Supporting Information

CONTENTS

Materials and Methods

1. Protein expression and purification:

1.1. GII.10 Vietnam026 P dimers

GII.10 Vietnam026 P domain (GenBank accession no. AF04671)[1] was expressed and purified following a slight modification[2] of a previously published protocol.[3] Briefly, the pMalc2X modified expression vector containing the codon-optimized gene encoding for GII.10 Vietnam026 P domain was transformed into *E.coli* BL21(DE3) cells (Novagen, Darmstadt, Germany) for protein expression. Transformed cells were grown for 3 h at 37 °C in modified Terrific Broth medium (12 g tryptone, 24 g yeast extract and 40 ml glycerol per liter culture) supplemented with M9 minimal medium components (0.5 g of NaCl, 3.3 g of KH_2PO_4, 16.6 g of $Na_2HPO_4x12H_2O$, 1 g of NH_4Cl, 1 ml of 1 M $MgSO_4$, 1 ml of 0.1 M $CaCl_2$ and 0.2 % glucose per liter culture), 0.4% casamino acids and 100 µg/ml ampicillin. Overexpression was induced with 1 mM isopropylthiogalactoside at OD_{600} between 1.2 - 1.5, and the incubation was continued at 17 °C for 44 to 48 h. To maintain the antibiotic pressure constant, 100 µg/ml ampicillin were added after the first 24 h. Cells were harvested by centrifugation at 9000 g for 15 min and the bacterial pellet was resuspended in 25 mM PBS buffer with 0.15 mM NaCl (pH 7.4). To the suspension 4 µl of 1 mg/ml Aprotinin and Leupeptin solutions (Roth), 25 µl of a 10 mg/ml chicken egg white lysozyme solution (Novagen) and 0.1 µl of a 25 U/µl Benzonase solution (Novagen) per gram of wet pellet were added, the suspension was incubated for 30 min at 4 °C and passed twice through a French Pressure cell at 14,000 psi followed by an ultracentrifugation at 125,000 g for 1 h. The protein was then purified as previously reported [3]. P domains were concentrated to 1.5 mg/ml and stored in 25 mM Tris-HCl, pH 7.4 and 0.3 M NaCl buffer at 4 °C. Average yield: 70 mg GII.10 Vietnam026 P dimers per liter culture.

1.2. Human norovirus VLPs

Samples of GII.10 Vietnam026[1] and GII.4 Ast6139[5-6] human norovirus VLPs used in this study were previously prepared. The GII.4 sample had already been subjected to binding studies using α-L-methyl fucopyranoside as ligand[4]. Both samples had been stored at 4 °C. The integrity of the preparations was controlled by electron microscopy (see below). The GII.10 sample had been stored for about two years ago and the GII.4 for about 4 years. The GII.10 sample had been kept in the absence of any HBGA ligands whereas the GII.4 sample had been stored in the presence of 1 mM methyl α-L-fucopyranoside.

1.3. Electron microscopy

VLP integrity was checked by electron microscopy (EM) as described in the following (Fig. S1). The VLP sample concentration was adjusted to 0.33 mg/ml in PBS buffer (pH 7.4). Formvar-carbon coated copper grids (Plano GmbH) were treated by glow discharge (Edwards Auto 306, Edwards) before sample addition. 5µl of each sample were applied to grids, incubated for 30s and aspirated afterwards. The grids were washed 3x with H_2O and then stained with 2% (m/v) uranylacetate in water (Merck) for 30s. After staining the grids were washed 3x with H_2O to remove excess uranylacetate. Samples were analyzed using a Jeol 1011 (Jeol) with iTEM 5.0 software (Olympus). The negative stains show homogeneously assembled particles in both cases. GII.4 Ast6139 particles appeared as small-size (T=1) particles, while the GII.10 were native-size (T=3) particles. We ascribe the differences in the appearance of the negative stains to the presence (GII.4) vs. the absence (GII.10) of methyl α-L-fucopyranoside under storage conditions.

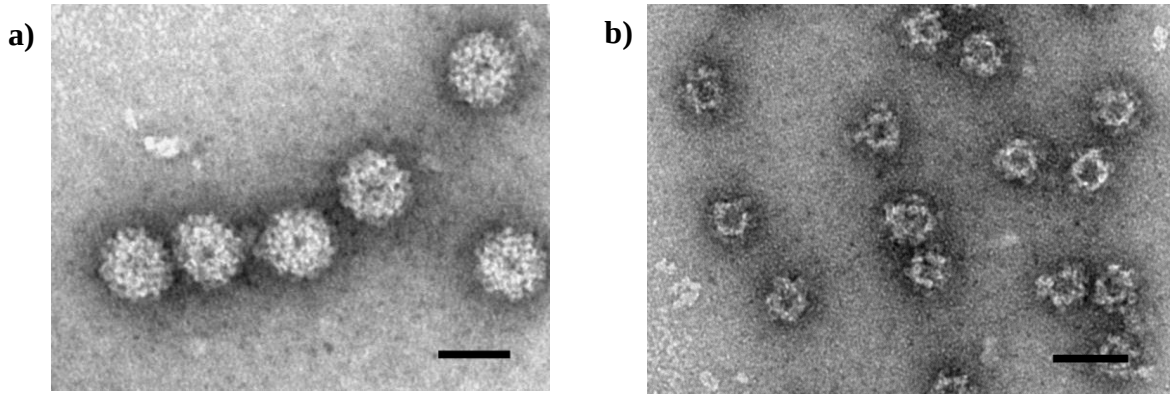


Fig. S1: Electron micrographs showing a) GII.10 Vietnam026 VLPs and b) GII.4 Ast6139 VLPs. The scale bar corresponds to 40 nm. The GII.4 VLPs had been stored in the presence of 1 mM methyl α-L-fucopyranoside.

