Infection with human noroviruses requires attachment to histo blood group antigens (HBGAs) via the major capsid protein VP1 as a primary step. Several crystal structures of VP1 protruding domain dimers, so called P-dimers, complexed with different HBGAs have been solved to atomic resolution. Corresponding binding affinities have been determined for HBGAs and other glycans exploiting different biophysical techniques, with mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy being most widely used. However, reported binding affinities are inconsistent. At the extreme, for the same system MS detects binding whereas NMR spectroscopy does not, suggesting a fundamental source of error. In this short essay, we will explain the reason for the observed differences and compile reliable and reproducible binding affinities. We will then highlight how a combination of MS techniques and NMR experiments affords unique insights into the process of HBGA binding by norovirus capsid proteins.

Interactions of human norovirus capsid proteins with HBGAs and experimental methods to obtain binding data

Noroviruses belong to the family of *Caliciviridae*, non-enveloped viruses with a single-stranded positive-sense RNA. Phylogenetically noroviruses are segregated into ten established genogroups that are further subdivided into genotypes [1]. Viruses of genogroups I, II, IV, VIII, and IX infect humans. Since 2002 GII.4 (genogroup II, genotype 4) norovirus strains have been responsible for most norovirus outbreaks. Noroviruses are the most common cause of acute gastroenteritis worldwide, often leading to epidemics that are difficult to contain. Frequent outbreaks with large case numbers causes substantial economic burden (Centers for Disease Control and Prevention (2021) Norovirus Worldwide: https://www.cdc.gov/norovirus/trends-outbreaks/worldwide.html) [2]. To date, there is no vaccine or antiviral therapy available.

It is well established that infection with human noroviruses critically depends on the attachment of virions to certain glycans, the so-called histo blood group antigens (HBGAs) [3–5]. HBGAs are abundantly present on epithelial cell surfaces in the form of glycolipids or glycoproteins, especially in the gastrointestinal mucosa. All HBGAs share the \(\alpha\)-L-Fuc-(1,2)-\(\alpha\)-Gal disaccharide (fucose \(\alpha\)-(1,2)-linked to galactose) as their core element. This so-called H-antigen can be extended with Gal (galactose) or GalNAc (N-acetylgalactosamine) residues by blood group glycosyltransferases, yielding blood group A or B antigens.

Infectious norovirus capsids consist of 180 copies of the major capsid protein VP1 arranged as 90 dimers in icosahedral symmetry. The outermost sphere of the viral capsid comprises the protruding domain (P-domain) of VP1 while the underlying sphere is made of the shell domain (S-domain).
Attachment to HBGAs is mostly mediated at the apical interface of two P-domains forming the dimer (P-dimer) as illustrated in Figure 1. Decrypting the molecular mechanisms behind this attachment process is desirable to better understand virus entry into host cells. Several crystal structures of complexes of human norovirus P-dimers with HBGAs ligands are now available [6–15], showing that for GII.4 type P-dimers L-fucose residues make the key binding interactions. Saturation transfer difference (STD) nuclear magnetic resonance (NMR) experiments monitoring binding of a variety of HBGAs ligands to P-dimers and to VP1-only virus-like particles (VLPs) complement these data and show that L-fucose is the minimal binding element presented by HBGAs [16].

Several biophysical techniques exist to determine binding affinities of ligands binding to proteins. Glycan ligand binding is often characterized by weak affinities with dissociation constants $K_D$ in the high $\mu$M to low mM range [17,18]. Detection and characterization of weak binding is a challenge, and not all experimental techniques are equally suited. The interactions between glycan ligands and norovirus capsid proteins are a biologically relevant example for such weak interactions that can be enhanced by multivalent ligand presentation.

A few studies have addressed this issue of multivalent presentation of HBGAs by, e.g. glycosphingolipids embedded in membranes [19–23]. Here, we focus on NMR and mass spectrometry (MS) methods to obtain quantitative binding data under weak binding conditions. The experimental techniques in question are native

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**Figure 1. Structures of VP1 and position of glycan binding.**

(a) Noroviruses are icosahedral shaped particles with a diameter of ca. 40 nm. The particles consist of 180 copies of the capsid protein VP1 forming 90 dimers (left: from yellow N-terminus to red C-terminus). Each VP1 consists of an N-terminal S-domain (yellow) and a C-terminal P-domain (red). The sketches are based on crystal structure data (PDB 1ihm). (b) Human norovirus P-dimers bind to HBGAs at the outermost tips at the interface. Shown here is the complex of a GII.4 Saga P-dimer with a blood group B trisaccharide (PDB 4 × 06, see also Figure 5 for the chemical formula).
Electrospray ionization mass spectrometry (ESI-MS), saturation transfer difference NMR spectroscopy (STD NMR), and chemical shift perturbation NMR experiments (CSP NMR).

