CH_3CN , 87% over two steps; (d) Ac_2O , DMAP, pyridine, 16 h, 88%; (e) $\text{TFA}/\text{H}_2\text{O}$ (3:2), DCM, 60%; (f) Ti_2O , pyridine, DCM, -25 °C, 1 h; (g) Et_2NH , DMF, -25 °C, 2 h, 65% over two steps; (h) CuSO_4 , sodium ascorbate, THF: H_2O , 36 h, 67%; (i) NaOMe, MeOH, 12 h; (j) NaOH, H_2O , 10 h, 91% over two steps.

position of lactose in **BG-2** to allow differential binding to viral strains. The synthesis of the α -2,6 analogue **BG-2** is shown in Scheme 1. Lactose septaacetate β -imidate **1** was reacted with a tetraethylene glycol spacer, 1-azido-(2-(2-ethoxy)ethoxy)ethanol to yield the beta isomer **2** in 74% yield. Saponification using NaOMe in MeOH was followed by benzylidene protection of the 4,6 hydroxyl groups to yield **3** in 87% over two steps. Reprotection of free hydroxyls with acetate groups followed by removal of the acetal furnished **4**. Next, the primary alcohol in **4** was selectively activated to the triflate and reacted with the known thio-*N*-acetylneuraminic acid, **5**, in the presence of diethyl amine to yield trisaccharide, **6**. The azide bearing trisaccharide, **6**, was reacted with a biotinylated scaffold carrying two alkyne functionalities **7**, in the presence of CuSO_4 and sodium ascorbate to give **8** in reasonable yield. A two-step procedure, saponification followed by deesterification resulted in the desired product, **BG-2**. This final product was purified using a Biogel P-2 column. Similarly, **BG-1** was synthesized and purified (see Supporting Information).

Next, we developed the carbohydrate analogue of a sandwich immunoassay (the virus is sandwiched between capture and fluorescent reporter molecules) on the surface of a waveguide-based optical biosensor that facilitates rapid and sensitive detection of biological agents.¹⁴ Briefly, the technology involves the use of planar optical waveguides comprising thin (~120 nm) high refractive index (RI) dielectric materials deposited on a substrate with a lower RI. Etched diffraction gratings provide facile means of coupling laser light into the thin waveguide film (see Figure 2). Although a majority of the incident light is present in the guided mode, the evanescent field extends out into the medium and excites the fluorescent reporter. Since the evanescent field fades with increasing distance from the surface, molecules > 200 nm from the surface are not excited eliminating background fluorescence in complex matrices. We have successfully demonstrated the application of the waveguide biosensor to the rapid and sensitive detection of nanomolar quantities of *B. anthracis* and breast cancer proteins in complex samples.¹⁴ Here, we demonstrate the capability of this assay platform to detect larger particles such as an intact virus.

The first set of experiments established the ability of synthetic glycans to capture viral particles, while a fluorescent monoclonal anti-HA antibody was used as reporter. These experiments establish

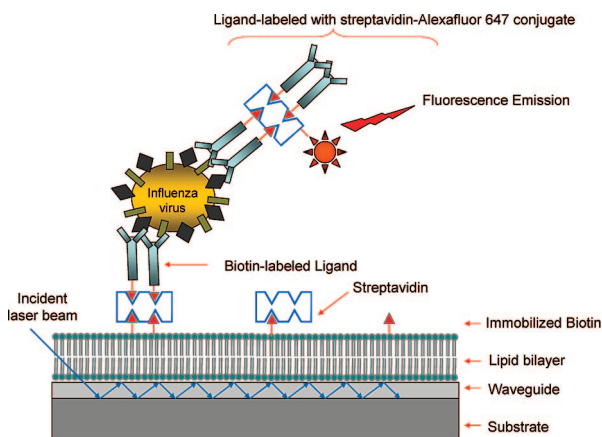


Figure 2. Schematic of the sandwich “carboassay” on a planar optical waveguide biosensor. A biotinylated carbohydrate ligand conjugate to fluorescent streptavidin is shown as the detector. Fluorescent antibodies as reporter were also used.

the efficient capture and provide a baseline for the follow up study, in which glycans were used both for capture and detection. Briefly, **BG-1** (or **BG-2**) was anchored onto the biosensor surface via streptavidin coupling. After washing, inactivated virus [A/Beijing/262/95 (H1N1) or A/Sydney/26/95 (H3N2)] from cell extracts was injected and incubated for 30 min. Following washing, injection of the fluorescently labeled reporter, Alexafluor-647 antibody or Alexafluor-647 streptavidin-(biotin-glycoconjugate)₄, gave the observed signal. The exact sequence of steps, without the virus, was performed to measure nonspecific binding (see Supporting Information).

