High-Throughput Screening

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Rapid Identification of the Receptor-Binding Specificity of Influenza A Viruses by Fluorogenic Glycofoldamers

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Abstract: *The re-emergence of influenza raises a global concern that viral pandemics can unpredictably occur. However, effective approaches that can probe the infection risk of influenza viruses for humans are rare. In this work, we develop a glycofoldamer that can rapidly identify the glycan-receptor specificity of influenza viruses in a high-throughput manner. The coupling of glycan receptors that can be recognized by hemagglutinin (a surface protein on the virion capsid of influenza) to a fluorogenic-dye foldamer produces the glycofoldamers with minimal fluorescence in aqueous solution. After interaction with human-infecting virus strains for only five minutes, the fluorescence intensity of the glycofoldamer is remarkably enhanced with a blue-shifted emission peak. The probes have also proven effective for the rapid identification of 1) the human- or bird-infecting properties of influenza viruses in a high-throughput manner and 2) the receptor-specificity switch of a virus strain by mutations.*

Influenza is an acute respiratory disease caused by influenza viruses. Type A influenza viruses such as H1N1, H3N2, and H5N1 circulate in human populations and induce seasonal outbreaks, annually contributing to thousands of human deaths and significant economic loss.[1,2] In 2013, human infections with H7N9 and H10N8 were reported in Shanghai and Nanchang (China), respectively.[3–12] As the re-occurrence of influenza viruses has led to concerns about new flu pandemics, methods for the effective surveillance and risk assessment of the human-infecting potential of viruses are in urgent demand.

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Hemagglutinin (HA) is a main surface protein of influenza viruses. Binding of HA to sialic acid (SA) terminated glycan receptors on the surface of host cells is the first crucial step of infection and transmission. It has been established that avian-adapted HA prefers the α2,3-linked sialylglycans commonly found in birds, whereas the human-adapted HA preferentially binds to the α2,6-linked regioisomer expressed in the upper respiratory tract of humans.[13] The switch in receptor-binding specificity is considered as a key determinant for virus interspecies transmission.[14,15] Therefore, effective techniques to determine the influenza virus receptor specificity are important for surveilling the human-infecting risk of influenza viruses. Conventional approaches to meet this goal rely on the hemagglutination/hemagglutinin inhibition assay, which requires strict quality control, long detection times, and is rather expensive. New elegant strategies, including the glyco-microarray[16–20] and other surface-based[21–24] and nanoparticle-based[25–27] analytical methods, have been developed for virus detection. However, several problems still exist. For example, whereas many solid-phase methods rely on the use of expensive equipment and reagents, nanoparticle-based sensors may face difficulties in sensor standardization. A simple and general means to identify the human-infecting risk of influenza viruses is thus urgently required.

Herein we report a unique glycofoldamer (a foldamer is a molecule that can fold into an ordered conformation in solution) for the homogeneous, rapid probing of the receptor-binding specificity of influenza viruses. Using lactose as a control (**1**; Figure 1a), a series of trisaccharide and pentasaccharide glycan receptors with avian specificity (Neu5Acα2,3Gal-β1,4Glc (**TO23**), Neu5Acα-2,3Gal-β1,4GlcNAc (**TN23**), and Neu5Acα-2,3Galβ-1,4GlcNAcβ-1,3Galβ-1,4Glc (**P23**)) and human specificity (Neu5Acα2,6-Gal-β1,4Glc (**TO26**), Neu5Acα-2,6Gal-β1,4Glc (**TN26**), and Neu5Acα-2,6Galβ-1,3GlcNAcβ-1,3Galβ-1,4Glc (**P26**); Figure 1)[17] were coupled to a pyrene-naphthalimide dyad produced by a straightforward synthetic procedure (see the Supporting Information, Scheme S1). Whereas the resulting glycofoldamers showed minimal fluorescence in their folded conformation, complexation with a selective virus in a homogeneous buffer solution rapidly unfolds the probe, leading to a sharp increase in the fluorescence intensity with a blue-shifted emission peak (Figure 1).[28] The glycofoldamer assay readout takes only five minutes.

