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Probing the receptor interactions of an H5 avian influenza virus using a baculovirus expression system and functionalised poly(acrylic acid) ligands

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Abstract—Influenza viruses attach to host cells by binding to terminal sialic acid (Neu5Ac) on glycoproteins or glycolipids. Both the linkage of Neu5Ac and the identity of other carbohydrates within the oligosaccharide are thought to play roles in restricting the host range of the virus. In this study, the receptor specificity of an H5 avian influenza virus haemagglutinin protein that has recently infected man (influenza strain A/Vietnam/1194/04) has been probed using carbohydrate functionalised poly(acrylic acid) polymers. A baculovirus expression system that allows facile and safe analysis of the Neu5Ac binding specificity of mutants of H5 HA engineered at sites that are predicted to effect a switch in host range has also been developed.

1. Introduction

The influenza virus is one of several viruses that utilise interactions between a viral surface protein (haemagglutinin HA) and specific cell surface oligosaccharides to initiate infection. Since the haemagglutinins of individual viral strains have differing affinities for specific sialyl oligosaccharides, recent studies have attempted to clarify the fine structure of the virus ligand.[1] Such studies have illustrated that, in general, human viruses show a preferential binding to saccharides bearing sialic acid (Neu5Ac) linked in the α-2,6 orientation, whereas avian viruses show a preference for sialic acid with an α-2,3 linkage.[2] In spite of these receptor preferences, some strains of avian influenza such as H5N1have proved able to infect and cause death in humans, cats, pigs and other mammals. This has raised concern that re-assortment between human and avian subtypes could generate viruses of pandemic potential.[3] In our current programme of work we were interested in studying the HA protein of the H5N1 avian influenza virus that has recently infected man (influenza strain A/Vietnam/1194/04) to probe the receptor specificity of the virus. Moreover, we wished to investigate whether a recombinant HA protein expressed from baculovirus infected insect cells would accurately reflect the binding properties of authentic H5N1 virus particles. In order to probe the receptor–ligand specificity, multivalent versions of the sialic acid containing carbohydrate ligands were required. A review of the literature indicated that dendritic macromolecules, polymerised liposomes and poly(acrylamide)/poly(acrylic acid) derivatives have all been functionalised with sialyl oligosaccharides to probe or inhibit influenza binding.[4] For example, poly(acrylic acid) and poly(acrylamide) derived glycopolymers displaying Neu5Ac-α-2,3-Lactose or Neu5Ac-α-2,6-Lactose/Lactosamine have been used widely to represent avian and human influenza receptors, respectively, and these systems were selected for the work described herein.[2,4]

Entry to Neu5Ac-α-2,3-Lactose-containing glycopolymers has previously involved anomeric functionalisation of the intact Neu5Ac-α-2,3-Lactose trisaccharide, to afford glycosylamine-1-*N*-glycyl derivatives that are suitable for attachment to the polymer.[4a] Whilst this three step protocol works well, particularly when the sialic acid-containing saccharides are either commercially

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available or can be isolated from natural sources, we were interested in comparing the feasibility of a modified, reverse approach. This would seek to introduce the sensitive Neu5Ac moiety to a disaccharide acceptor that had already been functionalised at the anomeric position with a chloroacetyl linker unit. If successful, the number of synthetic transformations to which the sensitive sialic acid containing trisaccharide would be exposed would be reduced. In addition, if protecting group strategies were appropriately selected, it was proposed that conversion of the chloroacetyl linker to the amino acetyl linker, that is required for attachment to the polymer, could be achieved using identical conditions to those required for global deprotection of the saccharide. Finally, it was anticipated that this approach could be particularly valuable when larger quantities of the saccharide material were needed than could be obtained from natural sources or commercial entities, broadening the applicability of this strategy. The retrosynthetic analysis for the approach developed herein is illustrated below (Fig. 1).

2. Results and discussion

In order to access the anomerically functionalised trisaccharide Neu5Ac-α-2,3-Lactose (**1**), regio- and stereoselective glycosylation of anomerically functionalised disaccharide acceptor (**8**) with free hydroxyls at C-3′ and C-4′ was required. It was postulated that diol (**8**) could be accessed by selective removal of the isopropylidene acetal from within (**7**) which could itself be accessed from lactose derivative (**2**) using established protecting group methodology (Scheme 1). Acetate esters were selected as hydroxyl protecting groups that were orthogonal to the isopropylidene acetal due to their ease of introduction and lability to treatment with ammonia: it was therefore anticipated that treatment of the acetate protected trisaccharide with ammonia would allow for both acetate deprotection and conversion of the chloroacetate linker unit to the aminoethyl linker in one-pot at a final stage of the synthesis.

Synthesis of the C-3 acceptor (**8**) commenced from β-azide (**2**) which could be readily prepared in multigramme quantities from lactose.[5] The hydroxyl groups at C-3′ and C-4′ were protected as the isopropylidene acetal by treatment of (**2**) with 2,2-dimethoxypropane and *p*-toluenesulfonic acid under thermodynamic conditions, to afford (**4**) in 68% yield. In addition, less than 10% of the kinetic product (**3**), where the C-4′ and 6′ hydroxyl groups were protected, was also recovered. Protection of the remaining free hydroxyl groups within (**4**) as acetate esters using acetic anhydride and pyridine afforded the desired fully protected azide (**5**) in 71% yield. Hydrogenation of the azide to afford the amine (**6**) was achieved in excellent yield using 10% Pd/C and hydrogen and the linker unit was successfully introduced as the *N*-chloroacetate (**7**) by reaction of the amine with chloroacetic anhydride. Removal of the isopropylidene acetal protecting group using a 9:1 mixture of TFA–water followed by precipitation of the product by addition of diethyl ether afforded the desired acceptor (**8**) in 86% yield.

Glycosylation of the acceptor (**8**) was then performed using the thioethyl sialic acid donor (**9**).[6] This was again designed to incorporate *O*-acetate protecting groups to allow global deprotection of the trisaccharide and simultaneous formation of the amine linker unit after glycosylation. Thus the acceptor was treated with the sialic acid donor under anhydrous conditions in acetonitrile with 1% dichloromethane at −40 °C. This afforded the desired trisaccharide (**10**) in 67% yield as a 2:1 mixture of the α- and β-anomers, which could be separated by careful column chromatography on silica gel, together with a sialic acid elimination product (**11**) (Scheme 2). The stereochemistry of the newly formed sialoside linkages was assigned based on the chemical shift of the H-3 equatorial proton within Neu5Ac, according to the guidelines outlined by Dabrowski et al.[7]

The protected Neu5Ac-α-2,3-Lactose (**10α**) was then treated with 35% aqueous ammonia in an attempt to effect removal of the *O*-acetate protecting groups and amination of the linker unit in one-pot. ^{1}H NMR spectroscopic analysis of the material thus produced indicated the loss of all the *O*-acetyl protecting groups, which had been represented by a group of singlets circa at 2 ppm. However, the conditions also resulted in the