2. NMR sample preparation

A. GII.10 Vietnam026 P dimers:

Samples were prepared in 25 mM Tris-d_{11}-HCl pH 7.4 containing 0.3 M NaCl and 100 µM TSP-*d4* as internal reference in D_2O. Methyl β-L-fucopyranoside and citrate were titrated to a sample containing 15 µM Vietnam026 P dimers, keeping the protein concentration and the pH constant during the whole titration. For methyl β-L-fucopyranoside, direct saturation of the ligand was observed over 20 mM ligand concentrations. For sodium citrate no direct irradiation was observed. A total of 10 and 13 data points were acquired for methyl β-L-fucopyranoside and sodium citrate, respectively.

B. VLPs:

Samples containing 35 nM (Vietnam026) and 61.3 nM (Ast6139) VLPs (3.12 µM and 5.5 µM binding sites respectively, considering a single binding site per VP1 dimer accessible at the starting of the titration) were prepared in PBS pH 7.4, 10% D_2O. DSS-*d6* 100 µM was used as internal reference. For Vietnam026 VLPs, methyl α-L-fucopyranoside was titrated keeping the VLPs concentration constant. For Ast6139 VLPs, the sample was diluted by 9.1 % at the end of the fourth binding event. A total of 30 and 23 data points were acquired for Vietnam026 and Ast6139 VLPs, respectively.

3. NMR experiments

NMR spectra were recorded on a Bruker AV III 500 MHz NMR spectrometer equipped with a TCI cryogenic probe. For STD NMR experiments, a train of 50 ms Gaussian-shaped radio frequency pulses with a field strength of 105 Hz separated by 1 ms for a total duration of 2 s was used during protein irradiation. The water signal was suppressed using an excitation sculpting sequence with gradients. The acquisition time was set at 2.34 s. Spectra were recorded at 282 K and analyzed using Topspin 3.1.

A. STD NMR experimental setting for P dimers:

An additional relaxation delay of 6 s was introduced at the beginning of the pulse sequence, resulting in a total delay between two on-resonance scans of 14.68 s. Unwanted protein signals were suppressed via a 30 ms spinlock filter. On- and off-resonances were placed at -4

ppm and 200 ppm, respectively. The number of scans was ranging from 1028 for the first titration step (300 µM ligand concentration) to 80 for the last step.

B. STD NMR experimental setting for VLPs:

Due to the low transverse relaxation times exhibited by VLPs no protein signal was observed, making spinlock unnecessary. To ensure > 92% relaxation of the protons in the binding site[7] and thus reduce errors during epitope mapping and K_D calculations, a 25 s delay was introduced, resulting in a total delay between two on-resonance scans of 33.68 s. On- and off-resonances were placed at -4 ppm and 300 ppm, respectively. The number of scans was ranging from 2304 for the first titration step (19 - 50 µM ligand concentration) to 16 for the last steps.

4. Binding models

For the analysis of NMR titration data we have used a simple model that reflect successive cooperative binding events (equation S1, identical with equation 1 of the main text):[2]

$$STD\,AF = \sum_{i=1}^{n} \frac{[L]^{h_i}}{K_{Di}^{h_i} + [L]^{h_i}} \times STD\,AF_{\max,i} \qquad \text{(S1)}$$

with K_{Di} being the microscopic dissociation constants for each binding event, $STD\ AF_{max}$ being the maximum change of the STD amplification factor, n being the number of distinct binding steps, and h_i being the Hill coefficient. Low R^2 were obtained when no h term was included for the fitting of the first binding event. The number of binding steps n is readily identified from the STD titration curves as explained below. The values used for global data analysis of STD NMR titration data and the ligand concentration *[L]* at which successive binding events are observed are summarized in Table S1 and corresponding binding curves are shown in Figs. S3-S6.

Ligand	**Protein**	***n***	**New binding event at *[L]* (mM)**
Methyl β-L-fucopyranoside	GII.10 Vietnam026 P dimers	2	4
Citrate	GII.10 Vietnam026 P dimers	3	2.7; 7
Methyl α-L-fucopyranoside	GII.10 Vietnam026 VLPs	6	0.42; 0.95; 3; 5; 15
Methyl α-L-fucopyranoside	GII.4 Ast6139 VLPs	6	0.42; 1.2; 2.43; 4.87; 15*

*Corresponding to 80.8; 233.2; 478.8; 970.2 and 2727 ligand excess.

Table S1: Number of binding steps observed by STD NMR titration experiments and ligand concentrations at which were observed. See also Fig. S3-S6.

The number of binding events was determined from the titration curves (Figs. S3-S6 and Fig. 1 of the main text) by visual inspection. In the case of the VLPs due to small STD AF differences for the three first binding steps, 95% prediction bands were calculated. They clearly unravel the presence of three steps in the binding curves which cannot be explained by uncertainty in the measurements (fig S2).

