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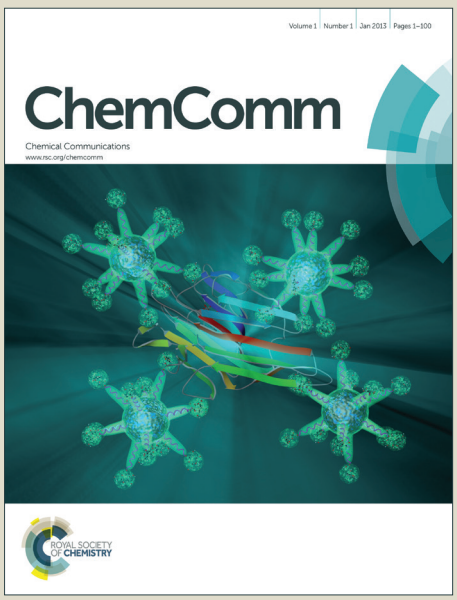

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ARTICLE

Determination of receptor specificities for whole influenza viruses using multivalent glycan arrays

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Influenza viruses bind to mucosal glycans to gain entry into a host organism and initiate infection. The target glycans are often displayed in multivalent arrangements on proteins; however, how glycan presentation influences viral specificity is poorly understood. Here, we report a microarray platform approximating native glycan display to facilitate such studies.

Pathogens rely on molecular recognition events at the cell surface to gain entry into a host organism.[1] As such, many pathogens have evolved to exploit glycans that are abundantly distributed on target epithelial tissues to initiate infection.[2] Influenza A virus (IAV) offers a prime example of this evolutionary adaptation. Its virion displays trimeric hemagglutinin (HA) proteins that bind to sialoside glycans presented on host glycoproteins, called mucins, to facilitate virion internalization. IAV also maintains tetrameric neuraminidase (NA) enzymes that cleave sialic acids to allow detachment from the cell surface. NA, a receptor-destroying enzyme, works in careful balance with HA proteins to enable newly produced viruses to leave infected cells and propagate infection. Another likely role for NA is to free bound virus from secreted host mucins, which can present glycans similar to those on the underlying tissue and serve as protective decoys covering mucosal epithelia and sequestering viruses to prevent infection.[3]

While terminal sialic acid is a carbohydrate residue universally recognized by all influenza strains, the nature of its attachment to the underlying glycan structures of glycoproteins determines viral specificity for distinct host species.[4] In the human upper airway, such glycans display sialic acid residues linked predominantly via α2-6 glycosidic linkages, while the lower respiratory tract and the secreted mucin decoys are rich in α2-3 sialoglycans.[5] In contrast, most influenza viruses populating both wild and domesticated birds show preference for α2-3 linked sialic acids, which is prevalent in avian gastro-intestinal epithelia.[6] Mutations in the viral HA binding site that switch selectivity from α2-3 to α2-6 sialoglycans is a prerequisite for interspecies transfer and can be indicative of a newly acquired ability of avian viruses to infect humans.[7,8,9] As such, screening tools to identify changes in influenza glycan specificity have been utilized for early indication of virus transmissibility and assessment of potential pandemic risks.

While individual sialoglycan structures are important determinants of influenza binding, the spatial presentation of these glycans may play a major role in the determination of IAV receptor specificity. The virus relies on avidity effects to compensate for the weak affinity and low selectivity of HA binding to individual sialoglycan structures (K_d ~ 2 mM).[10] Mucosal barriers are composed primarily of mucins, which are large protein scaffolds densely decorated with sialoglycans (Fig 1).[11] The multivalency of glycan display in mucins is matched by the high density of HA on the surface of influenza virions (~200-1000 copies of HA trimers per virus),[12,13] resulting in specific high-avidity binding of the virus to the mucosal membranes. It is known that increasing the surface density of glycans can result in altered selectivity of lectins for their glycan ligands;[14,15] however, the parameters that define how multivalency affects viral binding and specificity have not yet been fully established. High-throughput screening platforms will be needed to systematically interrogate the binding of whole viruses to sialoglycans presented in a manner that resembles their organization in mucosal barriers.

