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Binding of Glycans to the SARS CoV-2 Spike Protein, an Open Question: NMR Data on Binding Site Localization, Affinity, and Selectivity

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Dedicated to Professor Hans Paulsen on his 100th birthday.

Abstract: We have used NMR experiments to explore binding of selected glycans and glycomimetics to the SARS CoV-2 spike glycoprotein (S-protein) and to its receptor binding domain (RBD). STD NMR experiments confirm binding of sialoglycans to the S-protein of the prototypic Wuhan strain virus and yield dissociation constants in the mM range. The absence of STD effects for sialoglycans in the presence of the Omicron/BA.1 S-protein reflects a loss of binding as a result of S-protein evolution. Likewise, no STD effects are observed for the deletion mutant Δ143-145 of the Wuhan S-protein, supporting localization of the binding site in the N-terminal domain (NTD). The glycomimetics Oseltamivir and Zanamivir bind weakly to the S-protein of both virus strains. Binding of blood group antigens to the Wuhan S-protein cannot be confirmed by STD NMR. Using \(^{1}H,\(^{15}N\) TROSY HSQC based chemical shift perturbation (CSP) experiments we exclude binding of any of the ligands studied to the RBD of the Wuhan S-protein. Our results put reported data on glycan binding into perspective and shed new light on the potential role of glycan-binding to the S-protein.

Introduction

The interaction of sialoglycans and ABO(H) blood group antigens with the spike glycoprotein (S-protein) of SARS CoV-2 has been studied by several groups\(^{[1-9]}\), leading to the hypothesis that specific glycans serve as attachment factors\(^{[4, 8]}\). In the case of MERS CoV it has been convincingly shown that sialic acid residues may serve as attachment factors\(^{[10]}\). However, the situation is less clear for SARS CoV-2\(^{[2, 9]}\), with one study even concluding that sialic acid removal by sialidases promotes infection\(^{[9]}\). This controversial situation is partially due to inconsistent results from glycan-binding experiments. Here, we have used NMR experiments to shed new light on the binding of sialoglycans, blood group oligosaccharides, and glycomimetics (Scheme 1) to the S-protein and to the receptor binding domain (RBD) of the S-protein.

Scheme 1: Structural formulas of glycans and glycomimetics probed for binding to the SARS CoV-2 S-protein by NMR.
Results and Discussion

In a previous study we used CSPs to identify the heparin binding site on the RBD and to obtain dissociation constants for heparin and fondaparinux[3]. These results motivated us to extend the testing of binding of other glycan ligands to the S-protein and to the RBD using NMR. To this end, we have probed binding to the S-proteins of the prototypic Wuhan strain, the Omicron/BA.1 (B1.1.529) variant and the deletion mutant Δ143-145 of the Wuhan strain using STD NMR[11] experiments. Binding to uniformly 2H,15N-labeled Wuhan RBD[2, 5, 12] has been explored employing chemical shift perturbation (CSP) experiments.

STD NMR experiments provide sialoglycan binding epitopes. Binding of sialoglycans to the S-protein has been reported by several laboratories[2, 4-6, 13-15]. Whereas two studies find that sialic acid binds to the RBD[4, 15] other studies could not confirm binding of sialoglycans to the RBD[2, 5-6]. One study[6] reports a cryo-EM structure of a 3'-sialyllactose derivative bound to the S-protein of SARS CoV-2. This sialic acid binding pocket is located on the N-terminal domain (NTD) of the S-protein, supporting those studies that could not identify binding of sialoglycans to the RBD. In comparison, the sialic acid binding pocket of the S-protein of MERS CoV is also positioned in the NTD but at a distinct location[16]. To better characterize binding of sialoglycans to the S-protein we performed STD NMR experiments employing 3'-sialyllactose (3'SL) and 6'-sialyllactose (6'SL) as ligands. Of note, the protein background including the glycan signals of the heavily glycosylated S-protein did not interfere with the sharp signals of excess ligand used to determine STD effects (Fig. S1). The resulting binding epitopes match well with published data obtained employing 13C-labeled sialoglycans as ligands[5] and underscore the importance of the sialic acid residue for binding (Fig. 1). More specifically, Unione et al.[2] synthesized 13C-labeled 3'-sialyl-N-acetyllactosamine (3'SLN) and 6'-sialyl-N-acetyllactosamine (6'SLN), corresponding to 3'SL and 6'SL used in our study. 13C-labeling of the Neu5Ac and the galactose pyranose rings allowed acquisition of STD 1H,13C-HSQC spectra[11, 17] with very high S/N. This elegant approach provided ligand binding epitopes including protons that in 1D spectra cannot be evaluated due to signal overlap. Nevertheless, the spectra presented here complement the published binding epitope in that the epitope is extended towards the reducing residue (Glc) and the N-acetyl group of the terminal sialic acid.