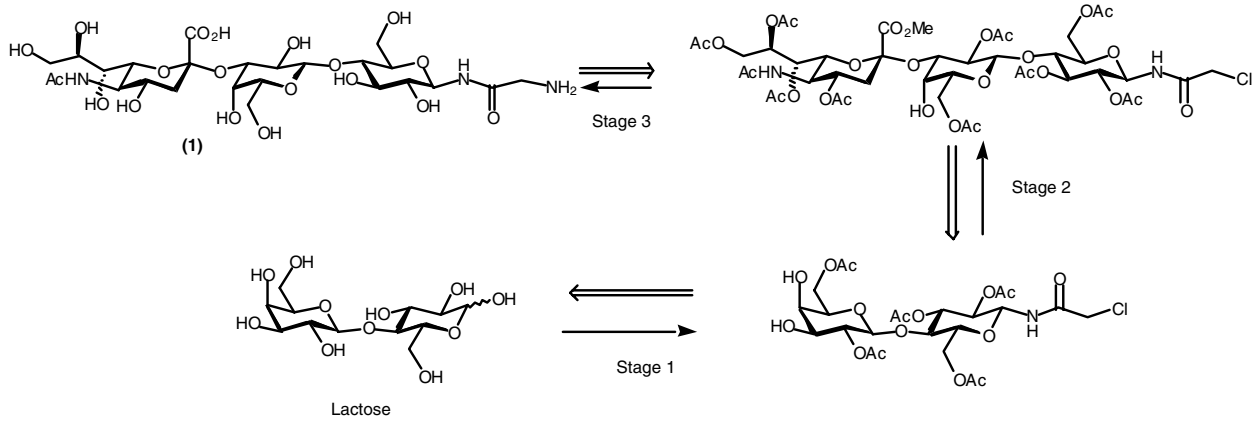
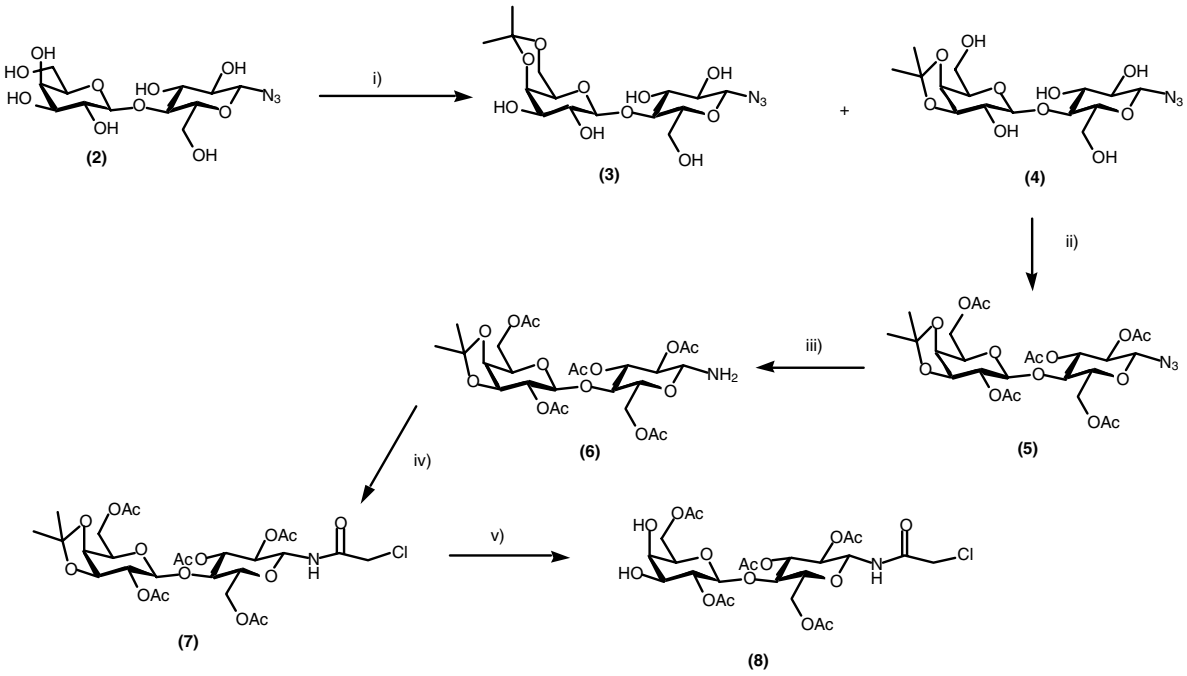
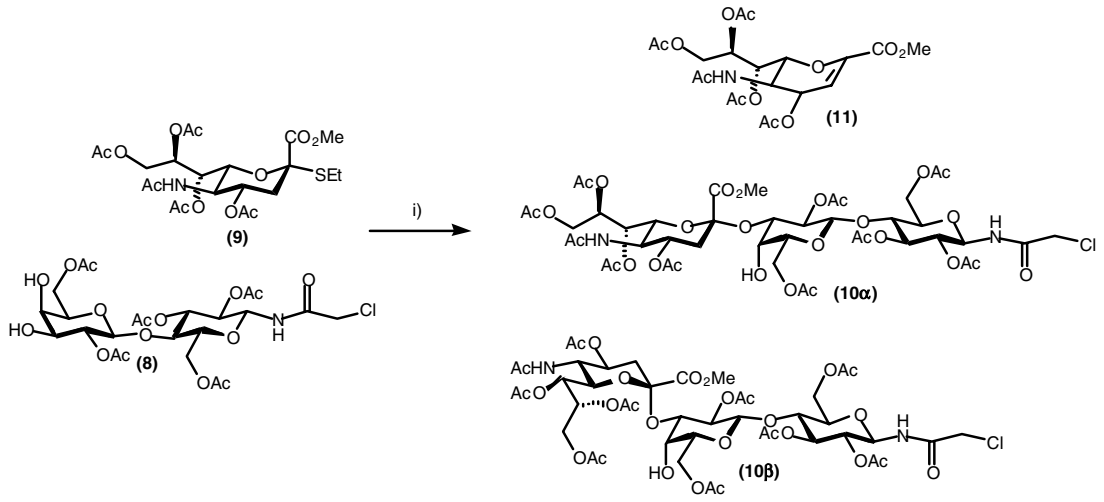


Figure 1. *Stage 1*, early introduction of the anomer linker and acetate protecting groups. *Stage 2*, introduction of sialic acid with common acetate protecting groups. *Stage 3*, one-pot conversion of chloroacetate linker group and removal of acetate protecting groups.



Scheme 1. Reagents and conditions: (i) 2,2-dimethoxypropane, *p*-toluenesulfonic acid, DMF, 40 °C, 48 h, (**3**) 10%, (**4**) 68%; (ii) acetic anhydride, pyridine, rt, 16 h, 71%; (iii) 10% Pd/C, H_2, triethylamine, methanol, THF, rt, 16 h, 81%; (iv) 1 M $NaHCO_{3(aq)}$, chloroacetic anhydride, ethyl acetate, 0 °C, 30 min, 65%; (v) trifluoroacetic acid, water, diethyl ether, rt, 1 min, 86%.

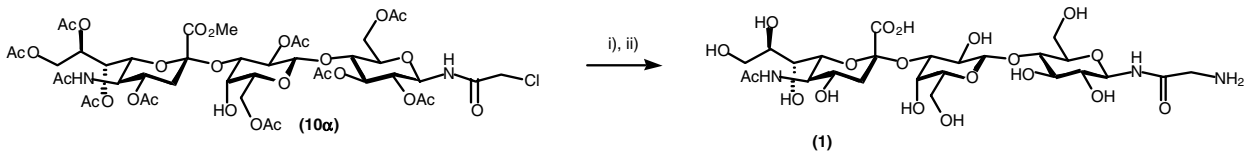


Scheme 2. Reagents and condition: (i) NIS, trifluoroacetic acid, 3 Å molecular sieves, acetonitrile, dichloromethane, −40 °C, 2 h, 67%.

loss of the sialic acid *N*-acetyl group as determined by the lack of a singlet in the ^{1}H NMR spectrum at 2 ppm. Subsequently, milder conditions of 10% aqueous ammonia were used to give a mixture of aminated material with partial removal of the *O*-acetyl protecting groups. This crude material was then treated with a methanolic solution of sodium methoxide to remove any remaining *O*-acetyl groups. The carboxylic acid functionality was restored by addition of 1 M sodium hydroxide to the reaction without prior work-up and this afforded the desired 3′ oligosaccharide (**1**) in yields of 80% without any need for purification of the reaction intermediates (Scheme 3).

This therefore illustrates that our modified synthetic approach involving introduction of sialic acid to acceptors that have already been functionalised at the anomeric position is an efficient alternative strategy to that previously reported.[4a]

To allow the virus binding results in this study to be compared with those from other workers,[4] poly(*p*-nitrophenylacrylate) (**12**) was used as the activated supporting polymer. This was prepared according to literature procedures via AIBN initiated polymerisation of *p*-nitrophenyl acrylate[8] and the precipitate was filtered and dried to give poly(*p*-nitrophenylacrylate) polymers

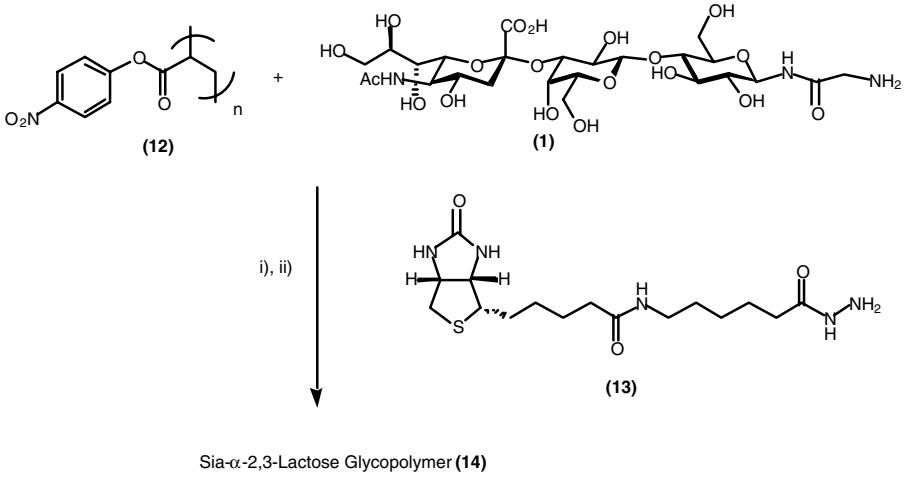


Scheme 3. Reagents and conditions: (i) 10% aqueous ammonia, rt, 16 h; (ii) NaOMe, MeOH, rt, 18 h then 1 M NaOH, rt 18 h, 80%.