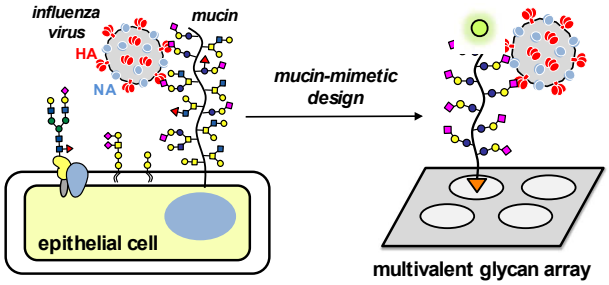

Figure 1. Influenza A viruses engage sialoside glycans arranged on epithelial mucins to initiate infection. A mucin-mimetic microarray platform can serve as a tool to investigate how parameters such as glycan structure, valency and surface density influence binding and specificity of the pathogen.

Glycan microarrays have emerged as a powerful tool for determining the ligand specificities of glycan-binding proteins (GBPs).[16,17,18] In a traditional format, individual glycan structures are immobilized on the array surface to create a multivalent ligand display that can elicit sufficiently strong

binding by GBPs. This technology has enabled important studies that provided key insights into the glycan specificity of HAs derived from influenza strains involved in recent pandemics;[19] yet, surprisingly few studies have been reported using these platforms to obtain information about the binding of intact viruses.[20,21,22,23] One limitation of the current glycan array technology is the lack of control over glycan presentation. This is compounded by the difficulties associated with the characterization of the arrays, including the determination of parameters such as surface density and spatial distribution of glycans after immobilization. Recent studies comparing different glycan array platforms have revealed that distinct glycan grafting strategies could influence their recognition by GBPs.[24] Perhaps more importantly, the two-dimensional glycan display in the current microarray format is limited in its ability to recapitulate the three-dimensional glycan presentation on cell surface glycoproteins, thus, obscuring higher-order binding events (and their physiological consequences) between the multivalent glycoconjugates and their oligomeric receptors, such as the influenza virion.

To address this limitation, a new generation of glycan microarrays have begun to emerge, where individual glycan structures are displayed on synthetic multivalent scaffolds that approximate the presentation of glycans in native glycoproteins.[25] These platforms that use synthetic neo-glycoproteins,[26] glycodendrimers,[27] or glycopolymers[28] as mimetics of the various glycoconjugates found on the surfaces of cells are beginning to reveal the subtle effects of three-dimensional glycan presentation on their recognition by GBPs.[29,30] Inspired by this work and the rich history of linear glycopolymers as soluble probes for analysing influenza binding,[31,32,33] we have developed, and report here, a microarray that utilizes glycopolymers to create a presentation of sialoglycans resembling their native display on mucosal membranes and allows for the interrogation of glycan binding preferences of intact influenza viruses.

To create a microarray platform, which could potentially accommodate a large repertoire of glycan structures found in mucins, we designed a polymer scaffold that can be rapidly assembled into glycopolymers while circumventing the challenges associated with carbohydrate synthesis or pre-functionalization. We have previously reported an acrylamide polymer decorated with pendant *N*-methylaminooxy groups that is primed for direct attachment of unmodified glycans available from natural or commercial sources.[34] Such α-heteroatom nucleophiles are known to react with the reducing terminus of various glycans producing stable *N*-glycopyranosides.[35] Here we describe the use of this strategy to generate glycopolymers displaying sialoglycans that can be recognized by influenza.

Using the RAFT technique,[36] we first prepared polymer precursor **1** with well-defined lengths (DP ~ 200) and narrow chain length distributions (DI ~ 1.18), carrying reactive *N*-methylaminooxy side-chains.[34] The polymer was end-functionalized with an azide group for covalent conjugation on cyclooctyne-coated glass and a tetramethylrhodamine (TAMRA) fluorophore for quantification of the extent of glycopolymer immobilization to the microarray surface. Ligation of glycans to **1** (1.1 equiv. of glycan per reactive side-chain) proceeded smoothly under acidic conditions (1 M sodium acetate buffer, pH = 4.5) at 50 °C for 72 hrs, affording lactose (**2a**), 3'-sialyllactose (α2-3, **2b**), and 6'-sialyllactose (α2-6, **2c**) glycopolymers in high yields. (see ESI) The lower ligation efficiency observed for 3'- and 6'-sialyllactose (45%) compared to lactose (70%) is likely due to the larger size of these glycans and to charge repulsion due to the presence of carboxylate groups in the sialic acid residues.