Native ESI-MS is a highly effective methodology, whose gentle nature allows the study of noncovalent biomolecular interactions, such as protein–ligand complexes, under near-physiological conditions [24]. Implementing native MS for precise quantification of binding stoichiometry requires a tight control of experimental conditions, as to avoid distortion of mass spectra due to solution and gas-phase processes. For example, non-specific interactions between analytes (clustering) may occur in the electrospray because of free ligands within the same droplets as free or ligand-bound protein, which then dries down to the protein surface upon droplet evaporation. This mechanism is independent of protein size and occurs at increased ligand concentrations [25,26]. To circumvent this, Sun et al. [27] developed a direct method, which adds appropriate reference proteins. These proteins do not bind specifically to any components of the solution and serve to monitor non-specific clustering.

STD NMR experiments [28] allow to identify and characterize the binding of small ligand molecules, typically with a molecular mass well below 1 kDa, to large receptor molecules, usually proteins. Briefly, resonances of the receptor protein are saturated by a cascade of radio frequency pulses. This saturation is passed to ligands binding to the protein, causing attenuation of respective ligand NMR signals. Since a large excess of ligand over protein is used and because free ligands can ‘store’ saturation received during the bound state this saturation accumulates in free ligands. The corresponding signal attenuation is read out in difference spectra only displaying signals of binding ligands and discriminating binding from non-binding ligands. The relative size of a difference signal depends on the proton-environment of that ligand proton in the binding pocket, yielding ligand binding epitopes [29,30]. Furthermore, STD intensities measured as a function of ligand concentration allow the determination of dissociation constants [29,31]. The method is well suited to detect weak binding into the mM range of dissociation constants [32].

Protein-NMR experiments allow direct observation of the effects of ligand binding since chemical shifts of protein protons in the binding pocket are perturbed. These chemical shift perturbations (CSPs) upon binding [33] are recorded employing heteronuclear correlation experiments that provide fingerprints of the protein backbone. Chemical shifts of protein protons attached to backbone $^{15}$N nuclei serve as sensitive probes for changes in the chemical environment such as changes in pH, buffer composition, temperature, or ligand binding. Under conditions where pH, buffer composition, and temperature are kept constant this technique allows detection of binding of ligands with affinities ranging from tight binding with dissociation constants in the pM range to very weak binding in the higher mM range. Mapping of such CSPs on the protein directly yields binding site topologies. CSP titration experiments provide quantitative binding data, i.e. dissociation constants $K_D$. Of note, the absence of CSPs proves the absence of binding interactions, which means the method is essentially free of false negatives. Because of the applicability under weak binding conditions, NMR experiments have been important for the identification and quantification of glycan–protein interactions [32,34,35]. An advantage of using NMR experiments is that the experiments can be performed under physiological conditions [32].

All three methods have been employed to determine affinities for HBGA binding to norovirus P-dimers or to VLPs [36–41]. In the following, we review the efforts that have been made to compile quantitative data for binding of HBGAs to primarily GII.4 P-dimers.

## Binding affinities from native MS and from STD NMR titrations

### Overview of reported affinity data

To quantitate binding of HBGAs to norovirus P-dimers native MS and STD NMR titrations have been used. The first study reporting quantitative dissociation constants $K_D$ was based on STD NMR titrations [14] of P-dimers of the GII.10 Vietnam norovirus strain with synthetic HBGAs. $K_D$ values were derived for $\alpha$-L-Fuc ($\alpha$-l-fucose), $\beta$-L-Fuc ($\beta$-l-fucose), citrate, $\alpha$-L-Fuc-(1,2)-D-Gal (H-disaccharide), and blood group A trisaccharide, as well as for a trisaccharide $\beta$-L-Fuc-(1,2)-$\beta$-D-Gal-(1,4)-D-GlcNAc. It must be noted that this trisaccharide is not H-type 2 trisaccharide. H-type 2 trisaccharide carries an $\alpha$-l-fucose instead of a $\beta$-l-fucose leading to a completely different molecular shape. The dissociation constants $K_D$ were given as follows: 390 $\mu$M ($\beta$-L-Fuc-(1,2)$\beta$-D-Gal-(1,4)$\beta$-D-GlcNAc), 420 $\mu$M (H-disaccharide), 460 $\mu$M (citrate), and 460 $\mu$M (l-fucose).