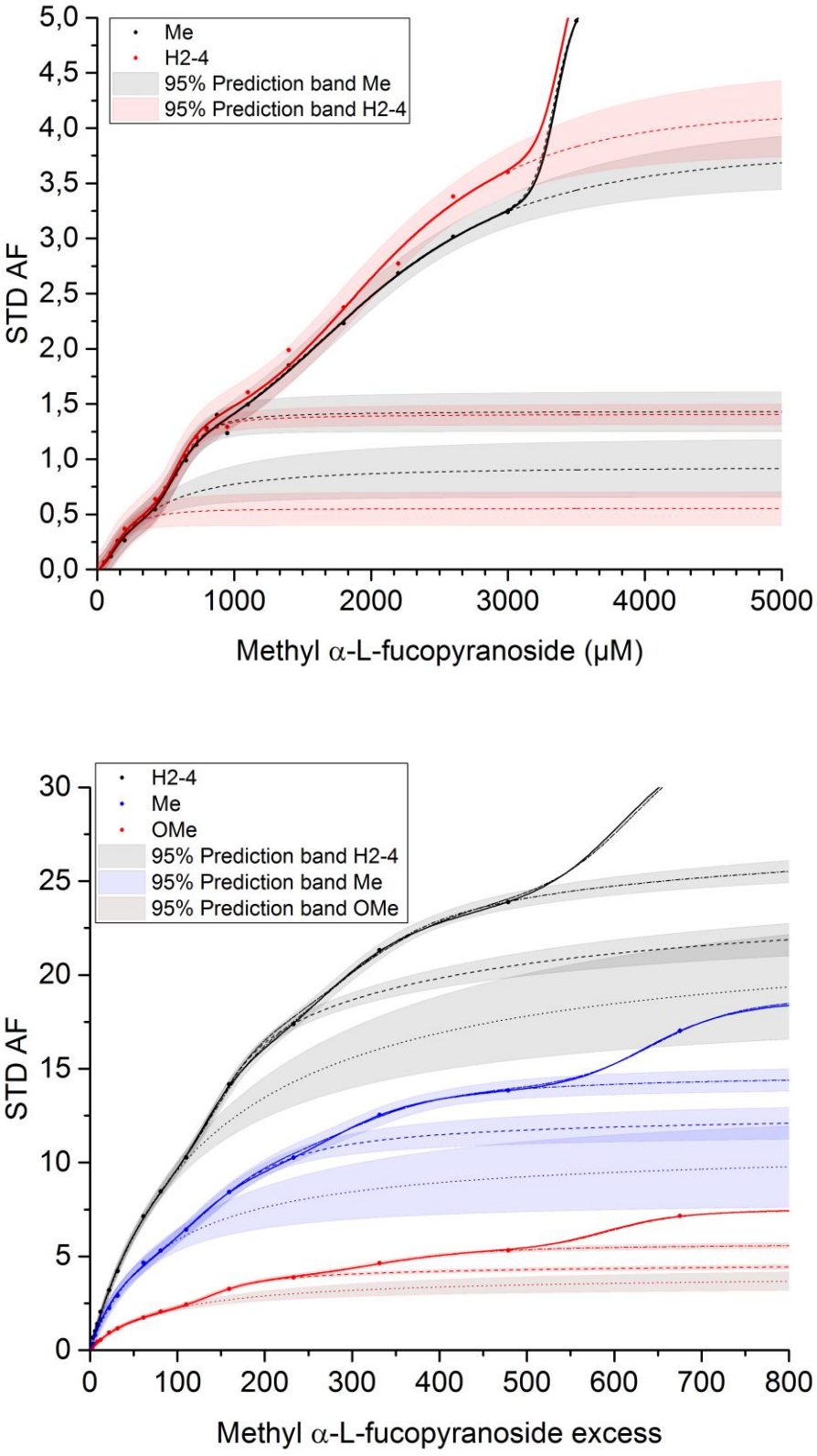


Fig S2: Plots showing 95% prediction bands for the first three binding steps for GII.10 Vietnam026 VLPs (top) and GII.4 Ast6139 VLPs (bottom). From prediction bands it becomes clear that successive steps cannot be included in the previous prediction bands with a 95% confidence.

5. Correction of free ligand concentrations

For STD NMR titrations of Vietnam026 P dimers with methyl β-L-fucopyranoside we used the assumption that the actual ligand concentration [L] is approximated by the total ligand concentration $[L]_{tot}$. This is justified by the conditions of STD NMR experiments where the ligand is always present at large excess over the receptor protein, which always leads to a small fraction of bound ligand molecules. The approximation $[L] = [L]_{tot}$ becomes invalid when low ligand to protein ratios are studied. This was the case for the titration of Vietnam026 P dimers with citrate (K_{D1}) and for the VLPs titrations (K_{D1}). Because of the much lower K_D value a correction of [L] was required for the first part of the titration curves. To correct for this we have employed the law of mass action to calculate the actual values of [L]. In these simple cases, the correction is achieved by substituting the free ligand concentration [L] as well as the free protein concentration [P] by the corresponding differences between the total ligand or protein concentration $[L]_{tot}$ and $[P]_{tot}$ and the concentration of the protein-ligand complex [PL]. This yields a quadratic equation that can easily be solved for [L].