Printing of the resulting glycopolymers on cyclooctyne-coated slides[34] produced microarrays of increasing glycopolymer densities. Using a robotic spotter, glycopolymers **2a-c** dissolved in a printing buffer (0.005% Tween 20 in PBS) were dispensed at a range of increasing glycan concentrations (1 μM to 10 mM) and the resulting arrays were stored at 4 °C overnight to allow sufficient time for glycopolymer grafting via the strain-promoted azide-alkyne cycloaddition[37] to proceed. The slides were then washed (0.1% Triton X-100 in PBS) to remove excess unbound material and the immobilized TAMRA-labelled glycopolymers were imaged using a fluorescence scanner to obtain an image of the resulting glycopolymer microarray (Fig 3A). A plot of fluorescence emission intensity at λ_{max} = 535 nm as a function of glycan concentration during printing (Fig 3B) indicates that the density of glycopolymers on the microarray surface can be modulated and is a function of both the polymer concentration in the printing buffer as well as the structure of the pendant glycans. While the maximum surface density for 3'- and 6'-siallylactose polymers was achieved at glycan concentrations of ~ 5 mM, the lactose glycopolymer did not reach surface saturation over the entire range of printing concentrations. This is not surprising, since the larger size and negative charge of the sialoglycans is expected to limit the accessibility and, thus, grafting efficiency of the glycopolymer chains to the substrate.

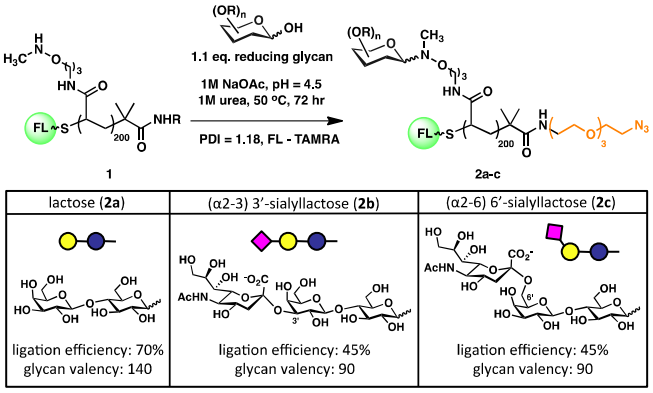

Figure 2. Glycopolymer synthesis. Condensation of reactive polymer **1** with reducing glycans yielded fluorescent glycopolymers **2a-c** primed with an azido-group for immobilization on cyclooctyne-coated microarray substrates.

The resulting density variant glycan arrays were then evaluated for binding by a set of lectins with known glycan specificities. The slides were incubated for 1 hr with *Maackia amurensis* agglutinin (MAA)[38] and *Sambucus nigra* agglutinin (SNA)[39] with preference for 3'- and 6'-sialoglycans, respectively, as well as *Ricinus communis* agglutinin (RCA-120)[20] that recognizes terminal galactose residues. Figure 3C shows that the arrayed glycopolymers were recognized selectively by these lectins according to the structures of their pendant glycans (for experimental details see ESI).

Once the selectivity of the surface-bound glycopolymers toward lectins was established, the arrays were tested for binding of influenza viruses. Whole H1N1 (A/Puerto Rico/8/34) and H3N2 (A/Aichi/2/68) viruses were incubated on the array for 1 hr at ambient temperature. Thereafter, the slides were washed (PBS), fixed (2% paraformaldehyde in PBS, 30

min), and probed with anti-H1 (A/California/06/09) and anti-H3 (A/Shandong/9/99) antibodies for 30 min. Immunostaining with secondary antibodies appropriately labelled to emit fluorescence at λ_{max} = 645 nm was used to detect viruses that remained bound to the microarray surface.

We observed robust binding for both viruses according to their established glycan specificities (Fig 4).[40,41] H1N1 (A/Puerto Rico/8/34) is known to engage both 3'- and 6'-sialoglycans and, accordingly, this virus bound to both sialoglycan polymers **2b** and **2c** in the microarray (Fig 4A). In contrast, the H3N2 (A/Aichi/2/68) virus, which recognizes exclusively 6'-sialoglycans, engaged only the 6'-sialyllactose glycopolymer **2c** (Fig 4C). Importantly, neither virus bound to the lactose glycopolymer **2a**, which lacks sialic acid residues, or the polymer backbone alone (for full microarray see ESI).