The pyrene-naphthalimide dyad was synthesized by coupling pyrene (**p**) to a naphthalimide derivative (**n**) with a flexible alkyl chain (Scheme S1). Then, an aminoxy handle was installed at the naphthalimide moiety by a click reac-

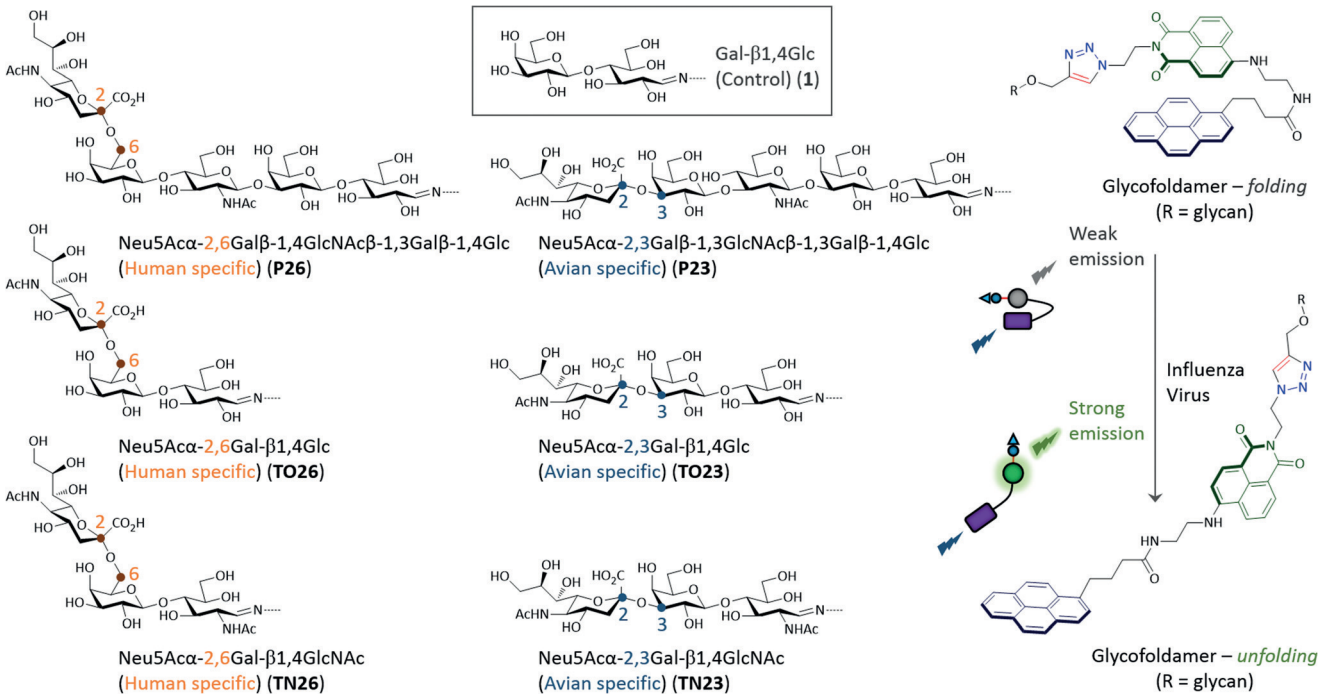

Figure 1. Structures of the glycofoldamers and illustration of their fluorescence response to influenza A virus. The illustration shows that the folded probes could unfold to fluoresce upon interaction with an influenza virus.

tion,[29,30] offering a reaction site for glycans.[16–20] The desired glycofoldamers **TO26**, **TN26**, **P26**, **TO23**, **TN23**, **P23**, and **1** (control) were produced in good yields through oxime ligation (Scheme S2 and Figure 1). Four virus strains, including A/California/08/2009 (H1N1pdm09, human-specific), A/Beijing/353/89 (H3N2, human-specific), A/Anhui/1/2013 (H7N9, human/avian-dual-specific), and A/environment/Dongting Lake/Hunan/3-9/2007 (H10N8, avian-specific) were used for the detection. The assay was carried out by coating the glycofoldamer (1 μM, 0.05 M phosphate buffered saline (PBS), pH 7.4) on a microplate, followed by the addition of virus strains (3.2 HAU per 50 μL^{-1}; where HAU (hemagglutinating unit) is equal to approximately 5–6 logs of viruses). Then, the microplate was shaken for 5 min, and the fluorescence intensity of the glycofoldamers was recorded by a microreader (Syxergy H4, BioTek USA) in a homogeneous, high-throughput manner.