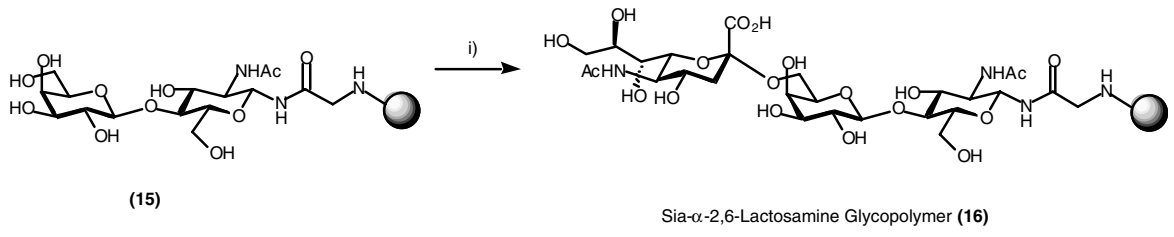
with a weight average of approximately 20 kDa and a polydispersity of 1.33, as determined by GPC in DMF. A solution containing 50 μmol of the conjugating polymer (**12**) in DMF was then treated with a solution containing 10 μmol of trisaccharide (**1**) in DMSO. In addition, 1 mol % of biotin hydrazide (**13**) was added as a biological marker that would allow the polymer to be visualised in the biological assays (Scheme 4). Treatment of the mixture with triethylamine gave rise to a bright yellow solution, which was attributed to the concomitant formation of *p*-nitrophenol. After 24 h at 40 °C the reaction mixture was cooled and the resulting conjugate was modified by saponification with NaOH. The polymeric glycoconjugate was isolated using size exclusion (Sephadex LH-20®) column chromatography to afford a glycopolymer (termed 3′SL) (**14**) that is believed to be representative of the avian influenza virus receptor.

The synthesis of glycopolymer (**16**) (termed 6′SLN) containing Neu5Ac-α-2,6-Lactosamine, that is representative of a human influenza virus receptor, was achieved by enzyme mediated glycosylation of a biotinylated lactosamine polyacrylic acid conjugate (Glycotech, USA) (Scheme 5).[2b] Thus an α-2,6-sialyltransferase enzyme (Calbiochem) was utilised to effect regio- and stereoselective glycosylation of the C-6′ hydroxyl of a lactosamine disaccharide polyacrylic acid conjugate (**15**) forming the desired trisaccharide (**16**) in one step. This was again isolated using size exclusion (Sephadex LH-20®) column chromatography.

Influenza viruses representing typical human or avian strains were next tested for their binding to the glycoconjugates to verify that the synthesized 3′SL and 6′SLN adequately represent the avian and human host receptors, respectively. The human influenza strain A/Eng/492/95 (H3N2) is a clinical isolate that has undergone minimal laboratory passage. The avian influenza virus A/Duck/Singapore/3/97 (H5N3) was used as a representative avian influenza virus since it represents the majority of avian influenza viruses that have never been observed to infect humans. To analyse the receptor–ligand interactions, concentrated viruses were immobilized on ELISA plates and probed with a range of concentrations of each biotinylated glycoconjugate



Scheme 4. Reagents: (i) Et_3N; (ii) NaOH.



Scheme 5. Reagents: (i) sodium cacodylate, Brij 97®, CMP-Neu5NAc, NaN_3, α-2,6-sialyltransferase.

polymer. Bound polymer was detected following incubation with streptavidin horseradish peroxidase conjugate and developed using 3,3′,5,5′-tetramethylbenzidine (TMB) chromagenic substrate. From the binding curves obtained, Scatchard Plots were generated and the inverse of the gradient of the plot was expressed as the K_d (mM) as a measure of the affinity of each viral HA protein for the SA in each polymer.

When the avian influenza virus was compared to the human strains a clear difference in their receptor specificity was observed (Fig. 2 and Table 1). Thus A/Duck/Singapore/3/97 virus bound with strong affinity to the 3′SL but did not bind to the human-like receptor 6′SLN. In contrast, the human virus bound well to the 6′SLN, but also showed some affinity for the avian-like receptor 3′SL.

Next, recombinant H5 HA proteins were generated, as a means to develop facile and safe analysis of the Neu5Ac binding specificity of mutants of H5 HA engineered at sites predicted to effect a switch in host range. Thus, following amplification of the sequence encoding the mature HA polypeptide from the H5N1 influenza virus A/Vietnam/1194/04, that has recently been seen to infect humans,[3] recombinant HA protein was expressed in a 'soluble' form, fused to one of two peptide tags used for detection, the human Fc tag or a TAP (based on protein A from *Staphylococcus aureus*) tag.[9] Recombinant protein was expressed using the baculovirus system following infection of insect cells and secreted protein was detected in the supernatant of infected cells using a polyclonal anti-H5 serum, and with anti-human IgG (Sigma) for H5-Fc or with anti-protein A (Sigma) antibody for H5-TAP.[10]

Table 1. Binding affinity of recombinant proteins and influenza viruses to sialylglycopolymers

HA protein/virus	K_d (mM)	
	3′SL	6′SLN
A/duck/Singapore (avian H5)	0.5	nb
A/Eng/492 (human H3)	0.4	0.1
RG14	0.7	nb
H5 TAP	0.8	nb
H5 Fc	0.5	nb

nb, no detectable binding.

Recombinant proteins H5-Fc or H5-TAP were then assessed in the binding assay. In addition the recombinant virus known as RG14 was analysed. This virus is based on the A/PR/8/34 human vaccine strain but bears the surface antigens HA and NA (Neuraminidase) of A/Vietnam/1194/04. The HA protein has been genetically engineered to remove the virulence determinant at the cleavage site between HA subunits 1 and 2 that is associated with high pathogenicity in birds.[11] This mutation should not affect its receptor binding properties. Both expressed proteins bound well to the 3′SL which represents the avian host receptor and showed no affinity for the 6′SLN which represents the human host receptor (Fig. 3 and Table 1). Moreover, the calculated affinity constant K_d derived from Scatchard analysis was remarkably similar to that obtained using whole influenza virus particles of RG14 (Table 1, K_d for H5-Fc is 0.5 mM and for H-5TAP is 0.8 mM compared to K_d for RG14 of 0.7 mM). These data confirm receptor specificity for the 3′SL conjugate resides solely in the HA and also confirm that the baculovirus system we have developed allows for easy and safe analysis of the SA binding specificity of mutants of H5 HA engineered at

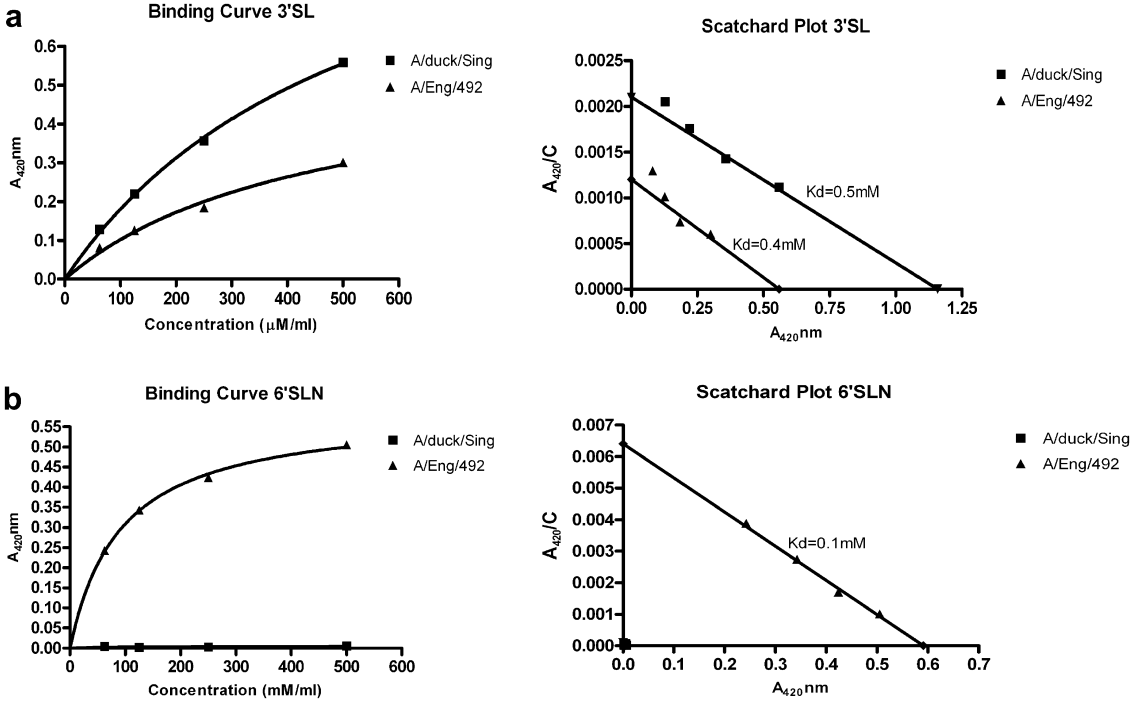


Figure 2. Binding curves and Scatchard Plot for (■) A/duck/Singapore/3/97 and (▲) A/Eng/492/95 with sialylglycopolymers (a) 3′SL-PAA and (b) 6′SLN-PAA. The data are means of two different experiments. The K_d are expressed in millimolar Neu5Ac.