In another study, affinities of a variety of HBGA ligands were measured [41] using a direct native MS approach [27]. In the original report, the affinities have been reported as association constants $K_A$. For the sake
of consistency and ease of comparability, we convert these values into dissociation constants $K_D$. For H-disaccharide ($\alpha$-L-Fuc-(1,2)-D-Gal) dissociation constants $K_D$ of 2.1 and 1.5 mM were determined for two slightly different P-dimer constructs of the GII.4 VA387 norovirus strain, being about a factor of five higher than determined by STD NMR titrations [14]. Since the HBGA binding sites of GII.10 Vietnam and of GII.4 VA387 are almost identical, one would expect more similar dissociation constants. The MS study [41] also reports that neither 3’-sialyllactose nor 6’-sialyllactose bind to P-dimers, in line with prior findings from STD NMR experiments [16].

A successive MS study addressed the binding of sialylated glycans as found in gangliosides to P-dimers of the GII.4 VA387 and VA115 strains [42]. Dissociation constants $K_D$ for a variety of sialylated glycans as found in gangliosides were reported to be in the $\mu$m range. For 3’-sialyllactose (GM3 gangliosides) a dissociation constant $K_D$ of 0.7 mM is reported. Binding of 3’-sialyllactose to P-dimers of a different strain, GII.4 MI001, was reported from our laboratories using a combination of native MS, STD NMR experiments, and surface plasmon resonance (SPR) [36], yielding dissociation constants for 3’-sialyllactose in the low mM range, in agreement with the previous results.

Another study into HBGA binding to human norovirus GII.4 Saga P-dimers from our laboratories [38] used a combination of NMR spectroscopy, native MS, and crystallography. It was observed that binding isotherms from STD NMR titrations exhibit steps. A crystal structure of GII.10 Vietnam P-dimers in complex with L-fucose showed four instead of the expected two bound fucose residues, and concentration-dependent co-crystallization reflected sequential occupation of the fucose binding pockets [13]. From native MS it was concluded that the GII.4 Saga P-dimers also exhibit four fucose binding pockets. Taken together, these observations led to the hypothesis, that binding of L-fucose is a stepwise process with negative cooperativity. NMR-based chemical shift titrations using uniformly $^2$H,$^1$N-labeled Saga P-dimers at first sight revealed no steps of corresponding binding isotherms, but denoising the data using singular value decomposition (SVD) revealed different components that matched the hypothesis of a stepwise process. At the time an assignment for the backbone resonances was not available. Step-shaped binding isotherms from STD NMR titrations were also observed for related systems [36,37].

Inspection of the data published revealed discrepancies that were addressed in follow up studies in our laboratories [43–45]. It turned out that the experimental approaches employed by us, and others contain hidden traps that are easy to fall into.

First, it turned out that the data leading to our hypothesis of cooperative binding were only seemingly consistent due to two main causes. For one, it turned out that native MS results were biased by secondary structure-induced glycan clustering [43]. On the other hand, spontaneous deamidation of a highly conserved asparagine residue (Asn373) and its quantitative conversion into an iso-aspartate residue over time led to mixtures of different protein species with different HBGA affinities. This resulted in extra components in the SVD analysis that falsely reflected consistency with the steps observed in STD NMR titrations [45]. Finally, the full backbone assignment had been instrumental for the identification of spontaneous Asn373 deamidation [45] that largely abolishes HBGA binding and obstructs interpretation of CSP titration data. Second, we showed that sialic acid containing glycans such as ganglioside head groups do not bind to human or murine norovirus P-dimers [44]. The reason for this again lies in the secondary structure-induced glycan clustering [43]. A third point concerns the dissociation constants reported so far by us, and others. Some of the data reported differed by more than one order of magnitude. For the STD NMR based data the ‘hidden trap’ is the occurrence of steps in the respective titration curves, and for MS-based data the ‘hidden trap’ again lies in structure-induced glycan clustering. In the following, we address the methodological limitations in more detail and illustrate some of the cases.