Shown in Figure 2 is the fluorescence change of the seven glycofoldamers in the presence of different virus strains. An evident fluorescence enhancement was observed for the glycofoldamers for selective virus strains (H3N2 (Figure 2a) and H1N1pdm09 (Figure 2b) with **TO26**, **TN26**, and **P26**, and H10N8 (Figure 2c) with **TO23**, **TN23**, and **P23**), whereas the presence of an unselective strain hardly caused the fluorescence to increase. In addition, the control probe **1** without the sialyl moiety induced minimal fluorescence changes towards all of the tested virus strains, suggesting the

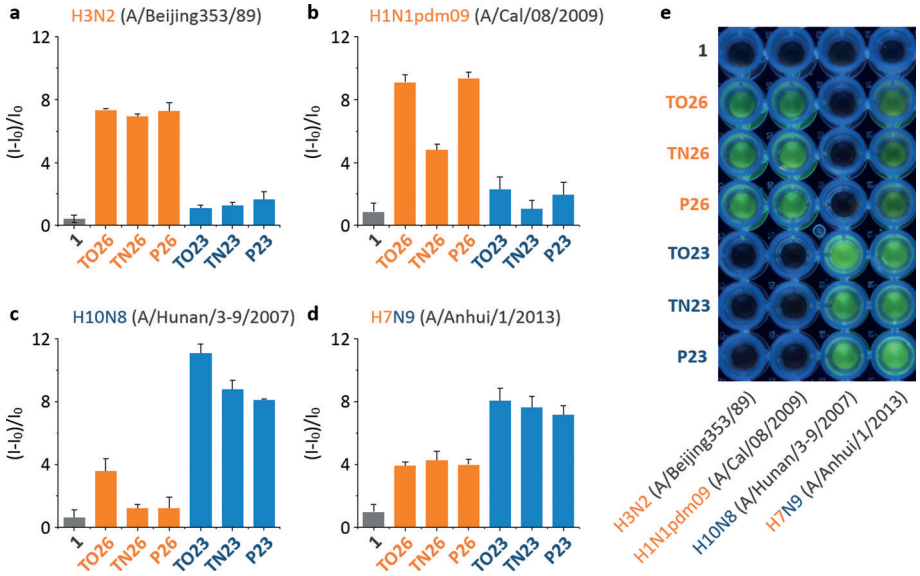

Figure 2. Fluorescence response of glycofoldamers (1 μM, 0.05 M PBS, pH 7.4, $\lambda_{ex} = 345 \pm 5$ nm) to a) H3N2 (A/Beijing353/89), b) H1N1pdm09 (A/California/08/2009), c) H10N8 (A/Hunan/3-9/2007), and d) H7N9 (A/Anhui/1/2013). I and I_0 are the fluorescence intensity of a probe in the presence and absence of a virus, respectively; virus titer: 3.2 HAU 50 μL^{-1}. b) Naked-eye fluorescence detection of influenza A viruses (8.0 HAU 50 μL^{-1}) in the presence of glycofoldamers (1 μM, 0.05 M PBS, pH 7.4) excited with a portable UV lamp.

good glycan-receptor specificity of the glycofoldamers. Notably, in addition to the avian-selective (2,3-selective) glycofoldamers, the presence of H7N9 (Figure 2d) also caused an obvious fluorescence enhancement of the human-selective (2,6-selective) probes. This is in agreement with previous reports suggesting the human-infecting potential[3,6] of H7N9. We also showed that the receptor-binding specificity of the influenza viruses can be determined with the naked eye upon irradiation with a simple UV lamp (Figure 2e). Both the 2,3- and 2,6-glycofoldamers fluoresced in the presence of H7N9, which is in agreement with the spectroscopic analysis (Figure 2d). These results preliminary demonstrate that the glycofoldamers can rapidly identify the receptor specificity of viruses in a homogeneous solution.

We determined the sensitivity of the glycofoldamers for influenza viruses in a subsequent titration assay. The limit of detection (LOD) for the viruses was calculated ($3\sigma_b/k$, where σ_b is the standard deviation of the probe solution in the absence of a virus, and k is the slope of the regression curves shown in Figure S1). The lowest LOD of the probes was determined to be 0.08 HAU 50 μL^{-1}. Whereas this sensitivity is better than or comparable to those of previously reported methods for HA detection, including surface plasmon resonance, quartz crystal microbalance, high-performance liquid chromatography, and gold-nanoparticle-based assays, the readout time of our method appeared to be the fastest (for details, see Table S1). Although ELISA is more sensitive than our probe, it relies on antibody-based immunoassays, which involve the sluggish and costly production of antibodies. In addition, the homogenous detection method of our glycofoldamer is simpler than solid-phase-based methods.