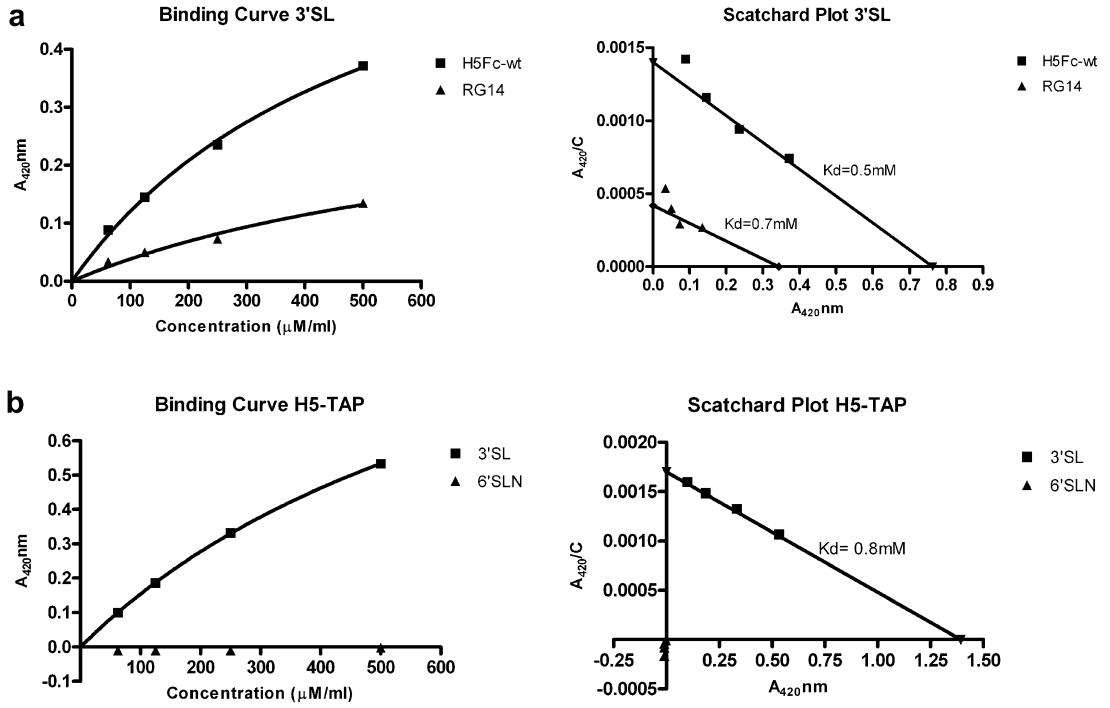


Figure 3. Binding curves and Scatchard Plot for (■) H5Fc and (▲) RG14 with sialylglycopolymer 3′SL-PAA (a); binding curve and scatchard plot for H5-TAP (b) with sialylglycopolymers 3′SL-PAA (■) and 6′SLN-PAA (▲). The data are means of two different experiments. The K_d are expressed in millimolar Neu5Ac.

sites predicted to effect such a switch in host range. The receptor binding affinity results are in agreement with a previous report using the inactivated H5N1 virus A/Vietnam/1194/04,[12] and a complimentary approach using glycan arrays to probe the specificity of a recombinant H5 HA protein derived from the influenza virus A/Vietnam/1203/04 that was isolated from humans during the same H5N1 outbreak in 2004.[1c]

3. Conclusions

A new and efficient route for the synthesis of 3′SL has been exemplified in this work, and new biological data generated herein have improved our understanding of the influenza host range. Using the glycoconjugates generated it proved possible to measure differences in relative affinity of influenza virus HA proteins for α-2,3- or α-2,6-linked terminal Neu5Ac. Baculovirus-mediated expression of recombinant HA proteins was shown to be a valid path for analysis of the receptor binding properties of HA. This study has contributed to a longer term programme which is now in progress to engineer mutants of H5 HA that potentially display altered binding properties and that can be expressed in a safe and facile system.

4. Experimental

4.1. General methods

Reagents were purchased from Aldrich, Fisher and Lancaster and used as supplied. Tetrahydrofuran was dried by distillation from sodium-benzophenone ketyl under nitrogen. Dichloromethane was dried by distillation from calcium hydride under nitrogen.

Thin layer chromatography was performed using Merck silica gel pre-coated aluminium plates. Plates were observed using a sulfuric acid dip (4% sulfuric acid in ethanol), a vanillin dip (1 g vanillin and 1 mL H_2SO_4 in 100 mL EtOH) or UV fluorescence (λ_{max}, 254 nm). Column chromatography was carried out using silica gel of particle size: 40–63 μm using head pressure by means of hand bellows.

^1H and ^{13}C NMR spectra were recorded in either chloroform-*d*, MeOH-*d* or D_2O and referenced to residual solvent peaks or to trimethylsilane as an internal standard using a Bruker AC250 (250 MHz) or a Bruker AC400 (400 MHz) spectrometer. Chemical shifts are quoted in parts per million (ppm), coupling constants are recorded in Hertz to the nearest 0.5 Hz.

Infrared spectra were recorded on a Perkin-Elmer 1720-X series Fourier Transform spectrometer as thin films, Nujol mulls or KBr discs, all absorptions are quoted in cm^{-1}.

Mass spectra data were recorded on a V.G. Micromass 70–70F spectrometer under chemical ionisation using ammonia or on a Finnigan MAT 95 under chemical ionisation.

GPC analysis was conducted using a Polymer Laboratories PL-GPC 220 high-temperature chromatography using PL mixed gel columns and RI detection.

Poly(*p*-Nitrophenyl acrylate) was run at 60 °C as a solution in GPC grade DMF (1 mg mL^{-1}) using PL Easy-Cal polystyrene calibrants. Glycopolymers were run at 40 °C as a solution in HPLC grade water (0.5 mg mL^{-1}) using PL Easy-Cal polyethyleneglycol calibrants.

Melting points were obtained using an electrothermal digital melting point apparatus and are uncorrected.

Specific optical rotations were recorded in chloroform or methanol. Optical activities were determined using a Perkin-Elmer 341 polarimeter at a wavelength of 589 nm.

