Glycan Microarrays

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Recognition of Sialylated Poly-*N*-acetyllactosamine Chains on *N*- and *O*-Linked Glycans by Human and Avian Influenza A Virus Hemagglutinins**

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The initial stages of an influenza A virus infection are mediated by the binding of the viral hemagglutinin (HA) to sialylated glycan receptors on host epithelial cells.[1] The specificity of the HA is believed to be a key determinant of viral host range.[2] While all 16 influenza HA subtypes are found in avian viruses, only three are found in viruses adapted to humans (H1, H2, and H3), each resulting in a major pandemic. HAs from avian and human viruses are characterized by their preference for α2-3 and α2-6-linked sialic acids, respectively. Studies now suggest that other elements of sialoglycan sequence are also important factors of HA specificity that contribute to the species barrier.[3] Recently, human and swine respiratory epithelial cells were shown to express sialylated N-linked glycans with extended poly-*N*-acetyllactosamine (poly-LacNAc) chains.[4] Poly-LacNAc chains are Galβ1-4GlcNAcβ1-3 tandem repeats that extend *N*- and *O*-linked glycans of glycoproteins and contribute to the biology mediated by glycan-binding proteins.[5] Sasisekharan and co-workers have suggested that human HAs bind preferentially to extended α2-6 sialosides and may be critically important for viral adaptation to humans.[4a,6]

Studies on the preference of influenza HAs for extended glycans have employed synthetic sialosides that are linear terminal fragments of natural *N*- and *O*-linked glycans, which differ in their core structure and are often branched.[4a,7] To more fully address the influence of poly-LacNAc chains on HA specificity in the context of natural glycans, we have synthesized a series of sialylated poly-LacNAc structures on intact *O*- (**4**–**9**, **16**–**21**) and *N*-linked glycan (**10**–**12**, **22**–**24**) cores (Figure 1). These sialosides were incorporated into a custom glycan microarray alongside the linear terminal fragments (**1**–**3**, **13**–**15**) for analysis of specificities of human and avian influenza HAs.

Several groups have reported chemical and chemoenzymatic syntheses of poly-LacNAc structures.[8] For synthesis of extended natural *N*- and *O*-linked glycans, our strategy relied on enzymatic elaboration of advanced core structures. The α2-3 and α2-6 sialoside targets comprised *O*-linked cores

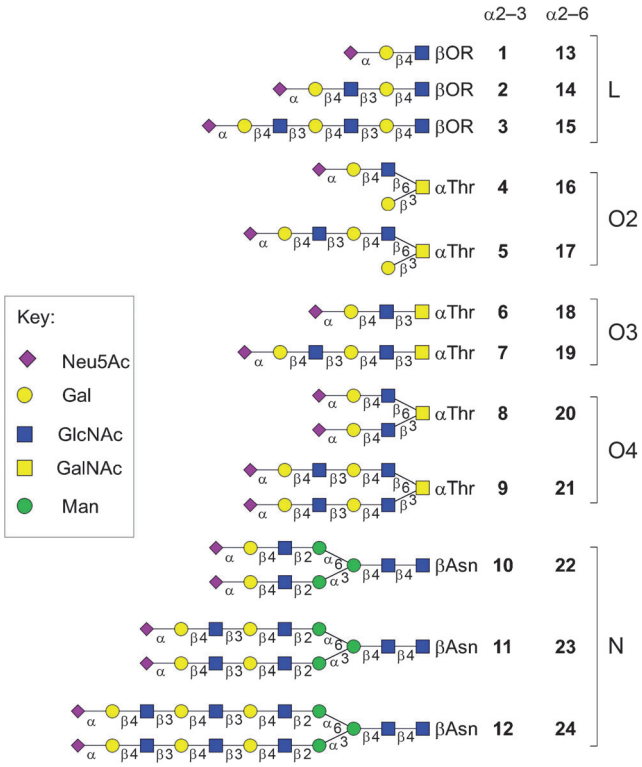

Figure 1. Structures of sialylated poly-LacNAc linear (L) fragments (**1**–**3**, **13**–**15**) and the same sequences elaborated on *O*-linked glycan cores 2–4 (O2, O3, O4; **4**–**9**, **16**–**21**) and *N*-linked (N) glycan cores (**10**–**12**, **22**–**24**). α2-3 and α2-6 indicate the linkage of the sialic acid moiety. Neu5Ac = sialic acid, GlcNAc = *N*-acetylglucosamine, GalNAc = *N*-acetylgalactosamine, R = ethyl amine.