Next, we used fluorescence spectroscopy to elaborate the glycofoldamer–influenza virus interaction in detail. The control probe **1** and a selective lectin, peanut agglutinin (PNA), were first employed to investigate the binding (Figure S2). We observed that the UV/Vis absorption band of **1** (**p-n** dyad) was red-shifted compared to unconjugated **p** and **n** (Scheme S1) in PBS (Figure S2a). Upon excitation at 345 nm (a typical pyrene excitation wavelength), we observed an emission peak centered at 531 nm, characteristic of naphthalimides,[31] for **1** in a range of organic solvents. In contrast, a red-shifted emission peak at 565 nm was observed in PBS (Figure S2b). The red-shifted absorption and emission bands probably suggest that conjugated **n** and **p** units are closely stacked in aqueous solution owing to the hydrophobicity of the dyes.[32–34] Once unstacked, Förster resonance energy transfer (FRET) could occur from **p** to **n** owing to the good overlap between the emission band of the former and the absorption band of the latter (Figure S2c), giving the **n** emission (531 nm) upon excitation of **p** (345 nm). Figure S2d shows that the emission band of **1** in PBS is gradually blue-shifted with an increase in temperature, suggesting that the foldamer could unstack upon changes in the external environment. We also observed that with increasing PNA, the emission of **1** (531 nm) intensified in a concentration-dependent manner (Figure S2e). This observation probably suggests that complexation of **1** with PNA could unfold the probe, enhancing the fluorescence of the naphthalimide. The fluorescence reached equilibrium within five minutes (Figure S2f). We also found that the fluorescence response was selective for PNA and another *N*-acetyl galactosamine/galactose selective lectin (soybean agglutinin, SBA) over other unselective proteins (Figure S2g).

We further analyzed the binding between glycofoldamers (**TO26** and **TO23** were selected) and the viruses by fluorescence spectroscopy. We observed a concentration-dependent fluorescence enhancement (531 nm) upon incubating **TO26** with H1N1pdm09 (Figure 3b) and H3N2 (Figure S3a) and upon incubating **TO23** with H10N8 (Figure 3a), H5N1 (Figure S3b), and H7N9 (Figure S3c). The fluorescence changes of all probes reached equilibrium within five minutes (Figure 3c and Figure 3d). As the switch of the receptor-binding specificity is crucial for influenza virus interspecies transmission,[14,15] we tested whether the mutation of an avian-

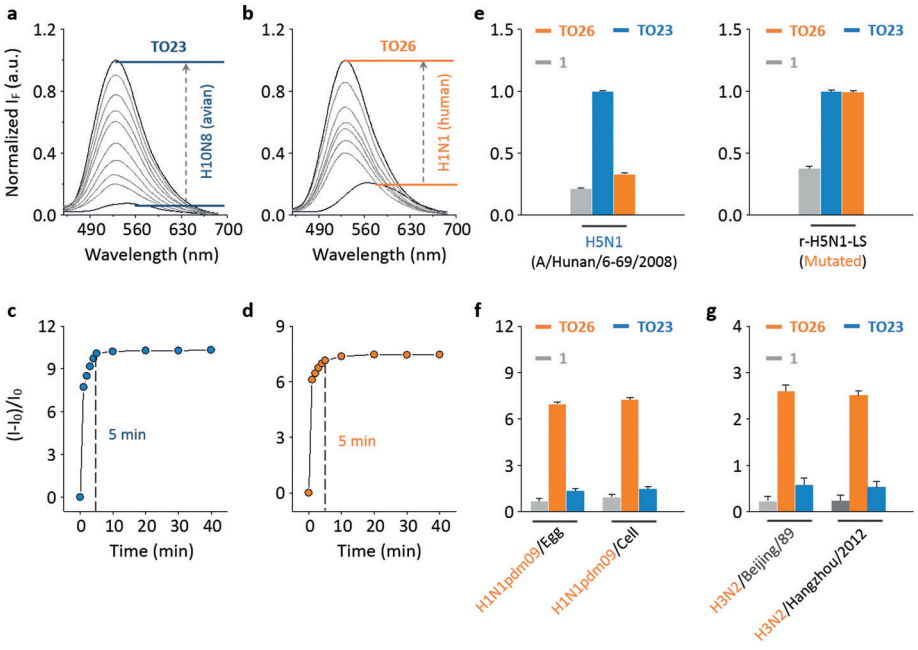

Figure 3. Fluorescence titration of a) **TO23** (2 μM) in the presence of increasing concentrations of H10N8 (A/Hunan/3–9/2007; 0–6.4 HAU 50 μL^{-1}) and b) **TO26** (2 μM) in the presence of increasing concentrations of H1N1pdm09 (A/California/08/2009; 0–6.4 HAU 50 μL^{-1}). The changes in the fluorescence intensity of c) **TO26** (2 μM) in the presence of H1N1pdm09 (A/California/08/2009; 6.4 HAU 50 μL^{-1}) and d) **TO23** (2 μM) in the presence of H10N8 (A/Hunan/3-9/2007; 6.4 HAU 50 μL^{-1}) as a function of time. e) Normalized fluorescence intensity of the glycofoldamers (2 μM) in the presence of H5N1 (A/environment/human/6-69/2008) and a mutated strain. Fluorescence enhancement of glycofoldamers (2 μM) in the presence of f) egg-cultured or cell-cultured H1N1pdm09 and g) H3N2 isolated in 1989 (A/Beijing353/89) or 2012 (A/Hangzhou/A101/2012).