STD NMR titrations reveal low-affinity binding of sialoglycans. The determination of dissociation constants from STD NMR titration curves followed an established protocol that fits the law of mass action to STD NMR titration curves of individual ligand protons[18, 22]. Each ligand proton typically yields a slightly different dissociation constant due to differences in relaxation times and distances to protein protons in the binding pocket. The protocol assumes that the lowest KD value is closest to the "true" value, which might be slightly lower. The dissociation constants KD for binding of 3'SL and 6'SL to the S-protein are in the mM range (Fig. 2 and Table S1), consistent with binding affinities obtained for the binding of sialoglycans to other sialic acid binding proteins[23-35]. To further substantiate these results we performed an initial growth analysis[21] of STD NMR titration data for 6'SL (cf. Fig. S3-S6 and Table S2). Extrapolation to zero saturation time essentially removes spurious contributions from ligand rebinding or from varying T1 relaxation times of individual ligand protons[21]. It turns out that both approaches, the determination of KD values using STD NMR titrations at a single saturation time of 2s, and the initial growth analysis that allows back-extrapolation to zero saturation time provide the same results within the error margins. In fact, this is not unexpected since it has been noted before that the degree of discrepancy between KD values obtained via the two methods depends on the size of KD, with differences shrinking with increasing KD values, i.e., with decreasing affinity[21, 36].

![Figure 1](image-url)
A deletion mutant allows identification of the sialic acid binding site. We also tested sialoglycan binding to the S-protein of the Omicron/BA.1 variant of concern, which has several mutations in the NTD. No saturation transfer is observed for 3'SL in the presence of the BA.1 S-protein (Fig. 3; additional sections of the spectra are shown in Fig. S7), matching results of a previous NMR study\cite{15} and indicating that the capability of binding to sialoglycans has been lost in the course of the evolution of the virus. Based on the topology of the sialic acid binding site as identified by cryo-EM\cite{8} we prepared a deletion mutant Δ143-145 of the S-protein of the Wuhan strain. No STD effects are observed for 3'SL in the presence of this deletion mutant (Fig. 3), showing that the deletion alone eliminates sialic acid binding and directly confirming the localization of the site in the S-protein of the Wuhan strain. Interestingly, the sialic acid binding site overlaps with amino acids 140–158 of the so-called "NTD supersite", which is recognized by all known NTD-specific neutralizing mAbs\cite{40,41}, suggesting that immune escape led to a loss of sialic acid recognition.

Oseltamivir and Zanamivir bind to the S-proteins. We then probed binding of two glycomimetics, Oseltamivir and Zanamivir, which are known as sialidase inhibitors to treat influenza infections. STD NMR experiments show that both compounds bind to the Wuhan S-protein as well as to the S-proteins of the Omicron/BA.1 variant and of the deletion mutant (Figs. S7-S10), suggesting that both glycomimetics bind to a site that is different from the sialic acid binding site. Therefore, binding to Oseltamivir serves as a positive control in the sense that other binding sites are unaffected by the mutations that disrupt the sialic acid binding site. We determined a value of 4 mM for the dissociation constant $K_D$ for Oseltamivir binding to the Wuhan S-protein (Fig. S9). To validate the conclusion that Oseltamivir and Zanamivir bind to a different binding site we performed a competition STD NMR experiment with Oseltamivir and 6'SL in the presence of Wuhan S-protein. The size of the STD amplification factors $STD_{Af}$ of 6'SL (30 mM) were unaffected by addition of 8 mM Oseltamivir, which agrees with the presence of different binding sites (Fig. S11). Finally, no chemical shift perturbations (CSPs) were observed in $^{1}H,^{15}N$ (TROSY) HSQC experiments upon addition of Oseltamivir or Zanamivir to uniformly $^{2}H,^{15}N$-labeled or $^{15}N$-labeled RBD (Fig. S12), excluding binding of the two glycomimetics to the RBD.