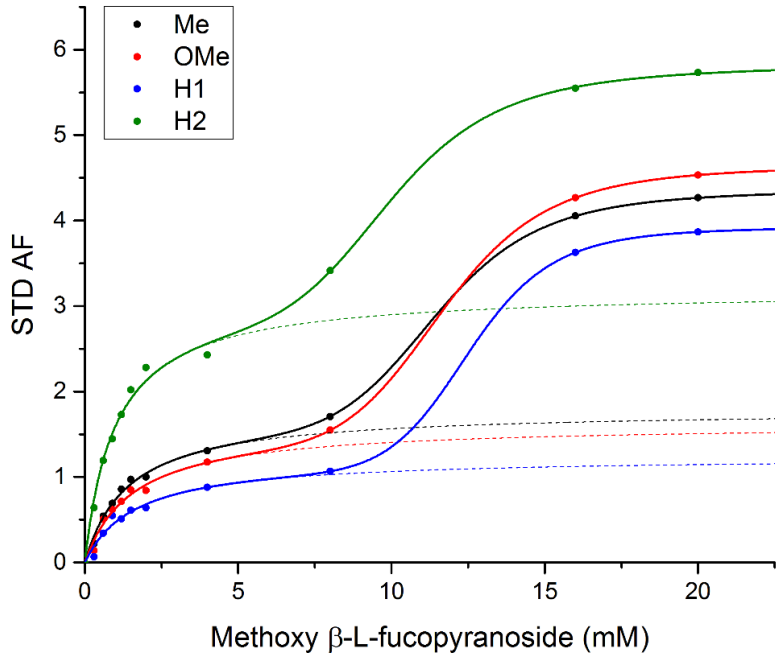
6. Non-linear squares fitting

The STD enhancements were expressed as the STD amplification factor (AF), described by equation (S2), which is identical with equation (2) in the main text:

$$STD\,AF = \frac{I_0 - I_{sat}}{I_0} \cdot ligand\,excess \qquad \text{(S2)}$$

with I_0 and I_{sat} being the signal intensities in the off- and on-resonance spectra, respectively. STD AF were plotted against ligand concentration or ligand excess and fitted to the corresponding binding model using Origin 2015 software package. K_D values obtained for each protein and ligand are listed below. In all the cases solid lines result from fitting the corresponding equation to the experimental data. Dashed lines indicate theoretical curves that would be observed if there were not any successive binding events.

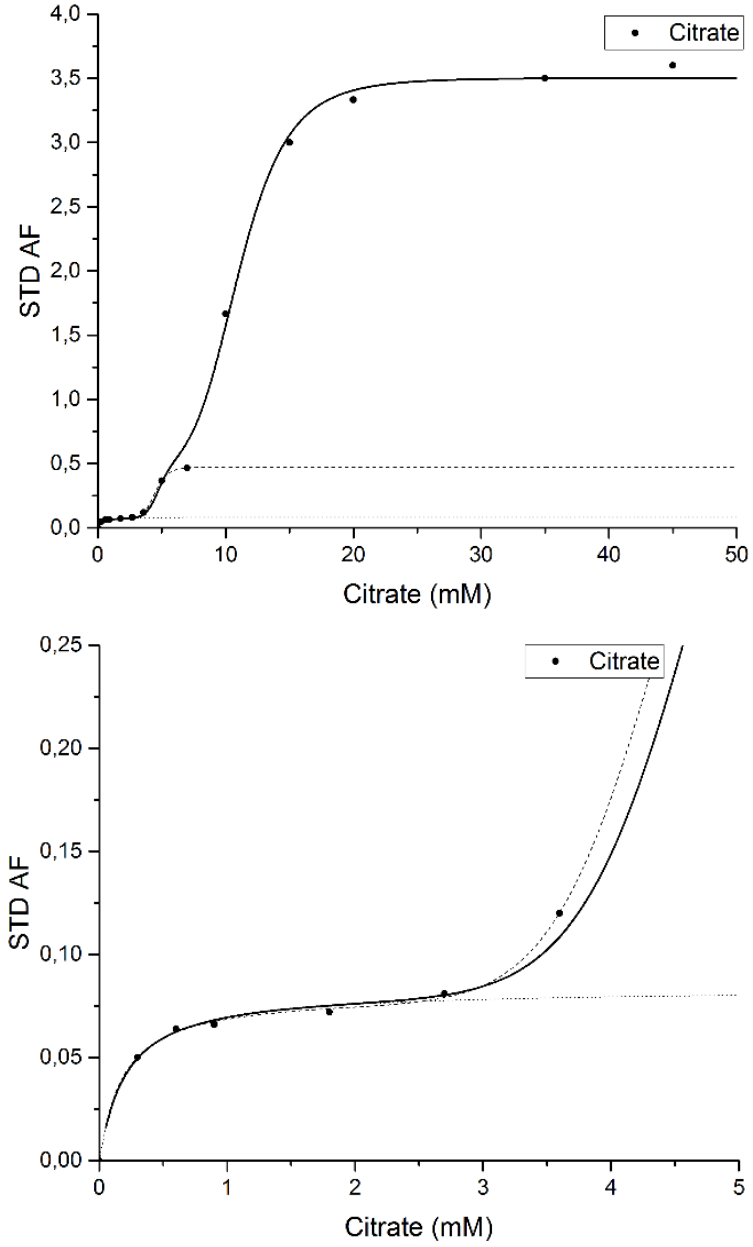
A. GII.10 Vietnam026 P dimers titrated with methoxy β-L-fucopyranoside:



Signal	K_{D1} (mM)	K_{D2} (mM)	*h*	R^2/χ^2
Me	1.43 ± 0.22	11.51 ± 0.52	7.00 ± 0.94	0.9988/0.0025
OMe	1.56 ± 0.36	11.73 ± 0.61	7.11 ± 1.18	0.9982/0.0044
H1	1.63 ± 0.53	12.55 ± 1.48	9.46 ± 5.19	0.9973/0.0049
H2	0.99 ± 0.21	10.04 ± 0.96	5.83 ± 2.1	0.9967/0.0113

Fig. S3 and Table S2: STD NMR titration curves for GII.10 Vietnam026 P dimers 15 μM titrated with methyl β-L-fucopyranoside. Shown is the STD amplification factor as a function of ligand concentration. The table contains corresponding K_Ds and *h* values. Equation S1 without correction for free ligand concentration was used.

B. GII.10 Vietnam026 P dimers titrated with sodium citrate:



Signal	K_{D1} (mM)	K_{D2} (mM)	K_{D3} (mM)	h_1	h_2	R^2/χ^2
Citrate	0.20 ± 0.03	4.8 ± 0.39	11.04 ± 0.18	9.44 ± 0.20	5.73 ± 0.60	0.9974/0.0058

Fig. S4 and Table S3: Complete STD NMR titration curve for GII.10 Vietnam026 P dimers (15 µM) titrated with citrate (top). Zoom of the first binding event (bottom). The table shows corresponding K_Ds and h values. Shown is the STD amplification factor as a function of ligand concentration. Equation S1 with correction for free ligand concentration (see text above) was used for the calculation of $_{KD1}$.

C. GII.10 Vietnam026 VLPs titrated with methyl α-L-fucopyranoside:

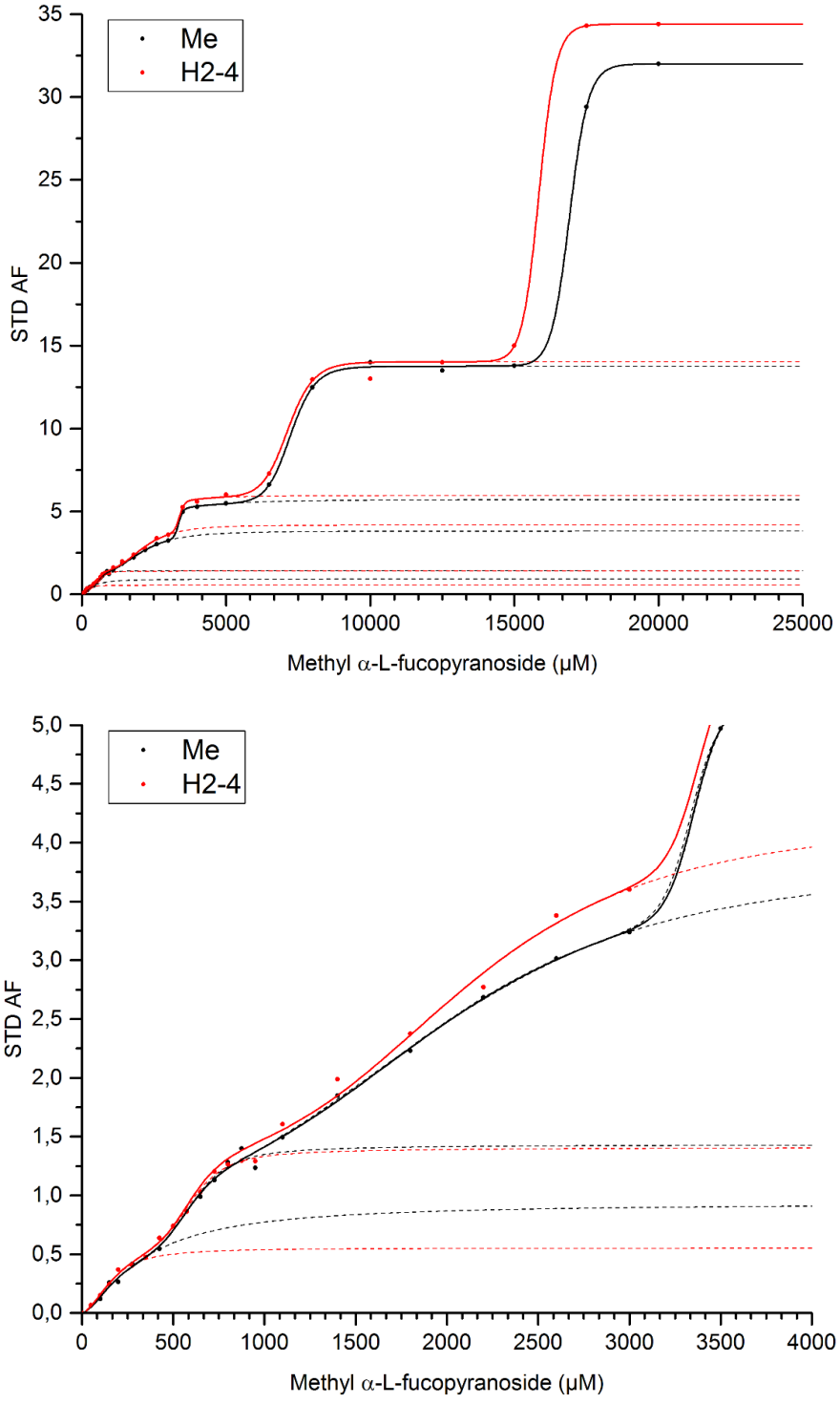


Fig. S5: Complete STD NMR titration curve for GII.10 Vietnam026 VLPs (3.12 µM binding sites) titrated with methyl α-L-fucopyranoside (top), and zoom of the first two binding events (bottom). Shown is the STD amplification factor as a function of ligand concentration.