**Protein secondary structure affects glycan clustering in native MS**

Several groups applied native ESI-MS to P-dimer–glycan interactions [38,41]. Results varied remarkably, calling into question whether the selection of reference proteins affects data interpretation. Our initial experiments utilized a small reference protein, as we could not detect any binding with a mid-sized protein, while others reported $\mu$M affinities, and attributed this to residual glycan-binding affinity of the tested protein. Since clustering is supposedly independent of size, the choice of the reference protein ought not to affect the results if the conditions are sufficiently gentle. Notably, the direct MS approach was originally developed for high-affinity interactions, where the clustering effect is much lower, which may have masked a size influence of the reference proteins. Therefore, glycan clustering was examined again with different reference proteins. Small proteins such
as myoglobin, ubiquitin, and cytochrome c did indeed exhibit less glycan clustering when compared with alcohol dehydrogenase (ADH) [43] (Figure 2). Interestingly, the $K_D$ from STD-NMR could be reproduced using ADH as reference protein. However, the signals of the remaining specifically bound glycans on the P-dimer fall within the range of the standard deviation, i.e. they are no longer significant. Closer inspection revealed that not only size affected clustering, but also $\beta$-sheet content, which increases with size. $\alpha$-helices on the other hand did not have significant impact on this. We hypothesized that the hydroxyl-group rich glycans bond in net-like structures with the $\beta$-sheets of the reference proteins. $\beta$-sheets are labile during ESI [46],

![Figure 2. Comparison of clustering on various reference proteins with P-dimers.](image)

Clustering in native MS with 500 $\mu$M HBGA B type 1 tetrasaccharide (HBGA B), all spectra contain P-dimer and different reference proteins. From top to bottom: cytochrome c (cyt c), green fluorescent protein (GFP), carbonic anhydrase (CA), alcohol dehydrogenase (ADH). It is evident that the clustering under otherwise identical conditions is vastly different for the four proteins. Figure reproduced under creative commons attributions license from [43].

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which could facilitate intercalation with glycans. This effect has such a strong impact that non-binding proteins with a high number of β-sheets exhibit more or similar glycan 'interactions' than the actual P-dimer. Additionally, protein flexibility turned out to be an important factor as well. The highly flexible but low affinity deamidated P-dimer showed similar or increased glycan interaction compared with the wild-type protein. So even a reference protein with the same number of β-sheets could prove difficult for low-affinity glycan binding and should thus be carefully selected to avoid errors [43,47]. Therefore, protein–glycan interactions are best compared with a proper glycan negative control, which has proven impossible to find except for glycan mimetics [48]. It can be assumed that the backbone of the glycan mimetics, which is closer to peptide structures, limits the intercalation effect leading to high clustering. This appears plausible as clustering is much lower for peptides [49].

**Saturation transfer difference NMR experiments are sensitive to bulk effects at high millimolar ligand concentrations**

STD NMR experiments have been used in the past to analyze the binding of ligands with dissociation constants in the μM to mM range. In the case of equal and independent binding pockets, i.e. a single-site binding model one expects normal Langmuir binding isotherms. In the case of binding of HBGA to norovirus P-dimers and VLPs the binding isotherms exhibit discontinuities [36–38]. One explanation would be the cooperativity of the binding process, but this has been ruled out by follow up experiments [45]. Likely, the discontinuous shape of the binding isotherms is due to bulk effects that occur at high mM ligand concentrations required for saturation of the low-affinity binding pockets. This is supported by the observation that the shape of the binding isotherms depends on pH and buffer (see Figure S2 of [38]). Although there is no exact explanation at hand yet, ignoring the discontinuities and performing an analysis based on a single-site binding model yields similar dissociation constants as derived from CSP titrations [45].

With this knowledge it is now also plausible why the first reported dissociation constants based on STD NMR titrations [14] were almost two orders of magnitude below the values obtained later from CSP titrations [44,45]: The first step of the binding isotherm has simply been taken as the endpoint of the titration yielding dissociation constants that match the corresponding $K_{D1}$ values reported for the first step of the binding isotherm [38].

It is well established that STD NMR intensities strongly depend on the dissociation rate constants of a ligand–protein complex [29,50]. Therefore, the observed effects may reflect subtleties of the binding process not accessible by other methods. Although the steps impeded the analysis of dissociation constants it may be worthwhile to study such effects more systematically.

**Chemical shift perturbation NMR experiments redefine the glycan recognition code for noroviruses**

Complementary to the above discussed STD NMR and other ligand-observed techniques protein NMR experiments allow direct observation of ligand binding by recording CSPs upon binding [33]. A bottleneck for employing CSP measurements is the availability of stable-isotope labeled protein together with the requirement of an assignment of all protein signals as far as possible. For small proteins with a molecular mass below ca. 25 kDa uniform labeling with $^{15}$N is sufficient to record two-dimensional backbone $^{1}H,^{15}N$ HSQC spectra. For large proteins such as the norovirus P-dimers with a molecular mass of ca. 70 kDa the experimental effort to obtain assignments of NMR resonances still is a challenge [51,52], and recording backbone $^{1}H$ fingerprints requires additional labeling of the protein with deuterium ($^{2}H$). Once this hurdle has been taken $^{1}H$ fingerprints are straightforward to acquire, in this case by recording $^{1}H,^{15}N$ TROSY HSQC spectra [53]. With such a full assignment in hands, we were able to study HBGA binding to norovirus P-dimers by 'NMR fingerprinting', i.e. by observing CSPs of backbone $^{1}H$ upon addition of ligand (Figure 3), yielding the topology of the binding pocket as well as long-range effects of binding [45].