Figure 2. STD NMR spectra and corresponding off-resonance spectra of sialoglycans reflecting binding to the S-protein of the prototypic SARS CoV-2 Wuhan strain (left), and corresponding binding isotherms with dissociation constants $K_D$ from STD NMR titrations (right). The rows show spectra and binding isotherms of (a) 6'SL (30 mM) and (b) 3'SL (30 mM). S-protein concentrations were 21 μM (a) and 19 μM (b). Negative controls at 30 mM ligand concentrations in the absence of S-protein are shown in Fig. S2. Binding isotherms, proton labels and $K_D$ are color coded, e.g., the H$_3$ Neu5Ac has a green color corresponding to the green binding isotherms. The binding isotherms were obtained by fitting Eq. 2, which results from the law of mass action to the experimental STD $w$s show spectra and binding isotherms of 6'SL (a) and 3'SL (b) at 30 mM ligand concentrations in the absence of S-protein. The size of the STD amplification $STD_{Af}$ of 6'SL (30 mM) were unaffected by addition of 8 mM Oseltamivir, which agrees with the presence of different binding sites (Fig. S11). Finally, no chemical shift perturbations (CSPs) were observed in $^{1}H,^{15}N$ (TROSY) HSQC experiments upon addition of Oseltamivir or Zanamivir to uniformly $^{2}H,^{15}N$-labeled or $^{15}N$-labeled RBD (Fig. S12), excluding binding of the two glycomimetics to the RBD.
ABO(H) blood group antigens do not bind to the Wuhan S-protein. Some studies have linked the risk of infection with SARS CoV-2 to the ABO(H) blood group type. Therefore, it has been hypothesized that binding to blood group antigens is causative for this effect, and experimental data from native mass spectrometry seem to verify this hypothesis[4]. However, other studies did not confirm binding of ABO(H) blood group antigens to the S-protein[7, 42], suggesting that the correlation between ABO(H) type and risk is linked to neutralization by anti-ABO(H) antibodies[7, 42]. To clarify this inconsistency, we probed binding of type I blood group A and B tetrasaccharides, and of the H disaccharide to the RBD and/or to the S-protein of the Wuhan strain using NMR experiments. In 1H,15N-TROSY-HSQC experiments employing uniformly 15N-labeled RBD no CSPs were observed upon addition of ABO(H) oligosaccharides, even at higher mM ligand concentrations. This unambiguously shows that none of the blood group oligosaccharides binds to the RBD (Fig. 4). Importantly, CSP NMR experiments are essentially free of false negatives[43]. Therefore, complete lack of CSPs is a very strong indicator of "no binding". Binding to the S-protein was tested using STD NMR experiments. We could not observe any STD effects above a level of signal intensities that would allow quantification (see Figs. S13 and S14), ruling out specific binding of ABO(H) type oligosaccharides to the Wuhan S-protein. The discrepancy between our NMR-based results and the MS-based data[4] may be explained by glycan clustering during electrospray ionization that strongly affects the results of native MS studies, depending on the reference protein used[44-47]. Taken together, our data show that the effects of blood group type on infection and transmission are not due to direct binding of blood group antigens to the S-protein and indirectly support the hypothesis that anti-ABO(H) antibodies may contribute to virus neutralization[7].
Sialoside affinities put into perspective. The dissociation constants for the binding of sialosides to the S-protein (Fig. 2, Tables S1 and S2) that we have derived from STD NMR titrations (see above) are in the mM range. This contrasts with an NMR binding study that applied a different data acquisition and analysis strategy for measuring saturation transfer, essentially fitting an eight-parameter binding model to STD intensities measured at different ligand and protein concentrations. The study reports a dissociation constant $K_D$ of 32 $\mu$M for the binding of 3'SL to the S-protein. This differs by a factor of ca. 300 from the $K_D$ value determined in this study. That analysis also yielded association and dissociation rate constants of $6300$ $M^{-1}s^{-1}$ ($k_{on}$) and $0.2$ $s^{-1}$ ($k_{off}$), respectively. Similar values were obtained in that study from kinetic analysis of SPR experiments where sialic acid was immobilized on the chip surface.