4.1.1. 3,4-*O*-Isopropylidene-β-D-galactopyranosyl-(1,4)-β-D-glucopyranosyl azide (4) and 4,6-*O*-isopropylidene-β-D-galactopyranosyl-1,4-β-D-glucopyranosyl azide (3). β-D-Galactopyranosyl-(1,4)-β-D-glucopyranosyl azide (**2**)[6] (3.78 g, 10.3 mmol) and *p*-toluene sulfonic acid (38 mg, 0.21 mmol) were dissolved in DMF under an inert atmosphere and heated to 40 °C. The reaction mixture was treated with 2,2-dimethoxypropane (5.67 mL, 46 mmol) and stirred at 40 °C for 48 h. The reaction mixture was quenched by the addition of NEt_3 (1.97 mL) and the solvent was removed in vacuo to give a mixture of starting material and product as a colourless solid. Purification by column chromatography on silica gel eluting with 8:1 chloroform-methanol gave a mixture of 3,4-*O*-isopropylidene- and 4,6-*O*-isopropylidene-protected material (3.51 g, 84%) as a colourless solid. Further purification by column chromatography on silica gel eluting with 6:2:1 ethyl acetate–^{i}propanol–water gave the desired product (**4**) (4.04 g, 68%) as a colourless solid, 4,6-*O*-isopropylidene-β-D-galactopyranosyl-1,4-β-D-glucopyranosyl azide (**3**) was also recovered (0.16 g, 10%) as a colourless solid. For (**4**), m.p. 179.8–181.1 °C; $[\alpha]_D^{20}$ 1.28 (*c* 0.335, MeOH); υ_{max} (MeOH, thin film) 3378, 2119, 1651, 1384, 1245 and 1073 cm^{-1}; δ_H (250 MHz, MeOD) 4.56 (1H, d, *J* 8.5, Glc-C(1)*H*), 4.43 (1H, d, *J* 7.5, Gal-C(1)*H*), 4.21–4.18 (2H, m, Glc-C(6)*H*, Gal-C(4)*H*), 3.95–3.84 (3H, m, Glc-C(6)*H*, Gal-C(6)H_2), 3.64–3.51 (6H, m, Glc-C(3)*H*, Glc-C(4)*H*, Glc-C(5)*H*, Gal-C(2)*H*, Gal-C(3)*H*, Gal-C(5)*H*), 3.24 (1H, app.t, *J* 8.5, Glc-C(2)*H*), 1.55 (3H, s, $C(CH_3)_2$), 1.32 (3H, s, $C(CH_3)_2$); δ_C (63 MHz, $CDCl_3$) 105.1 (Gal-*C*(1)H), 100.6 ($C(CH_3)_2$), 92.3 (Glc-*C*(1)H), 79.7 (*C*HOH), 79.0 (*C*HOH), 76.8 (*C*HOH), 74.9 (Glc-*C*(2)H), 73.7 (*C*HOH), 71.9 (*C*HOH), 70.4 (Gal-*C*(4)H), 68.5 (*C*HOH), 64.1 (Glc-*C*(6)H), 61.8 (Gal-*C*(6)H); 30.0 ($C(CH_3)_2$, 19.1 ($C(CH_3)_2$); *m*/*z* (CI) 425 (MNH_4^+, 9%), 236 (14), 203 (100), 145 (19), 60 (20); Found 425.1888. $C_{15}H_{29}O_{10}N_4$ requires 425.1883. For (**3**) mp 165–168 °C; $[\alpha]_D^{20}$ 8.7 (*c* 1, $CHCl_3$); υ_{max} (MeOH, thin film) 3350, 2880, 2121, 1630, 1381 and 1071 cm^{-1}; δ_H (250 MHz, MeOD) 4.57 (1H, d, *J* 8.5, Glc-C(1)*H*), 4.42 (1H, d, *J* 7.5, Gal-C(1)*H*), 4.20–4.18 (2H, m, Glc-C(6)*H*, Gal-C(4)*H*), 3.94-3.85 (3H, m, Glc-C(6)*H*, Gal-C(6)H_2), 3.64–3.50 (6H, m, Glc-C(3)*H*, Glc-C(4)*H*, Glc-C(5)*H*, Gal-C(2)*H*, Gal-C(3)*H*, Gal-C(5)*H*), 3.22 (1H, app.t, *J* 8.5, Glc-C(2)*H*), 1.49 (3H, s, $C(CH_3)_2$), 1.41 (3H, s, $C(CH_3)_2$); δ_C (63 MHz, $CDCl_3$) 105.1 (Gal-*C*(1)H), 100.6 ($C(CH_3)_2$), 92.3 (Glc-*C*(1)H), 79.7 (*C*HOH), 79.0 (*C*HOH), 76.8 (*C*HOH), 74.9 (Glc-*C*(2)H), 73.7 (*C*HOH), 71.9 (*C*HOH), 70.4 (Gal-*C*(4)H), 68.5 (*C*HOH), 64.1 (Glc-*C*(6)H), 61.8 (Gal-*C*(6)H); 30.0 ($C(CH_3)_2$, 19.1 ($C(CH_3)_2$); *m*/*z* (CI) 407 (M^+, 36%), 392 (46), 203 (100), 188 (84), 146 (54); Found 407.1537. $C_{15}H_{25}O_{10}N_3$ requires 407.154.

4.1.2. 2,6-Di-*O*-acetyl-3,4-*O*-isopropylidene-β-D-galactopyranosyl-(1,4)-2,3,6-tri-*O*-acetyl-β-D-glucopyranosyl azide (5). 3,4-*O*-Isopropylidene-β-D-galactopyranosyl-β-(1,4)-glucopyranosyl azide (*4*) (1.71 g, 4.19 mmol) was dissolved in pyridine (40 mL, 0.5 mol) and was treated with acetic anhydride (5 mL, 0.05 mol). The reaction mixture was stirred at room temperature for 16 h and the solvent was then removed by co-evaporation with toluene. The resulting oil was re-suspended in the minimum volume of DCM. The DCM solution was washed twice with HCl (3 M), dried ($MgSO_4$) and the solvent was removed in vacuo to afford the acetylated product as a colourless foam. Purification by column chromatography on silica gel eluting with 1:1 ethyl acetate–hexane gave the desired product (**5**) as a colourless foam (1.84 g, 71%); mp 73.6–75.4 °C; $[\alpha]_D^{20}$ 8.60 (*c* 1.0, MeOH); υ_{max} (CH_2Cl_2, thin film) 3580, 2236, 1750, 1371, 1220 and 1054 cm^{-1}; δ_H (250 MHz, $CDCl_3$) 5.20 (1H, app.t, *J* 9.0, Glc-C(3)*H*), 4.87 (1H, app.t, *J* 9.0, Glc-C(2)*H*), 4.85 (1H, app.t, *J* 7.5 Gal-C(2)*H*), 4.63 (1H, d, *J* 9.0, Glc-C(1)*H*), 4.48 (1H, dd, *J* 12.0, *J*′ 1.5, Glc-C(6)H_2), 4.37–4.33 (1H, m, Gal-C(5)*H*), 4.36 (1H, d, *J* 7.5, Gal-C(1)*H*), 4.27 (1H, dd, *J* 12.0, *J*′ 7.5, Gal-C(6)*H*), 4.21 (1H, dd, *J* 12.5, *J*′ 5.0, Gal-C(6)*H*), 4.19-4.14 (2H, m, Gal-C(3)*H*), Glc-C(6)*H*), 3.93 (1H, app.t, *J* 7.5, Gal-C(4)*H*), 3.77 (1H, app.t, *J* 9.5, Glc-C(4)*H*), 3.74 (1H, ddd, *J* 10.0, *J*′ 4.5, *J*″ 1.5, Glc-C(5)*H*), 2.13 (3H, s, C(O)CH_3), 2.08 (3H, s, C(O)CH_3), 2.07 (3H, s, C(O)CH_3), 2.05 (3H, s, C(O)CH_3), 1.97 (3H, s, C(O)CH_3), 1.53 (3H, s, $C(CH_3)_2$), 1.31 (3H, s, $C(CH_3)_2$); δ_C (63 MHz, $CDCl_3$) 170.7 (*C*(O)CH_3), 170.4 (*C*(O)CH_3), 169.9 (*C*(O)CH_3), 169.5 (*C*(O)CH_3), 169.2 (*C*(O)CH_3), 110.8 ($C(CH_3)_2$), 100.5 (Gal-*C*(1)H), 87.5 (Glc-*C*(1)H), 76.7 (Gal-*C*(5)H), 75.6 (Glc-*C*(4)H), 74.9(Glc-*C*(5)H), 73.0 (Gal-*C*(3)H), 72.6 (Gal-*C*(2)H), 72.2 (Glc-*C*(3)H), 70.9 (2C, Glc-*C*(2)H, Gal-*C*(4)H), 63.1 (Gal-*C*(6)H_2), 61.9 (Glc-*C*(6)H_2), 27.3 ($C(CH_3)_2$), 26.1 ($C(CH_3)_2$), 20.8 (2C, C(O)*C*H_3), 20.7 (C(O)*C*H_3), 20.6 (2C, C(O)*C*H_3); *m*/*z* (CI) 635 (MNH_4^+, 24%), 287 (100), 229 (22), 85 (27); found 635.2488. $C_{25}H_{39}O_{15}N_4$ requires 635.2412.