**Reproducible dissociation constants are obtained from backbone chemical shift perturbations**

The availability of an almost complete backbone assignment of norovirus GII.4 Saga P-dimers and transfer of the assignment of binding-relevant amino acid resonances to highly homologous P-dimers of other norovirus strains allowed us to scrutinize the mentioned inconsistencies [44,45]. The remarkable structural similarity of
the HBGA binding sites in different GII norovirus P-dimers is illustrated in Figure 4 by the superposition of different crystal structures.

Binding isotherms from CSP titrations yielded dissociation constants for GII.4 Saga P-dimers up to more than one order of magnitude higher than reported in previous studies from our and other laboratories. Dissociation constants $K_D$ from CSP titrations for several synthetic HBGAs are compiled in Figure 5. For instance, H-disaccharide is a very weak binder with a dissociation constant of 30 mM [44], which is even higher than the value of 22 mM found for L-fucose [45]. Compared with the initially reported value of 420 μM from STD NMR titrations [14] this is a factor of 71, and compared with the value from native MS of 1.5 mM [41] it still is a factor of 20. A noticeable increase in affinity is seen for the transition to the B-trisaccharide, in accordance with water-mediated interactions of the $\alpha$-(1,3)-linked Gal residue with the HBGA binding pocket [12]. The addition of an $\beta$-(1,3)-linked GlcNAc residue at the reducing end of the B-trisaccharide yields the blood group B-type-1 tetrasaccharide and leads to a decrease in affinity.

![Figure 3. Example for a TROSY HSQC ‘fingerprint’.](image)

(a) $^1$H$^{15}$N TROSY HSQC spectra of [U-2H$^{15}$N]-labeled GII.4 Saga P-dimers in the absence and presence of B-trisaccharide. The insert is highlighting CSPs due to binding of B-trisaccharide to P-dimers. (b) HBGA binding pocket of GII.4 Saga P-dimers (pdb 4 × 06) highlighting the close proximity between the L-fucose residue and R345 causing the strong CSP shown in (a). Figure reproduced [44] under creative commons attributions license.

![Figure 4. The glycan binding pocket in GII noroviruses.](image)

Superposition of HBGA binding pockets of GII.10 Vietnam (orange, pdb 3q38) GII.4 VA387 (gray, pdb 2obt) and GII.4 Saga P-dimers (blue, pdb 4 × 06) in complex with blood group B trisaccharide. Highly conserved amino acids in contact with L-fucose, the minimal binding motif, are highlighted (GII.4 Saga numbering).
Norovirus P-domains do not bind to sialylated glycans

To our surprise, sialic acid bearing glycans such as gangliosides do not bind to human norovirus GII.4 P-dimers [44], including the initially studied VA387 strain [42] and the MI001 strain studied in our laboratory [36]. Moreover, extension of our studies into murine noroviruses, where binding of viruses to sialylated glycans via P-dimers has been postulated based on biological assays, unequivocally shows that there is no binding of MNV P-dimers to sialic acid bearing glycans, such as gangliosides [54–56]. We have put considerable effort into systematically varying the experimental conditions such as buffer, pH, and temperature to exclude that these are false-negative results. At the same time, we also showed that HBGAs do not bind to murine norovirus P-dimers. Taken together a revised picture of glycan recognition by norovirus P-dimers has emerged. This is highlighted by Table 1, contrasting the results from previous and current research.

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### Table 1. Summary of results from previous and current research into the binding of glycan ligands to norovirus capsid proteins (Human norovirus P-dimers of GII.4 and GII.10 genotypes and murine norovirus P-dimers)

| P-dimer           | Binding to HBGAs | Binding to sialylated glycans |
|-------------------|------------------|------------------------------|
|                    | Previous         | Current                      | Previous                  | Current                  |
| Human norovirus   | $K_D$ in the range of ca. 0.5–5 mM | $K_D$ in the range of ca. 2–30 mM | $K_D$ in the range of ca. 0.5–5 mM | No binding |
| Murine norovirus  | No binding       | No binding                   | Indirect evidence of binding from infection assays | No binding |

Figure 5. Glycan structures and dissociation constants.