For an analogous case of influenza virus hemagglutinin it has been demonstrated that kinetic analysis of SPR data with this setup, i.e., sialosides immobilized on the chip surface, yields artificially low $k_{off}$ and $K_D$ values. The cause is mainly multivalent binding of the trimeric hemagglutinin to the immobilized sialic acid residues and possibly rebinding. $K_D$ values were four orders of magnitude lower than measured by NMR. Competition SPR experiments then yielded $K_D$ values in the mM range as found by NMR, essentially proving that the kinetic analysis with the immobilized sialosides provides avidity and not affinity data.

We argue that the case of trimeric S-protein is analogous to the case of trimeric influenza virus hemagglutinin. Therefore, the reported SPR binding data should reflect avidity due to a mix of mono-, bi- and trivalent binding of trimeric S-protein to sialic acid residues presented within the dextran matrix of the sensor chip. It may be inferred that the corresponding monovalent interaction between a sialic acid residue and the sialic acid binding pocket is much weaker. This would agree well with our finding of dissociation constants in the mM range. Finally, previous studies have found that dissociation rate constants $k_{off}$ of the order of $0.1$ $s^{-1}$ can prevent the direct observation of STD effects. As clear STD effects are observed in the case of sialosides binding to the S-protein, it can be expected that $k_{off}$ is substantially larger. These discrepancies require further analysis.

**MD simulations support the overall picture of weak sialoside binding.** We performed MD simulations of sialic acid ($\alpha$-D-Neu5Ac) or 3'SL bound to the NTD of the S-protein, using the cryoEM model (PDB 7qur) as a starting structure. For comparison and as a positive control, we also performed MD simulations with the corresponding and well-studied complex of $\alpha$-D-Neu5Ac with the NTD of the MERS CoV S-protein, starting with the cryoEM structure (PDB 6q04). A comparison of the two structures 7qur and 6q04 based on an atom-atom contact analysis suggests fundamentally different binding modes of $\alpha$-D-Neu5Ac bound to the NTD of SARS CoV-2 vs. MERS CoV (Fig. S15). Restrained MD runs were performed for equilibration. Several replica MD runs were then produced with and without protein backbone restraints. The results of these simulations (Fig. 5) show that the complex of the SARS CoV-2 NTD and $\alpha$-D-Neu5Ac dissociates on average in much less than 100 ns without backbone restraints (Fig. 5a). Even in MD simulations with backbone restraints dissociation is observed typically in less than 100 ns (Fig. 5b). The complex of 3'SL are marginally more stable (Fig. S16). In contrast, the complex of $\alpha$-D-Neu5Ac bound to the NTD of the MERS CoV S-protein is stable for up to approximately 500 ns of unrestrained MD simulations (Fig. 5c).

These results lend credibility to the weak binding as described in our present study.

Figure 4. The SARS CoV-2 RBD does not bind ABO blood group antigens. The absence of any detectable CSPs in a $^{1}H$ TROSY HSQC spectrum of $^{15}$N-labeled RBD (102 $\mu$M, black contours) upon addition of a mixture of ABO oligosaccharides (red contours; type I blood group A tetrasaccharide and 0.2 M AB0 blood group antigens) or 3'SL bound to the NTD (shown as an overlay of holo and apo NTD). As expected, the 3'SL bound to the NTD shows a much weaker STD signal in comparison to the 3'SL bound to the NTD. The absence of any detectable CSPs in a $^{1}H$ TROSY HSQC spectrum of $^{15}$N-labeled RBD (102 $\mu$M, black contours) upon addition of a mixture of ABO oligosaccharides (red contours; type I blood group A tetrasaccharide and 0.2 M AB0 blood group antigens) or 3'SL bound to the NTD (shown as an overlay of holo and apo NTD). As expected, the 3'SL bound to the NTD shows a much weaker STD signal in comparison to the 3'SL bound to the NTD.
S-protein are essential for the direction of future studies into the biological role of glycans in SARS CoV-2 infection.

Experimental Section

Protein expression and purification
SARS CoV-2 receptor binding domain (RBD, GenBank ID YP_009724390.1, aa 319-527) was expressed and purified from inclusion bodies as explained elsewhere[6]. Briefly, E. coli BL21DE3 cells were transformed with pMal-c2x plasmid encoding an ampicillin resistance and a fusion protein consisting of the RBD and maltose-binding protein separated by two His-tags and a HRV3C cleavage site. Cells for synthesis of [L-15N] or [U-3H,15N]-labelled RBD were grown in H2O or D2O based minimal medium enriched with 3 gL−1 NH4Cl (Deutero) and 3 gL−1 glucose or deuterated glucose (Deutero), respectively. The culture was incubated at 37 °C and protein expression was induced using 1 mM IPTG at an OD600 of 0.5. Cells were harvested after 6 h by centrifugation. Pellets were stored at -70 °C.