The results of the A/Beijing/262/95 (H1N1) binding with **BG-1** are shown in Figure 3a and 3b. The top curve of Figure 3a shows the spectral response in relative fluorescence units (RFU) of the excited waveguide following exposure of the viral particles (10^8 particles/ml) to **BG-1**. The bottom curve of Figure 3a is the background signal. A response of 2500 RFU was obtained when virus was injected. **BG-1** conjugated to fluorescent streptavidin can also be used as a reporter because of the presence of over 100 copies of multimeric HA and NA on the virus providing multivalent binding enhancement. In this case, a discernible signal of >250 RFU with a signal-to-noise ratio of 3.8 was recorded. (Figure 3b) While signal intensities are considerably lower than an antibody reporter, these results demonstrate for the first time, an influenza capture and reporter pair entirely based on glycans. We anticipate that sensitivities can be improved by adjusting the multivalency and distances of sialic acids on the scaffold. Testing the ability of **BG-1** to bind to A/Sydney/262/95 (H3N2) yielded similar results, supporting our initial expectation that this presentation of sialic acid on the scaffold does not provide strain selective binding. We also tested the binding of the two strains to **BG-2**. While A/Sydney/26/95 (H3N2) bound well to **BG-2**, we observed no detectable signal with A/Beijing/262/95 (H1N1) which demonstrates an absolute discrimination of this sialoside between these two specific strains. (Figure 3c) There are several possible explanations why A/Beijing/262/95 (H1N1) did not bind to **BG-2**. (a) Previous studies have been carried out with glycoconjugates that use 6'-sialyl-*N*-acetylglucosamine, whereas we have used 6'-sialyllactose. We have observed a similar behavior with Shiga toxins where the *N*-acetyl group at the 2 position plays a significant role in the discriminatory binding event.¹⁵ Indeed, modeling of the 6'-sialyl-*N*-acetylglucosamine with HAs of a different H1N1 strain, A/SouthCarolina/1/18, indicates hydrogen bonds from the NHAc to Asp 190 of HA,⁹ which should be absent in 6'-sialyllactose. (b) Differences between

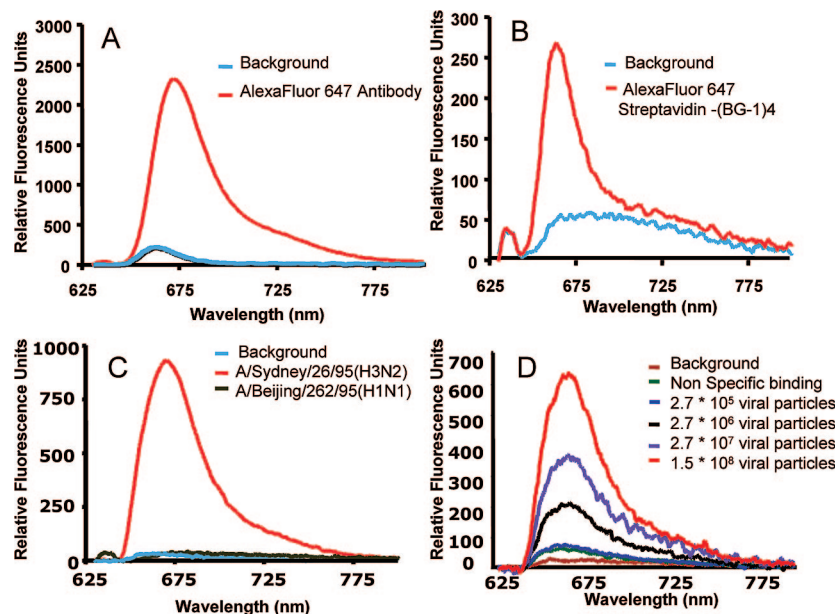


Figure 3. Influenza detection using (A) **BG-1** as capture and Alexafluor 647-antibody as reporter with A/Beijing/262/95 (H1N1). (B) **BG-1** as capture and Alexafluor-647-streptavidin-(**BG-1**)₄ as reporter with A/Beijing/262/95 (H1N1) and A/Sydney/26/95 (H3N2) using **BG-2**. (D) Limit of detection using **BG-2** as capture and Alexafluor-647-antibody as reporter with H3N2A/Sydney/26/95 (H3N2).

the NAs (N1 versus N2) could also contribute in the binding event. (c) The topology of the glycoconjugate adopts a conformation that the viral glycoproteins cannot access, thereby precluding A/Beijing/262/95 (H1N1) from binding. We also determined the detection limit. This nascent technology allows us to detect 10^6 viral particles/ml using the reporter antibody. (Figure 3d)

In summary, we have developed a modular strategy that allows for the development of synthetic *S*-sialosides. The molecules have been used as capture and reporter recognition elements for rapid detection of two strains of the influenza type A virus. The glycoconjugates are highly robust over extended time periods (>6 months) at ambient temperatures and require no refrigeration. One of the glycoconjugates, **BG-2** differentiates between A/Beijing/262/95 (H1N1) and A/Sydney/26/95 (H3N2) using a waveguide-based biosensor platform. While these initial studies are promising, it is important to note that one cannot extrapolate these results to suggest that **BG-2** will not bind to other sialic acid recognizing pathogens present in the respiratory tract or other strains. Several serotypes and strains within each serotype must be tested to explore the discriminatory potential of these thio-glycoconjugates. The binding of multiple and diverse classes of pathogens to sialic acid on host cell surfaces clearly poses challenges to carbohydrate-based differential detection. Subtle differences in binding affinities can be explored to derive selective binding as demonstrated in this limited preliminary study of two influenza type A strains belonging to two different serotypes. Currently, we are developing a focused glycan library comprising stable *S*-sialosides to evaluate the binding affinities of different strains of the Influenza virus and other pathogens. On the instrumentation side, we have extended our biosensor platform to demonstrate the capture of intact viral particles and are developing a multichannel adaptation of the optical biosensor. Advances, both in the availability of ligands and sensor refinement, will facilitate future exploration of selectivity, affinity, and discriminatory ability of our approach for respiratory infectious agents that share sialic acid binding to infect the host.

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Supporting Information Available: Details of the synthesis and the sandwich assay. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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