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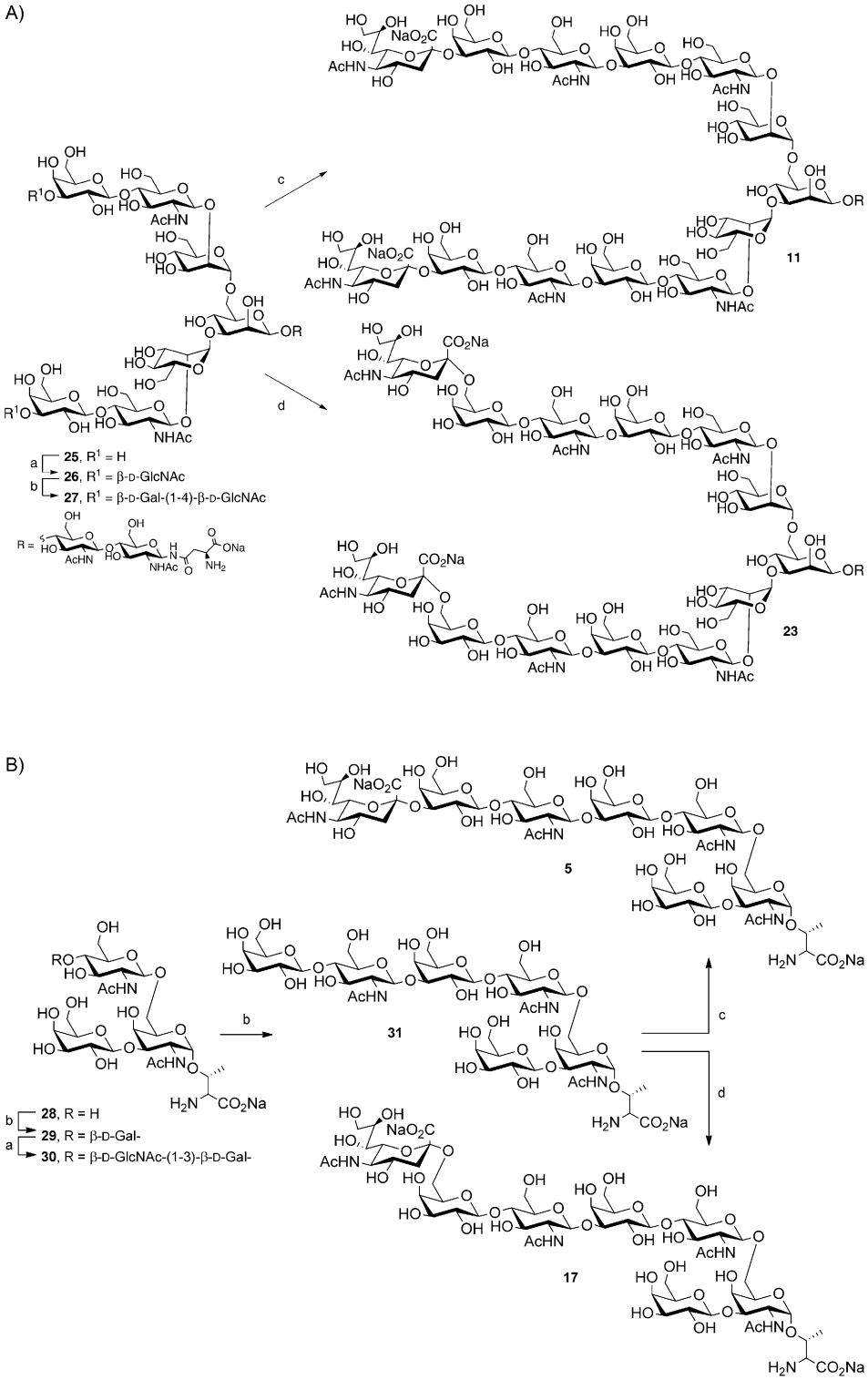
(cores 2–4) and *N*-linked cores with up to two and three LacNAc repeats, respectively. Representative syntheses for *N*-linked glycans (**11** and **23**) and *O*-linked glycans with core 2 (**5** and **17**) are described in Scheme 1. Key LacNAc extensions were attained by alternating reactions using recombinant *Helicobacter pylori* β1-3-*N*-acetylglucosaminyltransferase (β1-3GlcNAcT)[8b] and the bacterial β1-4-galactosyltransferase/UDP-4′-Gal-epimerase fusion protein (GalT-GalE).[9] Reaction of *N*-linked glycan **25** with UDP-GlcNAc (4 equiv) using enzyme β1-3GlcNAcT and subsequent treatment with UDP-Glc (4 equiv) and GalT-GalE allowed efficient construction of LacNAc on both antennae affording **27** (Scheme 1 A). Divergent sialylation of **27** using rat α2-3-sialyltransferase (rST3Gal-III) or human α2-6-sialyltransferase (hST6Gal-I), with CMP-Neu5Ac gave the desired α2-3-linked sialoglycan **11** and α2-6-linked sialoglycan **23**, respectively. The synthesis of *O*-linked cores 3–4 and of the tri-LacNAc *N*-linked glycans were conducted using similar conditions (Schemes S1–S6 in the Supporting Information).