4.1.3. 2,6-Di-*O*-acetyl-3,4-*O*-isopropylidene-β-D-galactopyranosyl-(1,4)-2,3,6-tri-*O*-acetyl-β-D-glucopyranosyl amine (6). Azide (**5**) (0.9 g, 1.46 mmol) was dissolved in methanol (15 mL) and treated with 10% Pd/C (0.45 g). The reaction mixture was stirred at room temperature under an atmosphere of hydrogen for 16 h. The reaction was then filtered through Celite® and the solvent was removed in vacuo to yield the product as a colourless foam. Purification by column chromatography on silica gel eluting with 2:1 ethyl acetate–hexane afforded the desired product (**6**) (0.86 g, 81%) as a colourless foam; mp 92.7–94.5 °C; $[\alpha]_D^{20}$ 37.6 (*c* 1, $CHCl_3$); υ_{max} ($CHCl_3$) 3408, 2989, 2941, 2124, 1745, 1434, 1372, 1230, 1158, 1135, 1045 cm^{-1}; δ_H (400 MHz, $CDCl_3$) 5.22 (1 H, app.t, *J* 9.5, Glc-C(3)*H*), 485 (1H, app.t, *J* 7.0, Gal-C(2)*H*),

4.74 (1H, app.t, *J* 9.5, Glc-C(2)*H*), 4.42 (1H, dd, *J* 12.0, *J′* 2.0, Glc- C(6)*H*), 4.34 (1H, d, *J* 7.0, Gal-C(1)*H*), 4.31 (1H, dd, *J* 11.5, *J′* 7.0, Gal-C(6)*H*), 4.29 (1H, dd, *J* 7.0, *J′* 4.5, Gal-C(5)*H*), 4.28 (1H, dd, *J* 11.5, *J′* 7.0, Gal-C(6)*H*), 4.15 (1H, d, *J* 9.5, Glc-C(1)*H*), 4.13 (1H, dd, *J* 12.0, *J′* 5.0, Glc-C(6)*H*), 4.13 (1H, dd, *J* 7.0, *J* 2.5 Gal-C(3)*H*), 3.93 (1H, app.t, *J* 4.0, *J′* 3.0, Gal-C(4)*H*), 3.69 (1H, app.t, *J* 10.0, Glc-C(4)*H*), 3.61 (1H, ddd, *J* 10.0, *J′* 5.0, *J″* 2.0, Glc-(5)*H*), 2.13 (3H, s, C(O)CH_3), 2.12 (3H, s, C(O)CH_3), 2.08 (3H, s, C(O)CH_3), 2.07 (3H, s, C(O)CH_3), 2.06 (3H, s, C(O)CH_3), 1.54 (3H, s, C(CH_3)$_2$), 1.32 (3H, s, C(CH_3)$_2$); δ_C (100 MHz, $CDCl_3$) 171.2 (*C*(O)CH_3), 171.0 (*C*(O)CH_3), 170.9 (*C*(O)CH_3), 170.3 (*C*(O)CH_3), 169.6 (*C*(O)CH_3), 100.9 (*C*(CH_3)$_2$), 111.2 (Gal-*C*(1)H), 85.0 (Glc-*C*(1)H), 77.3 (Gal-*C*(5)H), 76.9 (Glc-*C*(4)H), 74.2 (Glc-*C*(5)H), 73.4 (Gal-*C*(3)H), 73.1 (Gal-*C*(2)H), 73.0 (Glc-*C*(3)H), 72.8 (Glc-*C*(2)H), 71.3 (Gal-*C*(4)H), 63.5 (Gal-*C*(6)H), 63.0 (Glc-*C*(6)H), 27.7 (C(*C*H$_3$)$_2$), 26.5 (C(*C*H$_3$)$_2$), 21.3 (2C, C(O)*C*H$_3$), 21.2 (3C, C(O)*C*H$_3$)$_2$); *m*/*z* (CI) 532 (M-OC(O)CH_3, 17%), 288 (100), 229 (97), 169 (75), 127 (57); Found 532.2028. $C_{23}H_{34}O_{13}N$ requires 532.2030; Anal. Calcd for $C_{25}H_{37}O_{15}N$: C, 50.76, H, 6.30, N, 2.37; found: C, 50.02, H, 6.27, N, 2.18.

4.1.4. 2,6-Di-*O*-acetyl 3,4-*O*-isopropylidene-β-D-galactopyranosyl-(1,4)-2,3,6-tri-*O*-acetyl-*N*-chloroacetyl-β-D-glucopyranosyl) amine (7). Amine (**6**) (0.7 g, 1.18 mmol) was suspended in aqueous $NaHCO_3$ (1 M, 34 mL). Chloroacetic anhydride (2.4 g, 14 mmol) in ethyl acetate was added at 0 °C and the solution was stirred at 0 °C for 30 min. Acetic acid (0.04 mL, 0.7 mmol) was then added and the organic and aqueous phases were separated. The aqueous layer was washed twice with ethyl acetate, the organic layers were combined, washed with water, dried ($MgSO_4$), and the solvent was removed in vacuo to yield the crude product. Purification by column chromatography on silica gel eluting with 3:1 ethyl acetate–hexane afforded the desired product (**7**) (0.51 g, 65%) as a colourless foam; mp 115–118 °C; $[\alpha]_D^{20}$ 11.3 (*c* 1.0, $CHCl_3$); υ_{max} ($CHCl_3$, thin film) 3465, 1750, 1534, 1438, 1365, 1240 and 1050 cm^{-1}; δ_H (400 MHz, $CDCl_3$) 5.32 (1H, app.t, *J* 9.5, Glc-C(3)*H*), 5.16 (1H, app.t, *J* 9.5, Glc-C(1)*H*), 4.93 (1H, app.t, *J* 9.5, Glc-C(2)*H*), 4.85 (1H, app.t, *J* 7.5, *J′* 6.5, Gal-C(2)*H*), 4.41 (1H, dd, *J* 12.5, *J′* 1.0, Glc-C(6)*H*), 4.36 (1H, d, *J* 7.5, Gal-C(1)*H*), 4.33 (1H, dd, *J* 11.5, *J′* 5.0, Gal-C(6)*H*), 4.29 (1H, dd, *J* 11.5, *J′* 7.5, Gal-C(6)*H*), 4.20 (1H, dd, *J* 12.0, *J′* 4.0, Glc-C(6)*H*), 4.16-4.15 (2H, m, Gal-C(3)*H*, Gal-C(4)*H*), 4.06 (1H, d, *J* 15.5, C(O)CH_2Cl), 4.00 (1H, d, *J* 15.5, C(O)CH_2Cl), 3.93 (1H, ddd, *J* 7.5, *J′* 5.0, *J″* 1.5, Gal-C(5)*H*), 3.78-3.76 (2H, m, Glc-C(4)*H*, Glc-C(5)*H*), 2.14 (3H, s, C(O)CH_3), 2.12 (3H, s, C(O)CH_3), 2.08 (3H, s, C(O)CH_3), 2.07 (3H, s, C(O)CH_3), 2.06 (3H, s, C(O)CH_3), 1.54 (3H, s, C(CH_3)$_2$), 1.32 (3H, s, C(CH_3)$_2$); δ_C (100 MHz, $CDCl_3$) 171.1 (*C*(O)CH_3), 170.8 (*C*(O)CH_3), 170.5 (*C*(O)CH_3), 169.6 (*C*(O)CH_3), 169.2 (*C*(O)CH_3), 166.7 (*C*(O)CH_2Cl), 110.9 (*C*(CH_3)$_2$), 100.2 (Gal-*C*(1)H), 78.4 (Glc-*C*(1)H), 76.8 (Gal-*C*(3)H), 75.6 (Glc-*C*(5)H), 74.8 (Glc-*C*(4)H), 73.0 (Gal-*C*(4)H), 72.6 (Gal-*C*(2)H), 71.8 (Glc-*C*(3)H), 71.0 (Gal-*C*(5)H), 63.2 (Glc-*C*(2)H), 63.2 (Gal-*C*(6)H), 62.1 (Glc-*C*(6)H), 42.3 (C(O)*C*H$_2$Cl), 27.3 (C(*C*H$_3$)$_2$), 26.1 (C(*C*H$_3$)$_2$), 20.9 (2C, C(O)*C*H$_3$), 20.8 (C(O)*C*H$_3$), 20.7 (C(O)*C*H$_3$), 20.6 (C(O)*C*H$_3$); *m*/*z* (CI) 667 (M^+ (^{35}Cl), 43%), 652 (62), 590 (37), 303 (100), 287 (76); Found 667.1873. $C_{27}H_{38}O_{16}N^{35}Cl$ requires 667.1879.