specific virus strain (A/environment/Hunan/6-69/2008, H5N1) would enhance the fluorescence of **TO26**. The previously reported mutations Q226L and G228S were introduced into an H5N1 HA plasmid, and the mutated virus was rescued by reverse genetics in the background of A/Puerto Rico/8/1934, termed rH5N1-LS.[15,35,36] We observed that whereas wide-type H5N1 only increased the fluorescence of **TO23**, the mutated rH5N1-LS caused a fluorescence enhancement for both **TO23** and **TO26** (Figure 3e). These data suggest that the glycofoldamer can identify the human-infecting potential of mutated viruses. Incubation of the H1N1pdm09 strain cultured in cells caused a similar level of fluorescence enhancement to a strain cultured in eggs with **TO26** rather than **TO23** and **1** (Figure 3f). This suggests that our glycofoldamer can identify the receptor specificity of influenza viruses derived from different biological media. In addition to the earlier A/Beijing/353/89 H3N2 strain, we also used a contemporary H3N2 (A/Hangzhou/A101/2012) strain isolated from a clinical patient to interact with our probes. The result (Figure 3g) indicated that incubation of the strain with the probes only led to fluorescence enhancement for **TO26**, suggesting the human-receptor specificity of this strain. This result is similar to that observed for the H3N2 strain isolated in 1989.

Eventually, dynamic light scattering (DLS) and confocal laser scanning microscopy (CLSM) were applied to investigate the glycofoldamer–influenza virus interaction. The DLS data showed that whereas the particle size of **TO23** significantly increased after binding to H10N8 (avian-specific), there was no obvious change in the size of **TO26** after treatment with the virus (Figure 4a). In addition, CLSM images showed that the fluorescence emission of **TO23**, rather than **TO26**, was elicited by H10N8 (Figure 4b). A solid-state co-crystal structure has been resolved for a monovalent glycorhodamine probe with a lectin, where the glycoliand was shown to bind to the carbohydrate-recognition domain of the lectin while the rhodamine "tails" could interstack by π-stacking to induce lectin aggregation.[37] With this piece of evidence, we propose that the binding of a glycofoldamer to the hemagglutinin of a selective virus could unfold the probe, thereby releasing the pyrene tails, which could further interstack[28] to aggregate the virus particles. Although more evidence is needed to fully clarify the aggregation mode between probe and virus, our observation represents a unique example of virus aggregation induced by a single molecular probe.

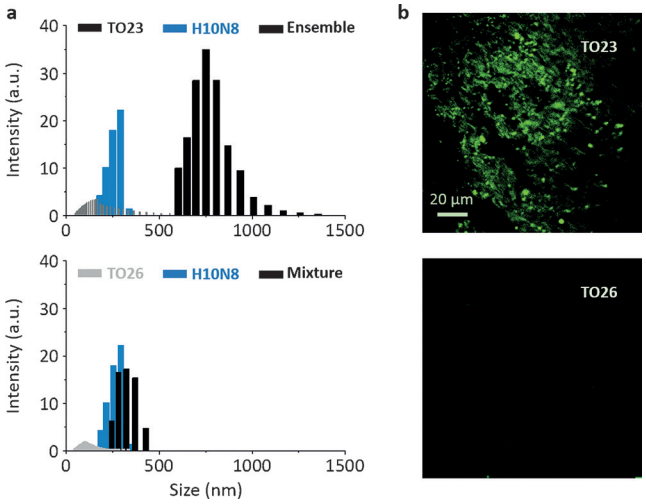

Figure 4. a) Dynamic light scattering of H10N8 (A/Hunan/3-9/2007, 4.0 HAU 50 μL^{-1}), **TO23** (2 μM), **TO26** (2 μM), and probe–virus mixtures. b) Confocal laser scanning microscopy of **TO23** (2 μM) and **TO26** (2 μM) in the presence of H10N8 (4.0 HAU 50 μL^{-1}).

In conclusion, we have developed a single-molecule probe for the rapid identification of the glycan-receptor specificity of a series of influenza virus strains in homogeneous solution. In particular, the glycofoldamer can effectively monitor the human-infecting risk of mutated virus strains, and it can be incorporated into common facilities for high-throughput virus detection with minimal technique requirements, which suggests its promise for the effective identification of the human-infecting risk of influenza viruses.

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