The β1-6 branch of core-2 *O*-linked glycans are commonly extended with poly-LacNAc. Initial galactosylation of **28** added Galβ1-4 to GlcNAc giving **29** (Scheme 1 B). As both branches of **29** present terminal Gal residues, two sites were potentially reactive for GlcNAc addition. Regioselective reaction on the β1-6 branch was anticipated, because the enzyme β1-3GlcNAcT demonstrates higher selectivity for Galβ1-4GlcNAc substrates. Thus, under controlled conditions using UDP-GlcNAc (2 equiv), selective elongation of the β1-6 branch was achieved to afford branched glycan **30**.[10] NMR spectroscopy and MS analysis confirmed addition of a single GlcNAc unit. The asialo di-LacNAc structure **31** was prepared by reaction of **30** with UDP-Glc catalyzed by fusion protein GalT-GalE. Finally, selective sialylation of **31** was performed with either enzyme rST3Gal-III or enzyme hST6Gal-I and CMP-Neu5Ac (2 equiv). Both sialyltransferases show preference for Galβ1-4GlcNAc substrates and



Scheme 1. A) Enzymatic transformations of **25** to **11** and **23**: a) enzyme β1-3GlcNAcT, UDP-GlcNAc; b) fusion protein GalT-GalE, UDP-Glc; c) sialyltransferase rST3Gal-III, CMP-Neu5Ac; d) sialyltransferase hST6Gal-I, CMP-Neu5Ac. B) Enzymatic transformation of **28** to **5** and **17**. See Scheme 1 A for conditions. UDP = uridine-5′-diphospate, CMP = cytidine monophosphate.

gave compounds **5** and **17**, respectively. The mono-sialylated products were confirmed by NMR spectroscopy and MS analysis.

The 24 glycans in the sialoside library (Figure 1) contain either the terminal Neu5Acα2-3Gal (**1**–**12**) or Neu5Acα2-6Gal (**13**–**24**) sequence. A glycan microarray was constructed from this library to study the binding properties of influenza A virus HA.[7a,11] The aglycone of each sialoside was equipped with a free amino group for direct printing on slides activated with *N*-hydroxy succinimide (Figure S1 in the Supporting Information). Recombinant HAs from selected avian and human influenza A viruses were then screened to assess the effects on HA binding of both length and presentation of sialylated poly-LacNAc.

As expected, the avian HAs preferentially recognized α2-3-linked sialosides (Figure 2 and Figure S2 in the Supporting Information). However, while hemagglutinin H4 (A/duck/Czech/1/56) bound strongly to nearly all α2-3 structures, other avian HAs showed more selective binding patterns. For instance, hemagglutinin H3 (A/duck/Ukr/1/63), a progenitor of the 1968 Hong Kong pandemic,[12] only bound the linear glycans **2** and **3** and the *O*-linked glycan **8** and *N*-linked glycan