4.1.5. 2,6-Di-*O*-acetyl-β-D-galactopyranosyl-(1,4)-2,3,6-tri-*O*-acetyl-*N*-chloroacetyl-β-D-glucopyranosyl amine (8). Isopropylidene acetal (**7**) (0.7 g, 1.05 mmol) was dissolved in 9:1 trifluoroacetic acid–water (1.8 mL). Diethyl ether (18 mL) was added and the resulting precipitate was filtered, and the solvent was removed *in vacuo* to give the desired product (**8**) (0.57 g, 86%) as a colourless solid; mp 194.5–196.7 °C; $[\alpha]_D^{20}$ 0.81 (*c* 0.97, $CHCl_3$); υ_{max} ($CHCl_3$, thin film) 3448, 1739, 1665, 1530, 1432, 1371, 1233, 1130 and 1049 cm^{-1}; δ_H (400 MHz, $CDCl_3$) 7.33 (1H, d, *J* 9.0, N*H*C(O)CH_2Cl), 5.31 (1H, dd, *J* 10.0, *J′* 8.5, Glc-C(3)*H*), 5.17 (1H, app.t, *J* 9.0, Glc-(1)*H*), 4.94 (1H, app.t, *J* 9.5, Glc-C(2)*H*), 4.91 (1H, dd, *J* 9.5, *J′* 8.0, Gal-C(2)*H*), 4.45 (1H, dd, *J* 12.0, *J′* 1.5 Glc-C(6)*H*), 4.34 (1H, dd, *J* 11.5, *J′* 6.0, Gal-C(6)*H*), 4.33 (1H, d, *J* 8.0, Gal-C(1)*H*), 4.26 (1H, dd, *J* 11.5, *J′* 6.5, Gal-C(6)*H*), 4.20 (1H, dd, *J* 12.0, *J′* 4.5, Glc-C(6)*H*), 4.08 (1H, d, *J* 15.5, C(O)CH_2Cl), 4.02 (1H, d, *J* 15.5, C(O)CH_2Cl), 3.91 (1H, d, *J* 3.0, Gal-C(4)*H*), 3.79 (1H, dd, *J* 4.5, *J′* 1.5, Gal-C(5)*H*), 3.70-3.67 (2H, m, Glc-C(4)*H*, CHO*H*), 3.67-3.62 (2H, m, Gal-C(3)*H*, Glc-C(5)*H*), 2.13 (3H, s, C(O)CH_3), 2.12 (6H, s, C(O)CH_3), 2.08 (3H, s, C(O)CH_3), 2.07 (3H, s, C(O)CH_3); δ_C (100 MHz, $CDCl_3$) 171.6 (*C*(O)CH_3), 171.2 (*C*(O)CH_3), 171.1 (*C*(O)CH_3), 170.7 (*C*(O)CH_3), 170.5 (*C*(O)CH_3), 167.2 (*C*(O)CH_2Cl), 100.7 (Gal-*C*(1)H), 78.4 (Glc-*C*(1)H), 75.9 (Glc-*C*(4)H), 74.7 (Glc-*C*(5)H), 73.2 (Gal-*C*(2)H), 72.6 (Gal-*C*(3)H), 72.3 (Gal-*C*(5)H), 72.2 (Glc-*C*(3)H), 70.4 (Glc-*C*(2)H), 68.5 (Gal-*C*(4)H), 62.5 (Gal-*C*(6)H), 62.0 (Glc-*C*(6)H), 42.2 (C(O)*C*H$_2$Cl), 20.9 (2C, C (O)*C*H$_3$), 20.8 (2C, C (O)*C*H$_3$), 20.6 (C (O)*C*H$_3$); *m*/*z* (CI) 627 (M^+ (^{35}Cl), 22%), 550 (37), 364 (63), 287 (73) 247 (100), 188 (84); Found 627.1567. $C_{24}H_{34}O_{16}N^{35}Cl$ requires 627.1566; Anal. Calcd for $C_{24}H_{34}O_{16}NCl$: C, 45.90, H, 5.46, N, 2.23; found C, 44.67, H, 5.45, N, 2.19.

4.1.6. 4,7,8,9-Tetra-*O*-acetyl-*N*-acetyl-α-D-neuraminic acid methyl ester-(2,3)-2,6-di-*O*-acetyl-β-D-galactopyranosyl-(1,4)-2,3,6-tri-*O*-acetyl-*N*-chloroacetyl-β-D-glucopyranosyl amine (10). Amine (**8**) (0.75 g, 1.2 mmol) was dissolved in acetonitrile (10 mL) and DCM (250 μL) under an inert atmosphere in the presence of 3 Å molecular sieves. 4,7,8,9-Tetra-*O*-acetyl-2-*S*-ethyl-2-deoxy-*N*-acetyl neuraminic acid methyl ester (**9**) (1.0 g, 1.85 mmol) and *N*-iodosuccinimide (0.80 g, 3.6 mmol) were added and the reaction mixture was cooled to −40 °C and treated with trifluoromethane sulfonic acid (500 μL, 0.5 mmol). After 2 h, the molecular sieves were removed by filtration and the reaction mixture was washed with water. The aqueous layer was washed twice with DCM. The organic layers were combined and washed twice with sat. sodium thiosulfate, dried ($MgSO_4$) and the solvent was removed in vacuo to give the crude product as a colourless solid. Purification by column chromatography on silica gel eluting with 3:2 toluene–acetone gave a mixture of α- and β-linked trisaccharide material (880 mg, 67%) as a colourless solid. Further purification by column chromatography on silica gel eluting with 4:2:1 chloroform–hexane–^{i}propanol gave the desired α-anomer

(**10α**) (591 mg, 45%) as a colourless foam; mp 131.5–133.2 °C; $[\alpha]_D^{20}$ 0.31 (*c* 0.67, $CHCl_3$); υ_{max} ($CHCl_3$, thin film) 3443, 1641, 1101 and 1054 cm^{-1}; δ_H (400 MHz, $CDCl_3$) 7.35 (1H, d, *J* 9.0, N*H*C(O)CH_2Cl), 5.52 (1H, d, *J* 3.0, Gal-C(4)*H*), 5.34–5.23 (3H, m, SA-C(7)*H*, SA-C(6)*H*, SA-C(4)*H*), 5.29 (1H, app.t, *J* 9.0, Glc-C(3)*H*), 5.21 (1H, app.t, *J* 9.0, Glc-C(1)*H*), 5.12 (1H, d, *J* 8.0, Gal-C(1)*H*), 5.04 (1H, dd, *J* 10.5, *J*″ 3.5, Gal-C(2)*H*), 4.97 (1H, app.t, *J* 9.5, Glc-C(2)*H*), 4.78 (1H, dd, *J* 12.5, *J*′ 2.5, Gal-C(6)*H*), 4.56 (1H, d, *J* 8.0, Gal-C(1)*H*), 4.46 (1H, dd, *J* 12.0, *J*′ 1.5, Glc-C(6)*H*), 4.14 (1H, dd, *J* 12.5, *J*′ 5.5, Gal-C(6)*H*), 4.07 (1H, dd, *J* 7.5, *J*″ 2.0, SA-C(5)*H*), 4.06 (1H, d, *J* 15.5, C(O)CH_2Cl), 4.04 (1H, dd, *J* 11.5, *J*′ 10.0, Glc-C(6)*H*), 4.00 (1H, d, *J* 15.5, C(O)CH_2Cl), 3.88-3.75 (4H, m, Glc-C(4)*H*, Glc-C(5)*H*, Gal-C(5)*H*, SA-C(8)*H*), 3.83 (3H, s, C(O)OCH_3), 3.57 (1H, dd, *J* 9.0, *J* 3.0, SA-C(9)*H*), 3.46 (1H, dd, *J* 9.0, *J*″ 8.0, SA-C(3)*H*), 2.86 (1H, s, Gal-C(4)O*H*), 2.64 (1H, dd, *J* 13.0, *J*′ 5.0, SA-C(3)*H*), 2.28 (3H, s, NHC(O)CH_3), 2.16 (3H, s, C(O)CH_3), 2.13 (3H, s, C(O)CH_3), 2.09 (3H, s, C(O)CH_3), 2.06 (3H, s, C(O)CH_3), 2.04 (3H, s, C(O)CH_3), 2.03 (3H, s, C(O)CH_3), 2.02 (3H, s, C(O)CH_3), 1.98 (3H, s, C(O)CH_3), 1.90 (3H, s, C(O)CH_3), 1.90–1.88 (1H, m, SA-C(3)*H*); δ_C (100 MHz, $CDCl_3$) 171.7 (*C*(O)CH_3), 170.9 (*C*(O)CH_3), 170.8 (*C*(O)CH_3), 170.7 (*C*(O)CH_3), 170.6 (*C*(O)CH_3), 170.4 (*C*(O)CH_3), 170.2 (*C*(O)CH_3), 169.8 (*C*(O)CH_3), 167.1 (*C*(O)), 166.7 (*C*(O)), 101.0 (Gal-*C*(O)H), 98.7 (SA-*C*(2)), 78.1 (Glc-*C*(1)H), 75.8, 74.6, 72.3 (Glc-*C*(4)H, Glc-*C*(5)H), Gal-*C*(5)H), 72.5 (2C, Gal-*C*(3)H, SA-*C*(7)H), 71.7, 71.0, 68.5 (SA-*C*(4)H, SA-*C*(6)H, SA-*C*(8)H), 70.6 (Glc-*C*(2)H), 69.6 (Gal-*C*(2)H), 68.6 (Glc-*C*(3)H), 67.4 (Gal-*C*(4)H), 62.6 (Gal-*C*(6)H), 62.1 (Glc-*C*(6)H), 60.6 (SA-*C*(9)H), 53.0 (C(O)O*C*H_3), 48.6 (SA-*C*(5)H), 42.3 (C(O)*C*H_2Cl), 36.7 (SA-*C*(3)H), 23.2 (NHC(O)*C*H_3), 21.1 (C(O)*C*H_3), 21.0 (C(O)*C*H_3), 20.9 (C(O)*C*H_3), 20.8 (C(O)*C*H_3), 20.7 (2C, C(O)*C*H_3), 20.6 (2C, C(O)*C*H_3), 20.5 (C(O)*C*H_3); *m*/*z* (CI) 1123 (MNa^+, 100%), 1121 (43) 1118 (35), 1101 (41).