	Fitting for K_{D1} to K_{D6}		Fitting for K_{D1} and K_{D2}	
Parameter	**Me**	**H2-4**	**Me**	**H2-4**
K_{D1} **(mM)**	0.22 ± 0.56	0.22 ± 0.14	0.23 ± 0.09	0.22 ± 0.04
K_{D2} **(mM)**	0.58 ± 0.1	0.60 ± 0.02	0.61 ± 0.03	0.56 ± 0.02
K_{D3} **(mM)**	2.05 ± 0.28	2.10 ± 0.13	-	-
K_{D4} **(mM)**	3.35 ± 0.06	3.37 ± 0.10	-	-
K_{D5} **(mM)**	7.24 ± 0.05	7.15 ± 0.09	-	-
K_{D6} **(mM)**	16.91 ± 0.31	15.85 ± 0.32	-	-
h_1	1.67 ± 2.49	1.61 ± 0.58	1.67 ± 0.49	1.61 ± 0.58
h_2	6.70 ± 9.42	6.77 ± 1.61	6.45 ± 2.65	6.80 ± 1.51
h_3	3.17 ± 1.35	3.82 ± 0.40	-	-
h_4	45.03 ± 16.02	40.53 ± 37.67	-	-
h_5	17.30 ± 1.22	16.26 ± 1.92	-	-
h_6	52.69 ± 30.62	53.92 ± 17.08	-	-
R^2/χ^2	0.9998/0.0134	0.9995/0.0398	0.9869/0.0029	0.9961/0.0009

Table S4: K_D values and h coefficients obtained from global fitting of K_{D1} to K_{D6}. K_{D1} and K_{D2} were also calculated fitting only locally to the corresponding ligand concentrations. This was done because of the large errors observed in the global fitting. These large errors must be attributed are due to the low weight of the first two binding events in the global fitting process. For the calculation of K_{D1} equation S1 with correction for free ligand concentration was used.