Cells were resuspended in 20 mM Tris buffer with 100 mM NaCl (pH 8) containing lysosome (Merck Millipore) and benzonase (Merck Millipore) and lysed using a high-pressure homogenizer (Thermo). The lysate was centrifuged to collect the insoluble inclusion bodies. Inclusion bodies were washed by incubation for 1 h at room temperature in 1 mL BugBuster reagent (Millipore) per 0.1 g inclusion body under moderate shaking. The supernaemant was removed by centrifugation (1h, 14,000 *g, 4 °C) and the purified inclusion bodies were stored at -70 °C.

The inclusion bodies were resolved in 20 mM Tris buffer, 8 M urea, 100 mM NaCl, 10 mM 2-mercaptoethanol (pH 8.5) and incubated for 5 h at room temperature under moderate shaking. Refolding was carried out by flash dilution (1:100) into cold 100 mM Tris buffer, 100 mM NaCl, 5 mM reduced glutathione (Roht), 1 mM oxidized glutathione (Roht) (pH 8.5) and subsequent incubation for 19 h at 4 °C under slight stirring. The fusion protein was purified by N-NTA affinity chromatography and cleaved by addition of HRV 3C protease. The solution containing MBP, RBD and HRV 3C was dialyzed against 100 mM Tris buffer, 100 mM NaCl, 5 mM reduced glutathione (Roht), 0.2 mM oxidized glutathione (Roht) (pH 8.5) prior to a second N-NTA affinity chromatography run yielding the RBD. The protein was concentrated, and the refolded protein was separated from aggregates and line widths measured (Table S3). We analyzed the protein-ionic acid complexes for short atom-atom distances indicating H-bonds, or lipophilic interactions (Table S3).

We then compared our results to cases of sialic acid-binding proteins, most of them viral proteins, where high-resolution crystal structure data and equilibrium dissociation constants are available. In all cases the dissociation constants were in the mM range (Table S3). The dissociation constants resulted from different experimental approaches ranging from the measurement of concentration-dependent NMR chemical shift changes of specific ligand signals upon binding and line-widths measurements [24-26, 35, 52] to integrating ligand electron densities from soaking crystals with increasing ligand concentrations[33-34], to CSP titrations using 1H, 15N-HSQC experiments [23, 29, 33-34]. We analyzed the protein-sialic acid complexes for short atom-atom distances indicating H-bonds, or lipophilic interactions (Table S3).

Our analysis gives no indication for an increased binding enthalpy of sialic acid bound to the SARS CoV-2 NTD as compared to the other proteins. To the contrary, the number of short-range heavy atom contacts was lowest for the complex of sialic acid with the SARS CoV-2 NTD. We conclude that the dissociation constants in the mM range reported here agree with results for other sialic acid-binding (viral) proteins and reflect binding under equilibrium conditions.

Conclusion

Weak binding to glycan ligands does not necessarily translate into a minor role in infection as the example of influenza virus convincingly demonstrates. However, for SARS CoV-2 the role of glycans is still disputed. We think that our findings of low affinity (mM) binding of sialylglycans, and no binding of ABO(H) blood group antigens to the S-protein, together with the observation that none of the glycan ligands studied interacts with the RBD of the S-protein are essential for the direction of future studies into the biological role of glycans in SARS CoV-2 infection.

NMR experiments - general information and sample preparation
NMR spectra were acquired on Bruker 600 MHz Avance III HD spectrometers equipped with a TCI cryogenic probe or with a room temperature probe. Spectra were processed and analyzed with TopSpin v3.6 (Bruker) if not stated otherwise. Spike proteins were concentrated and exchanged into D2O-based spike NMR buffer (20 mM Bis-Tris-d19, 100 mM NaCl, pH 7.3) using 0.5 ml Amicon Ultra centrifugal filters (10 kDa, Merck Millipore). Protein

Figure 5. Estimation of the relative stability of α-D-Neu5Ac complexes with SARS CoV-2 NTD (A, B) and MERS CoV NTD (C) based on MD simulations. The RMSD values of the ligand are classified into three categories. Each replica within a set starts from the same equilibrated molecular system but different random seed.