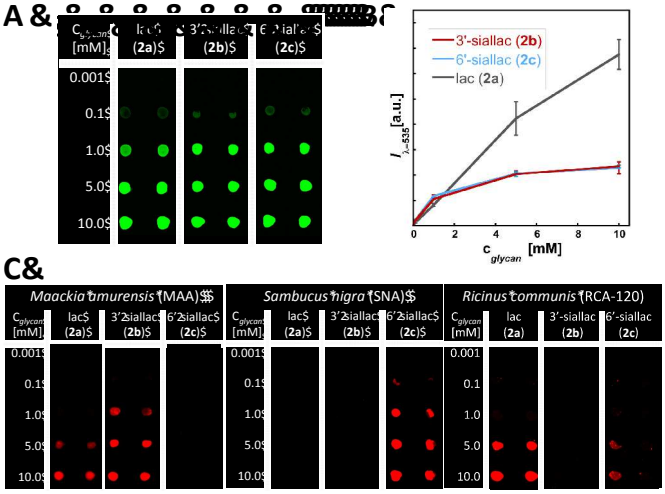

Figure 3. Density variant glycan microarray was fabricated by printing TAMRA-labeled glycopolymers **2a-c** at increasing glycan concentrations (c_{glycan} = 1 μM to 10 mM) (A). The negatively charged sialoglycan polymers **2b** and **c** showed comparable surface grafting efficiency (B). The arrayed glycopolymers were recognized by lectins according to the structure of their pendant glycans (C).

Our glycan array platform reveals additional information about viral binding as a function of glycan presentation at the surface. The fluorescence tag in the arrayed polymers allows for determination of the relative glycan densities across the microarray, and thus, for direct comparison of viral binding to the various sialoglycan presentations. As expected, the amount of virus bound in the microarray generally increases with increasing glycopolymer density (Fig 4B and D). Interestingly, while at lower surface densities, we did not observe significant preference of H1N1 binding for either glycoconjugate; the virus showed consistently enhanced binding to the more densely grafted 3'-sialyllactose polymers (Fig 4B). While we have yet to fully investigate this phenomenon and its biological relevance in the context of viral specificity, our observations suggests the possible role of glycan presentation in modulating the specificity of the pathogen.

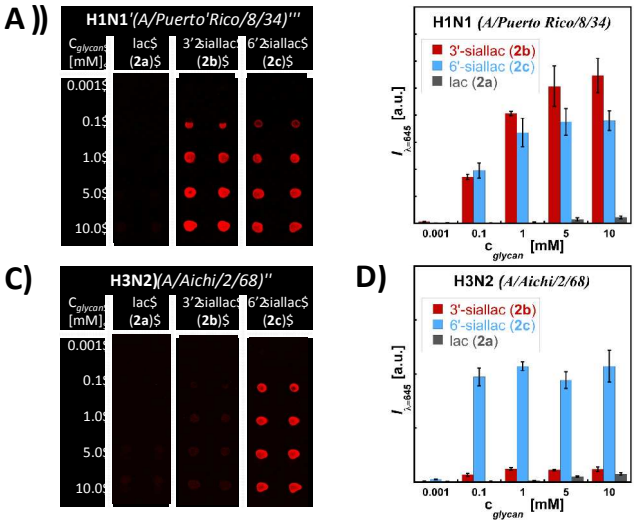

Figure 4. Intact influenza A viruses were introduced to the microarray, visualized using immunostaining of their HA proteins, and analysed for specificity toward individual glycopolymers. While H1N1 (A/Puerto Rico/8/34) virus bound to both sialoglycan polymers (A), it exhibited a preference for 3'-sialyllactose epitopes (B). The H3N2 (A/Aichi/2/68) virus bound exclusively to the 6'-sialyllactose glycopolymers (C and D), consistent with its known specificity.

The mucin mimetic array offers a convenient and quantitative analytical platform to systematically evaluate various parameters, such as glycan structure, valency, and surface density, which define the interactions of viruses with their glycoprotein ligands. In addition, the ease and modularity of the glycopolymer assembly offers rapid access to multivalent mucin-like ligands with a broad diversity of glycan structures that can be integrated within the microarray platform. The ability to systematically evaluate viral interactions in the context of multivalency of glycan presentation on mucosal membranes and the oligomeric state of viral glycan receptors may provide new insights into the mechanisms of the earliest stages of influenza entry as well as its infectivity and potential for interspecies transmission.

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Notes and references

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† Electronic supplementary information (ESI) available: Materials and methods, analytical data, and copies of NMR spectra for compounds **1, 2a-c**, and other synthetic intermediates. See DOI: 10.1039/c000000x

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