4.1.7. *N*-Acetyl-α-D-neuraminic acid-(2,3)-β-D-galactopyranosyl-(1,4)-β-D-*N*-glycyl-glucopyranosyl amine (1). Trisaccharide (10α) (87 mg, 0.08 mmol) was treated with 10% ammonia (3.5 mL) at room temperature for 16 h. The reaction was quenched by the addition of acetic acid (0.87 mL) and the solvent was removed in vacuo. The resulting colourless residue was dissolved in methanol (4 mL) under an inert atmosphere and treated with sodium methoxide at pH 10. After 18 h, NaOH (1 mL, 1 M) was added and the reaction mixture was stirred at room temperature for a further 18 h. The reaction mixture was neutralized to pH 7 with amberlyst® IR-120 and the amberlyst was removed by filtration. The solvent was removed in vacuo to give the desired product (**1**) (40 mg, 80%) as a colourless solid; mp 261–264 °C; $[\alpha]_D^{20}$ 4.5 (*c* 0.995, MeOH); υ_{max} (MeOH, thin film) 3444, 1750, 1371 and 1230 cm^{-1}; δ_H (400 MHz, MeOH) 5.18 (1H, app.t, J 9.5, Glc-C(1)H), 4.54 (1H, d, J 8.0, Gal-C(1)H), 4.29-3.53 (21H, m, Glc-C(2)H, Glc-C(3)H, Glc-C(4)H, Glc-C(5)H, Glc-C(6)H_2, Gal-C(2)H, Gal-C(3)H, Gal-C(4)H, Gal-C(5)H, Gal-C(6)H_2, SA-C(4)H, SA-C(5)H, SA-C(6)H, SA-C(7)H, SA-C(8)H, SA-C(9)H_2), 2.63 (1H, dd, J 13.5, J′ 4.0, SA-C(3)H), 2.01 (3H, s, NHC(O)CH_3), 1.98 (1H, dd, J 13.5, J′ 10.0, SA-C(3)H); δ_C (100 MHz, MeOH) 174.8 (C(O)), 170.8 (C(O)), 170.5 (C(O)), 105.2 (Gal-C(1)H), 104.1 (CC(O)OH), 82.1 (Glc-C(1)H), 82.0 (CH), 80.6 (CH), 70.3 (CH), 78.2 (CH), 76.6 (CH), 76.4 (CH), 76.3 (CH), 73.1 (CH), 72.8 (CH), 71.6 (CH), 71.5 (CH), 71.2 (CH), 64.3 (CH_2OH), 64.0 (CH_2OH), 54.0 (SA-C(5)H), 44.8 (CH_2NH_2), 41.8 (SA-C(3)H), 23.6 (C(O)CH_3); *m*/*z* (CI) 689 (M^+, 23%), 616 (45), 454 (63), 219 (74), 146 (100), 73 (56); Found 689.2494. $C_{25}H_{43}O_{19}N_3$ requires 689.2491.

4.1.8. Synthesis of poly(acrylic acid) conjugate of *N*-acetyl-α-D-neuraminic acid-(2,3)-β-D-galactopyranosyl-(1,4)-β-D-*N*-glycyl- glucopyranosyl amine (14). To a solution of poly (*p*-nitrophenyl acrylate) (**12**) (9.66 mg, 50 μmol) in DMF (500 μL, 6.45 mmol) was added, a solution of trisaccharide (**1**) (10 mg, 10 μmol) and biotin NH_2 (**13**) (1.4 mg, 3 μmol) in DMSO (100 μL, 1.41 mmol). The reaction mixture was treated with triethylamine (20 μL, 0.143 mmol) and stirred at 40 °C for 24 h. After this time the heat was removed and the reaction mixture was treated with NaOH (2 mL, 0.2 mmol) for 24 h. The solution was neutralized with 1 M HCl and purified by gel filtration on Sephadex® LH-20 eluting with 1:1 acetonitrile–water to give the desired product (**14**) (9 mg, 93%) as a pale brown solid; GPC (RI, H_2O) M_w 1403, M_n 1230, PD 1.1407.

4.1.9. Enzymatic synthesis of poly(acrylic acid) conjugate of *N*-acetyl-α-D-neuraminic acid α-(2,6)-lactose-*N*-acetate (16).[2b] The biotinylated poly(acrylic acid) conjugate of galactose-β-D-glucose-*N*-acetate (Glycotech, Gaithersburg MD, USA) (0.8 mg) was dissolved in sodium cacodylate (Sigma, UK) (500 μL, 50 mM, pH 6.5). This was added to a solution of Brij 97® (Sigma, UK) (6.25 μL, 10%), CMP-NeuNAc (Sigma, UK) (46.63 μL), sodium azide (Sigma, UK) (2.5 μL, 1%), sodium cacodylate (12.5 μL, 50 mM, pH 6.5) and recombinant α-2,6-sialyl transferase (Calbiochem) (59.63 μL, 50 mU). The mixture was incubated at 37 °C for 24 h. The product was isolated using a PD-10 Sephadex G-25M column (Amersham Biosciences) eluting with 0.3 M sodium phosphate buffer.

4.2. Recombinant baculovirus

Spodoptera frugiperda cells (Sf9) were grown in suspension in BioWhittaker Insect-Xpress serum-free media (Cambrex) supplemented with 2% foetal calf serum. They were infected with recombinant baculovirus expressing H5Fc or H5TAP and incubated at 28 °C. After 72 h the supernatant was harvested by centrifugation at 4000 rpm for 10 min to remove the infected cells. The soluble protein was concentrated by centrifugation at 4500 rpm for 4 h using Vivaspin columns (Viva Science, Sartorius Group). The yield of concentrated protein ranged from 500–700 μg/mL. Protein was aliquoted and stored at −80 °C.

4.3. Binding assay

The binding assay was performed in 96-well polystyrene plates. Wells were coated with 10 μg/mL of human IgG (for H5 TAP tag protein) or anti-human IgG (for H5 Fc tag protein) and incubated for 1 h at 37 °C or with purified virus corresponding to 64 HA units. Viruses were originally obtained from HPA Colindale, London, and were propagated in MDCK cells of embryonated chicken eggs. Assays involving virus were performed in the presence of zanamavir neuraminidase inhibitor to prevent viral NA digestion of the glycoconjugates. Plates were washed four times with PBS/0.5% Tween 20 and incubated overnight at 4 °C with 15 μg/mL H5 HA. Proteins were removed by aspiration and the plate washed four times. Polymers were added to the plate in duplicate wells at an initial concentration of 500 μM/mL and serially diluted in washing buffer. After incubation for 2 h at 4 °C plates were washed four times and incubated for 1 h at 4 °C with streptavidin/HRP (Oxford Biotechnology, UK) at 1:500 dilution. Peroxidase activity was detected using TMB (Europa Bioproducts Ltd., UK) and the reaction was stopped by the addition of 0.5 M of HCl. Absorbances were measured at 420 nm and the data were converted to Scatchard plots A_{420}/C versus A_{420}, where C is concentration of Neu5Ac, A_{420} is absorbency in the corresponding well. The association constants (K_{Ass} and K_d) of H5 HA proteins and virus complexes with sialylglycopolymers were inferred from fitting binding curves using GraphPad Prism Software version 4 and expressed in millimolar Neu5Ac.

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