Synthetic HBGAs and $K_D$s for binding to GII.4 Saga and GII.4 MI001 P-dimers [44,45]. The abbreviations (N,N), etc. refer to the deamidation status of Asn373. (N,N) stands for not deamidated, and (ID,ID) stands for fully deamidated, i.e. both Asn373 residues have been transformed into isoAsp residues.
Linking glycan binding, protein dynamics and quaternary structure

Native mass spectrometry reports directly on oligomeric state

Native MS has proven to be an apt methodology to investigate the relationship between the monomer and its twin-relative when affinities are in the low μM range, e.g. for antibodies and half antibodies [57]. Interestingly, the ratio of P-monomer to P-dimer seems highly dependent on several different factors such as strain and deamidation [47]. Deamidation appears to increase the monomer fraction. Notably, strain dependencies also occur on the level of noroVLPs out of the VP1 protein, both in terms of stability and assembly state, which may point to additional mechanisms for host cell attachment or entry [58,59].

Therefore, it makes sense that strain-dependencies should be regarded as a major influence on any attributes of noroviruses. Still, the impact of deamidation on dimerization is convincing and well supported by the increase in overall protein flexibility that can be observed in hydrogen–deuterium-exchange (HDX) MS, on which a closer look will be taken next.

HDX MS yields insights into protein dynamics

HDX MS is based on, as the name suggests, switching out hydrogen for deuterium atoms. Hydrogens of OH, NH, and SH groups are labile and naturally exchanging with other hydrogen atoms in the surrounding

Figure 6. HDX MS and MD on GII.10 Vietnam P-dimer in presence of glycans.
Top: Regions showing protection in presence of HBGA B trisaccharide or fucose in blue revealing protection also in regions distal from the binding site suggesting long-range effects. Bottom: overlay of crystal structures for various P-dimers in presence of glycans showing an extended loop in GII.10 Vietnam 026 (a). Whereas the loop is folded into a short helix in presence of glycans, it unfolds and flops around in absence of glycans as revealed by MD simulations (b). Figures reproduced under creative commons license attribution from [47].
solution. For HDX MS, these are replaced with an isotope of hydrogen: deuterium. The exchange with the heavier isotope results in a net mass gain of ∼1, which can be observed using MS. Usually, the exchange is measured on the peptide level to gather local structural information. Since side-chain hydrogens exchange extremely fast, only the backbone amide hydrogen-deuterium exchange is generally probed. This process obviously applies only for solvent-accessible hydrogens. The ratio of exchange is influenced by several factors such as pH, temperature, and the local environment of a given peptide [24,60]. Other relevant properties are conformational dynamics and flexibility of protein regions, as this may increase solvent-accessibility and thus accelerate deuterium exchange [61]. This of course includes ligand interaction, which makes HDX MS a most suitable tool to examine protein–glycan interaction.

Initial HDX experiments showed that the canonical binding site (G443, Y444) of the wildtype P-dimer is protected in the presence of either B trisaccharide or methyl α-L-fucopyranoside, but not when α-galactose is applied. Interestingly, protection like this could not be observed in fully deamidated P-dimers. As it turns out, the entire P2 domain, the structure most proximal to the binding site, is much more flexible than in the wild-type [45]. This also fits the aforementioned MS data on increased amounts of monomer in deamidated species. Notably, the higher exchange and flexibility in deamidated P-dimers cannot only be attributed to the presence of P-monomers, i.e. there is additional increased dynamics. As written before, strain-dependent differences may prove to be important. Thus, the structures of different GII strains were compared, with the result that all share the canonical binding site motif with little or no long-range effects, except GII.10 Vietnam 026 (Figure 6). For this specific strain, glycan interaction induced folding in a loop proximal to the binding site. This long-range effect is supported by MD simulations [47].

Deamidation in many GII.4 strains may point towards some important mechanism in infection. Under physiological conditions, deamidation usually has a half-life of 1.6 days, which implies mixed species are to be expected [45]. Partially deamidated dimers do still interact with glycans, which results overall in a more deprotective effect, thus exposing more residues to solvent [47]. Glycan interaction may, therefore, be a mechanism to open these partially deamidated P-dimers to access additional receptor bindings sites.