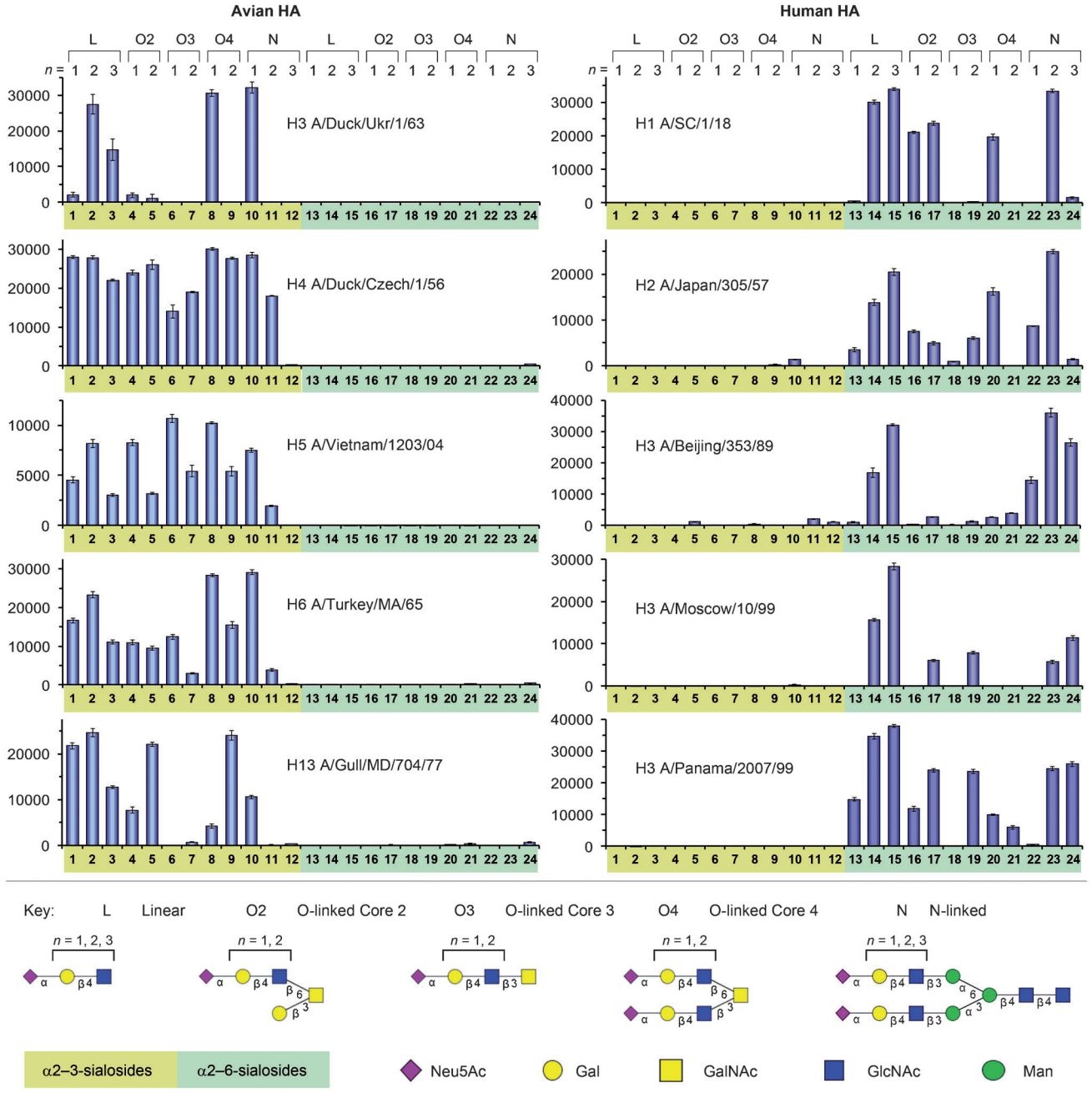


Figure 2. Glycan microarray binding analyses as measured by fluorescence intensity for avian and human influenza A recombinant hemagglutinins. All HAs were evaluated at 15 μg mL^{-1} except for A/SC and A/Beijing, which were evaluated at 150 μg mL^{-1}. See additional details in the Supporting Information.

10. Remarkably, all avian HAs, including H5 (A/Vietnam/1203/04), a highly pathogenic human isolate of the bird flu,[13] showed strong preference for short *N*-linked structures, thus binding strongly to **10**, and showed reduced or no binding to the longer glycans **11** and **12**.

Although human HAs demonstrated classic preference for α2-6 sialosides, they exhibited varied fine specificity for the extended *N*- and *O*-linked glycans (Figure 2 and Figure S2 in the Supporting Information). As reported, the human HAs bound best to the linear sialosides with di- and tri-LacNAc extensions (**14**–**15**).[4] Significantly, however, the same sequences were not uniformly recognized when presented on *N*- and *O*-linked glycan cores. For instance, while the H1 (A/SC/1/18) and the H2 (A/Japan/305/57) HAs bound strongly to the linear sialoside with the di-LacNAc extension (**14**), they bound poorly to the same sequence presented on glycans of core 3 (**19**) and core 4 (**21**). Surprisingly, these same two HAs exhibited strong binding to *N*-linked glycans with the di-LacNAc sequence (**23**) but dramatically reduced binding to the same sequence with the tri-LacNAc repeat (**24**).

In summary, we have synthesized a panel of novel glycans containing sialylated poly-LacNAc on intact *N*- and *O*-linked glycan cores as candidates of the natural glycan receptors of influenza viruses. While all avian and human virus HAs retained their basic specificity for α2-3 and α2-6 linkages, respectively, the *N*- and *O*-linked glycan cores differentially impacted the ability of individual HAs to recognize the sialic acid as a receptor. The lack of a consistent recognition pattern for human HAs suggests that the fine specificity of the virus for receptor(s) may drift under antigenic selective pressure, while the ability to bind to a subset of α2-6 sialosides is sufficiently retained to mediate infection and transmission. It should also be noted that the branched *N*-linked glycans and *O*-linked glycans with core 4, which were produced with our synthetic strategy, are symmetric di-sialylated glycans. However, glycans extended on a single branch also occur in nature.[4a,b] Thus, it will also be of interest to investigate the role of asymmetric glycans on influenza receptor biology.

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