We think that our findings of low affinity (mM) binding of sialylglycans, and no binding of ABO(H) blood group antigens to the S-protein, together with the observation that none of the glycan ligands studied interacts with the RBD of the S-protein are essential for the direction of future studies into the biological role of glycans in SARS CoV-2 infection.
concentrations were determined using the mean of two UV spectroscopy measurements at 280 nm with a molar extinction coefficient of 140960 M⁻¹cm⁻¹. Potential spike protein ligand molecules were titrated from highly concentrated stocks in spike NMR buffer with carefully adjusted pH values. 100 μM DSS-d6 was used as a spectral reference. RBD samples were diluted to 10:1 with a 100 % D2O solution for field-frequency lock, containing 5 mM DSS-d6 as a spectral reference. Potential RBD ligand molecules were titrated from highly concentrated stocks in RBD NMR buffer with carefully adjusted pH values for plotting binding isotherms.

Saturation times were 2 s using a cascade of 50 ms Gaussian pulses (40 dB) separated by 1 ms. The relaxation delay was set to 5 s and excitation sculpting was verified by acquisition of 1D proton NMR spectra on a Bruker 600 MHz Avance III HD spectrometer equipped with a TCI cryogenic probe using 16 scans, a relaxation delay of 5 s and excitation sculpting to remove water signals.

**Evaluation of STD NMR experiments at single saturation times**

The STD NMR spectra were acquired at 279 K using an off-resonance frequency of 200 ppm and on-resonance frequency of 4 ppm. The on-resonance spectrum has been chosen such that a sufficient S/N is obtained while safely avoiding direct irradiation of ligand signals and follows recommendations in the literature for STD NMR experiments with very large proteins[17, 20, 55]. Fig. S17 shows the dependency of the STD NMR signal intensity as a function of the on-resonance frequency in the case of the homo-trimeric S-protein of the Wuhan strain (MW ca. 500 kDa)[56]. Saturation times were 2 s using a cascade of 50 ms Gaussian pulses (40 dB) separated by 1 ms. The relaxation delay was set to 5 s and excitation sculpting was used for suppression of the water signal. The number of scans is given in Tab. S4.

**Evaluation of STD NMR experiments at single saturation times**

STD amplification factors (STDn) were calculated according to equation (1):

\[
STD_{n} = \frac{\text{Int}_{\text{sat}} - \text{Int}_{0}}{\text{Int}_{0}}
\]

with \(\text{Int}_{\text{sat}}\) being the intensity of a proton resonance signal in the off-resonance spectrum, \(\text{Int}_{0}\) being the intensity of a proton resonance in the on-resonance spectrum, \(L\) being the total ligand concentration, and \(P\) being the total protein concentration.

\(K_{0}\) and \(STD_{\text{max}}\) were then fitted using equation (2). Experimental \(STD_{n}\) values were measured as a function of the total ligand concentration \(L\) and the total protein concentration \(P_{r}\), yielding binding isotherms as shown in Fig. 2. Only protons with clearly resolved resonance lines were used for the fitting procedure. Using this approach, binding isotherms of individual protons yield different \(K_{0}\) values due to different relaxation times of the protons[18, 20, 56]. It is assumed that the lowest \(K_{0}\) value obtained reflects the best approximation to the “true” value, which may be slightly lower[20, 56].

\[
STD_{0} = \frac{1}{1 + \frac{K_{0}}{P_{r}}} = \frac{P_{r}}{1 + \frac{K_{0}}{P_{r}}} = STD_{\text{max}} = \frac{P_{r}}{1 + \frac{K_{0}}{P_{r}}}
\]

Fitting of equations was carried out using in-house python scripts employing the imfit and numpy libraries. The matplotlib library was used for plotting binding isotherms.

For epitope mapping, \(STD_{0}\) values were normalized by setting the largest \(STD_{n}\) of a specific ligand at 100%.