Ion mobility MS and glycan binding

Ion mobility mass spectrometry (IMMS) is another way to implement MS technology in protein–ligand interaction research. Here, the travel time of ionized proteins through an ion mobility cell are analyzed to monitor large conformational changes. Initially, the impact glycan-binding has on P-dimer structure was reported to be relatively high with an over 10% increase in arrival time [39]. Compared with the above-described data collected from HDX, NMR, and crystallography, which indicate more subtle changes, the reported effects appear too large. Unfolding processes may be the cause of this, though this remains unclear. Therefore, we revisited these measurements. IMMS is suitable for large changes in protein structure, therefore, arrival time differences above 3–5% are commonly recognized as significant. Here, changes of about 1.6% were determined for glycan-bound and non-bound GII.4 Saga dimers, as expected for the addition of a small molecule. Notably, this was independent of glycan selection [43]. These experiments imply that IMMS is not a suitable approach to decipher the subtle interplay at work here.

Conclusion

This review presents and compares previous and current research into the binding of norovirus capsid proteins to glycan ligands, employing MS, and NMR methodology. It is explained how previous studies have suffered from methodological limitations, not known at the time, and it is then described how combinations of the highly complementary MS and NMR technologies provide a robust toolset to unambiguously identify and characterize the weak binding of glycan ligands to receptor proteins in general.

Perspectives

- Detection and characterization of low-affinity protein–ligand interactions are a challenge and require careful control experiments. The combination of NMR and MS techniques is well suited to identify potential traps.
The glycan recognition code for human and murine norovirus P-dimers has been revised: Only human but not murine norovirus P-dimers bind to HBGAs, and sialic acid bearing glycans are neither recognized by human nor by murine norovirus P-dimers.

It is conceivable that glycans and other ligands are modulators that enable further virus–host interactions. This ought to be considered planning future experiments to identify additional interaction partners.

Competing Interests
The authors declare that there are no competing interests associated with the manuscript.

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Abbreviations
ADH, alcohol dehydrogenase; CSP NMR, chemical shift perturbation NMR experiments; CSPs, chemical shift perturbations; ESI-MS, electrospray ionization mass spectrometry; HBGAs, histo blood group antigens; HDX, hydrogen-deuterium-exchange; IMMS, ion mobility mass spectrometry; MS, mass spectrometry; NMR, nuclear magnetic resonance; STD NMR, saturation transfer difference NMR spectroscopy; STD, saturation transfer difference; SVD, singular value decomposition; VLPs, virus-like particles.

References
1. Chhabra, P., de Graaf, M., Parra, G.I., Chan, M.C., Green, K., Martella, V. et al. (2019) Updated classification of norovirus genogroups and genotypes. J. Gen. Virol. 100, 1393–1406. https://doi.org/10.1099/jgv.0.001318
2. Lopman, B.A., Steele, D., Kirkwood, C.D. and Parashar, U.D. (2016) The vast and varied global burden of norovirus: prospects for prevention and control. PLoS Med. 13, e1001999. https://doi.org/10.1371/journal.pmed.1001999
3. Huang, P., Farkas, T., Marionneau, S., Zhong, W., Ruvoen-Clouet, N., Morrow, A.L. et al. (2003) Noroviruses bind to human ABO, Lewis, and secretor histo-blood group antigens: identification of 4 distinct strain-specific patterns. J. Infect. Dis. 188, 19–31. https://doi.org/10.1086/375742
4. Marionneau, S., Ruvoen, N., Le Moullac-Vaidye, B., Clement, M., Cailléau-Thomas, A., Ruiz-Palacois, G. et al. (2002) Norwalk virus binds to histo-blood group antigens present on gastroduodenal epithelial cells of secretor individuals. Gastroenterology 122, 1967–1977. https://doi.org/10.1053/gast.2002.33661
5. Clausen, H. and Hakomori, S.-I. (1989) ABH and related histo-blood group antigens; immunochemical differences in carrier isotypes and their distribution. Vox Sang. 56, 1–20. https://doi.org/10.1111/j.1423-0410.1989.tb00404.x
6. Bu, W., Mamedova, A., Tan, M., Xia, M., Jiang, X. and Hegde, R.S. (2008) Structural basis for the receptor binding specificity of Norwalk virus. J. Virol. 82, 5340–5347. https://doi.org/10.1128/JVI.00125-08
7. Cao, S., Lou, Z., Tan, M., Chen, Y., Liu, Y., Zhang, Z. et al. (2007) Structural basis for the recognition of blood group trisaccharides by norovirus. J. Virol. 81, 5949–5957. https://doi.org/10.1128/JVI.00219-07
8. Bereszczak, J.Z., Barbu, I.M., Tan, M., Xia, M., Jiang, X. van Duijn, E. et al. (2012) Structure, stability and dynamics of norovirus P domain derived protein complexes studied by native mass spectrometry. J. Struct. Biol. 177, 273–282. https://doi.org/10.1016/j.jsb.2012.01.005
9. Chen, Y., Tan, M., Xia, M., Hao, N., Zhang, X.C., Huang, P. et al. (2011) Crystallography of a lewis-binding norovirus, elucidation of strain-specificity to the polymorphic human histo-blood group antigen. PLoS Pathog. 7, e1002152. https://doi.org/10.1371/journal.ppat.1002152
10. Tan, M., Xia, M., Chen, Y., Bu, W., Hegde, R.S., Meiller, J. et al. (2009) Conservation of carbohydrate binding interfaces: evidence of human HBGA selection in norovirus evolution. PLoS One 4, e5058. https://doi.org/10.1371/journal.pone.0005058
11. Singh, B.K., Leuthold, M.M. and Hansman, G.S. (2016) Structural constraints on human norovirus binding to histo-blood group antigens. mSphere 1, e00049-16. https://doi.org/10.1128/mSphere.00049-16