D. GII.4 Ast6139 VLPs titrated with methyl α-L-fucopyranoside:

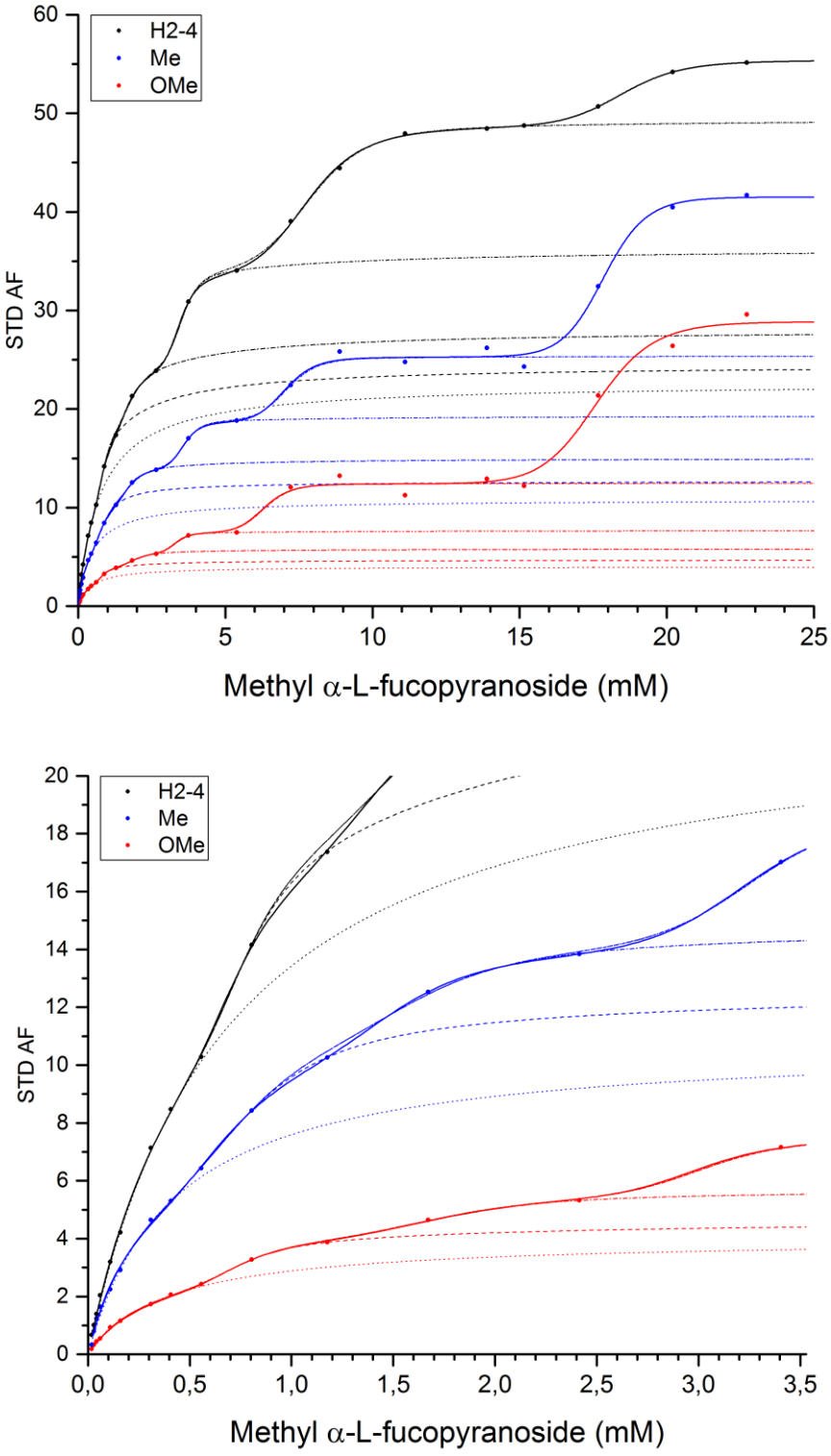
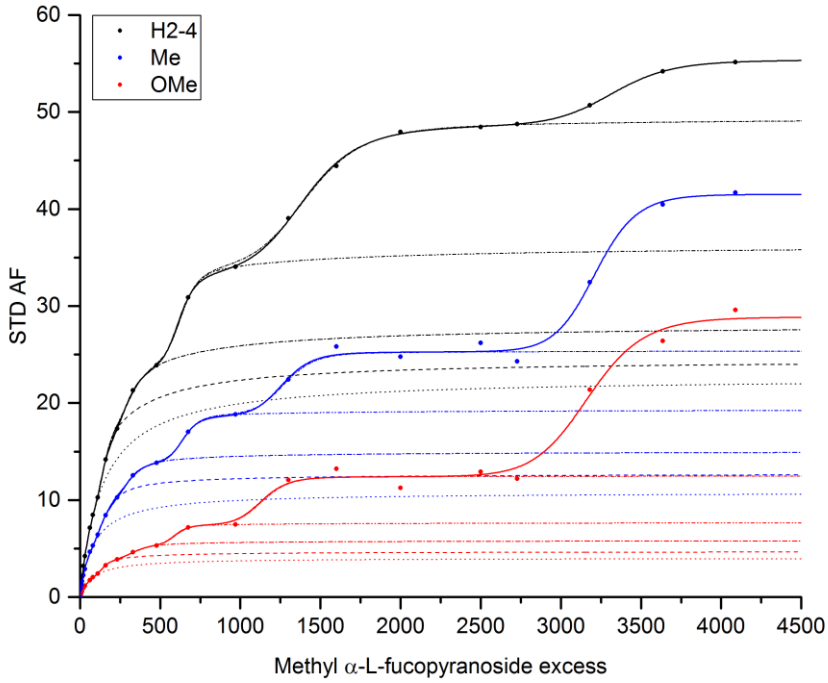


Fig. S6: Complete STD NMR titration curve for GII.4 Ast6139 VLPs (5.5 µM binding sites) titrated with methyl α-L-fucopyranoside (top) and detail of the two first binding events (bottom).



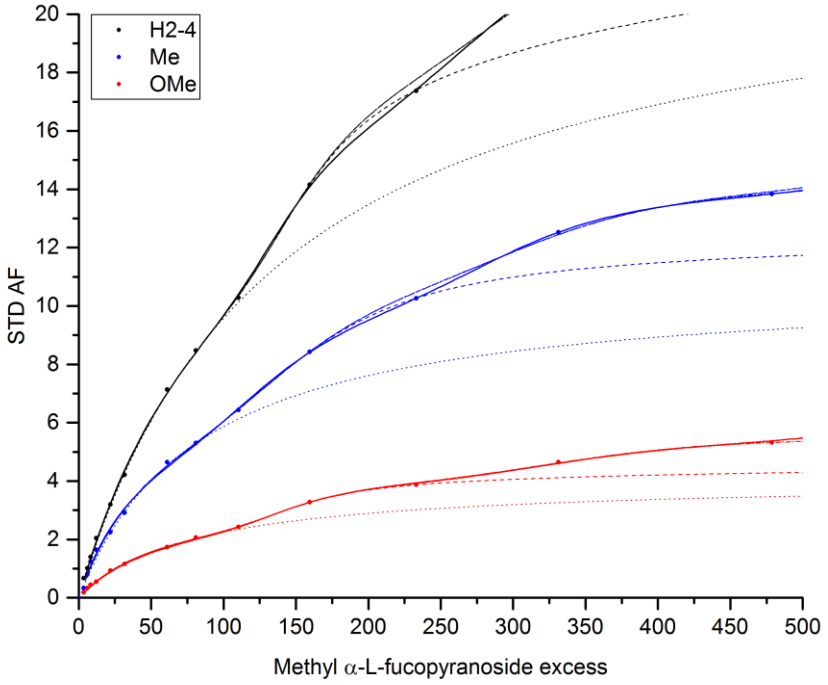


Fig. S6 (continued): Alternative representation of complete STD NMR titration curves for GII.4 Ast6139 VLPs (5.5 µM binding sites) titrated with methyl α-L-fucopyranoside (top) and detail of the two first binding events (bottom). Since VLPs were diluted during this titration, it is instructive to plot STD amplification factors as a function of ligand excess.