**Initial growth analysis of STD NMR titrations and calculation of dissociation constants \(K_{0}\)**

STD NMR spectra of 6'SL bound to the Wuhan S-protein were acquired with saturation times \(t_{\text{sat}}\) of 250 ms, 500 ms, 750 ms, 1000 ms, 2000 ms, and 4000 ms and at 6'SL concentrations of 3 mM, 8 mM, 15 mM, and 30 mM. The S-protein concentration was 18 μM. Except for the different saturation times, the experimental setup followed the protocol above. Eq. (3) was then fitted to the experimental values \(STD_{0}(\text{sat})\) yielding \(STD_{\text{max}}\) and \(K_{0}\).

\[
STD_{0}(\text{sat}) = STD_{0,\text{max}} \times (1 - e^{-\frac{K_{0}}{P_{r}}})\ (3)
\]

with \(STD_{0,\text{max}}\) being the maximum STD0 for a certain proton and \(K_{0}\) being the saturation rate constant. The initial slopes were than calculated using as \(STD_{0}(\text{sat}) = d(STD_{0,\text{max}})/dt = STD_{0,\text{max}}k_{\text{sat}}\). For error propagation, 1000 Monte Carlo iterations of \(STD_{0}(\text{sat})\) calculations were done by randomly drawing \(K_{0}\) and \(STD_{0,\text{max}}\) values from synthetic Gauss distributions estimated from the values and standard deviations resulting from the residuals of the fitting procedure of \(K_{0}\) and \(STD_{0,\text{max}}\). The 1000 synthetic \(STD_{0}(\text{sat})\) values per 6'SL concentration were then used to repeat fitting of dissociation constants according to equation (2). The mean and standard deviation of the 1000 obtained dissociation constants were used as final value and its error.

**1H,15N TROSY HSQC spectra for CSP NMR experiments**

For 

1H,15N TROSY HSQC spectra, the relaxation delay was set at 1.5 s. Spectra of 1H,15N-labeled RBD or were acquired using 2048 data points in \(t_{\text{p}}\) and 128 increments in \(t_{\text{m}}\). Spectra of 1H,15N-labeled RBD were acquired using 2048 data points in \(t_{\text{p}}\) and 256 increments in \(t_{\text{m}}\). The centers of the spectral windows were set at 4.7 ppm (F2) and 117 ppm (F1). The sweep widths were set at 16 ppm (F2) and 33 ppm (F1). The spectra were acquired at 298 K on a Bruker 600 MHz Avance III HD spectrometer with cryo probe. Number of scans were 24 for 1H,15N-labeled RBD and 32 for 15N-labeled RBD samples. Resonance positions of \(1^H,15N\) TROSY HSQC spectra were obtained with CCPNmr v2.4[60].

**Molecular Dynamics Simulations**

Starting structures of the SARS CoV-2 NTD domain were built using the graphical interface of YASARA[41] and employing the coordinates of PDB entry 7qur (residues 14-307). The NTD of the S-protein of MERS CoV is based on PDB entry 6q04 (residues 18-341). Ligands in low energy conformations were taken from an in-house library and positioned into the binding sites using the NeuAc residues present in the PDB entries as template.

The molecular complexes were solvated in 0.9% NaCl solution (0.15M) and simulations were performed at 310 K using periodic boundary conditions and the AMBER14 force field, which includes GLYCAM06[62] parameters for the carbohydrate residues. The box size was rescaled dynamically to maintain a water density of 0.996 g/ml. Simulations were performed using YASARA[41] with GPU acceleration in ‘fast mode’ (4 fs time step)[10] on ‘standard computing boxes’ equipped with one 12-core i9 CPU and NVIDIA GeForce GTX 1080 Ti. Each system was equilibrated for at least 10 ns with position restraints on the protein backbone atoms and distance restraints to keep the NeuAc in place. Multiple replica MD simulations were sampled for each equilibrated system using different random seeds. The stability of the complexes was monitored based on RMSD values of the NeuAc residue.

Conformational Analysis Tools (CAT, http://www.md-simulations.de/CAT/) was used for analysis of trajectory data, general data processing and generation of scientific plots. VMD[64] was used to generate molecular graphics.

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Keywords: Spike glycoprotein • STD NMR • Carbohydrate-protein interaction • Sialylglycan • Blood group antigens

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Sweet, but not so sweet - The SARS CoV-2 spike glycoprotein engages in weak interactions with sialoglycans but binding to ABO(H) blood group antigens cannot be confirmed by STD NMR experiments. Chemical shift perturbation experiments show that none of the glycans interacts with the receptor binding domain of the spike glycoprotein.