Since VLPs were diluted by 9.1 % at the end of the titration, equation S1 was modified in such a way that [L] is the ligand excess instead of the ligand concentration and K_{Di} is the ligand excess 'K_i' at which the ligand concentration corresponds to the true K_D value. The K_D values were calculated from the ratio of ligand excess to ligand concentration that was known for each titration step according to equation S3.

$$K_D(mM) = \frac{K_i}{ligand\,excess / [L]_{tot}(mM)} \quad \text{(S3)}$$

Were *ligand excess* and $[L]_{tot}$ correspond to the ones observed at the end of each binding step.

Parameter		**H2-4**	**Me**	**OMe**
K_1		111.16 ± 13.15	50.62 ± 32.45	66.27 ± 24.20
K_2		139.98 ± 8.58	137.92 ± 48.60	142.50 ± 60.52
K_3		288.67 ± 14.80	296.20 ± 64.12	340.19 ± 109.48
K_4		616.64 ± 7.73	640.83 ± 35.31	592.57 ± 117.57
K_5		1401.18 ± 10.15	1263.57 ± 38.42	1131.93 ± 75.11
K_6		3323.58 ± 28.36	3214.26 ± 20.56	3171.46 ± 31.14
Parameter	**(L:b.s.) / L [mM]**	**H2-4**	**Me**	**OMe**
K_{D1} **(mM)**	192.48	0.58 ± 0.07	0.26 ± 0.17	0.34 ± 0.12
K_{D2} **(mM)**	194.30	0.72 ± 0.04	0.71 ± 0.25	0.73 ± 0.31
K_{D3} **(mM)**	197.05	1.46 ± 0.07	1.50 ± 0.32	1.73 ± 0.55
K_{D4} **(mM)**	199.22	3.09 ± 0.04	3.22 ± 0.18	2.97 ± 0.59
K_{D5} **(mM)**	181.8	7.71 ± 0.05	6.95 ± 0.21	6.23 ± 0.41
K_{D6} **(mM)**	181.8	18.28 ± 0.15	17.68 ± 0.11	17.45 ± 0.17
h_1		1.00 ± 0.08	1.01 ± 0.16	1.00 ± 0.10
h_2		6.80 ± 0.12	4.11 ± 2.42	6.17 ± 1.33
h_3		7.00 ± 1.54	7.32 ± 2.12	7.03 ± 1.39
h_4		12.65 ± 1.92	13.57 ± 5.44	15.00 ± 1.80
h_5		8.55 ± 0.45	14.00 ± 7.43	14.00 ± 4.15
h_6		18.55 ± 2.38	25.03 ± 4.78	18.46 ± 3.82
R^2/χ^2		0.99930/0.02717	0.99816/0.30489	0.99426/0.4074

Table S5: K_Ds and *h* coefficients calculated for the global fitting of K_{D1} to K_{D6}. Equation S1 with correction for free ligand concentration was used for the calculation of K_{D1}.

Parameter		H2-4	Me	OMe
K_1		117.99 ± 28.93	59.82 ± 33.12	63.58 ± 12.97
K_2		150.46 ± 3.47	156.33 ± 14.98	145.07 ± 3.39
Parameter	**(L:b.s.) / L [mM]**	**H2-4**	**Me**	**OMe**
K_{D1} **(mM)**	192.48	0.61 ± 0.15	0.31 ± 0.17	0.33 ± 0.07
K_{D2} **(mM)**	194.30	0.77 ± 0.02	0.80 ± 0.08	0.75 ± 0.02
h_1		1 ± 0.05	1 ± 0.18	1 ± 0.07
h_2		6.81 ± 0.21	4.1 ± 2.42	6.17 ± 1.33
R^2/χ^2		0.99968/0.01033	0.99816/0.02049	0.99957/0.00070

Table S6: To compensate for large errors observed in the fitting, K_{D1} and K_{D2} were recalculated independently. This procedure was necessary because of the low weight of these first two binding events compared to the larger successive steps in the global fitting. Equation S1 with correction for free ligand concentration was used for the calculation of K_{D1} and K_{D2}.

		Saturation of binding sites [%]				
Protein	Ligand	Site 1 (Step 2)	Site 2 (Step 3)	Site 3 (Step 4)	Site 4 (Step 5)	Site 5 (Step 6)
GII.10 VLPs	**3**	68	61	59	59	68
GII.4 VLPs	**3**	58	63	62	61	66

Table S7: Saturation of binding sites as a function of ligand concentration. For this calculation it has been assumed that each step (cf. Figs. S2, S5 and S6) corresponds to the "release" of one new class of binding sites at the concentrations of ligands given in Table S1. The concentration of binding sites follows from the concentration of VLPs and assuming that each class of binding sites corresponds to one site per P-dimer yielding concentrations of binding sites of 3.1 μM (GII.10 VLPs) or 5.5 μM (GII.4 VLPs) as this is described in the Materials and Methods section. For the calculation of saturation levels the law of mass action was applied employing these concentrations of binding sites and the dissociation constants K_{Di} given in Table I of the main text. Interestingly, new binding sites are released at ligand concentrations that saturate about two thirds of the binding sites corresponding to the preceding step in each case.

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