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Yan, H., Lockhauserbaumer, J., Szekeres, G.P., Mallagaray, A., Creutznacher, R., Taube, S. et al. (2021) Protein secondary structure affects glycan clustering in native mass spectrometry. Life (Basel) 11, 554 https://doi.org/10.3390/life11060554

Creutznacher, R., Maas, T., Ogriszek, P., Wallmann, G., Feldmann, C., Peters, H. et al. (2021) NMR experiments shed new light on glycan recognition by human and murine norovirus capsid proteins. Viruses 13, 416 https://doi.org/10.3390/v13030416

Mallagaray, A., Creutznacher, R., Duffer, J., Mayer, P.H.O., Grimm, L.L., Orduña, J.M. et al. (2019) A post-translational modification of human Norovirus capsid protein attenuates glycan binding. Nat. Commun. 10, 1320 https://doi.org/10.1038/s41467-019-09251-5

Seo, J., Hoffmann, W., Warnke, S., Bowers, M.T., Pagel, K. and von Helden, G. (2016) Retention of native protein structures in the absence of solvent: anisotropy indicates an avenue to NMR structures of very large biological macromolecules in solution. Proc. Natl Acad. Sci. U.S.A. 94, 12366 https://doi.org/10.1073/pnas.94.23.12366

Pervushin, K., Riek, R., Wider, G. and Wüthrich, K. (1997) Attenuated T2 relaxation by mutual cancellation of dipole coupling and chemical shift anisotropy indicates an avenue to NMR structures of very large biological macromolecules in solution. Proc. Natl Acad. Sci. U.S.A. 94, 12366 https://doi.org/10.1073/pnas.94.23.12366

Taube, S., Perry, J.W., Yetming, K., Patel, S.P., Auble, H., Shu, L. et al. (2009) Ganglioside-linked terminal sialic acid moieties on murine macrophages function as attachment receptors in a strain-dependent manner. J. Virol. 86, 5584–5593 https://doi.org/10.1128/JVI.06854-11

Taube, S., Jiang, M. and Wobus, C.E. (2010) Glycosphingolipids as receptors for non-enveloped viruses. Viruses 2, 1011–1049 https://doi.org/10.3390/v2041011

Taube, S., Perry, J.W., Yetming, K., Patel, S.P., Auble, H., Shu, L. et al. (2009) Ganglioside-linked terminal sialic acid moieties on murine macrophages function as attachment receptors for murine noroviruses. J. Virol. 83, 4092–4101 https://doi.org/10.1128/JVI.02245-08

Rose, R.J., Labrijn, A.F., van den Bremer, E.T., Loverix, S., Lasters, I., van Berkel, P.H. et al. (2011) Quantitative analysis of the interaction strength and dynamics of human IgG4 half molecules by native mass spectrometry. Structure 19, 1274–1282 https://doi.org/10.1016/j.str.2011.06.016

Pogan, R., Weiss, V.U., Bond, K., Duffer, J., Krisp, C., Lykoty, N. et al. (2020) N-terminal VP1 truncations favor T = 1 norovirus-like particles. Vaccines (Basel) 9, 8 https://doi.org/10.3390/vaccines9010008

Ronja, P., Carola, S., Rudolph, R., Grant, H. and Charlotte, U. (2018) Norovirus-like VP1 particles exhibit isolate dependent stability profiles. J. Phys.: Condens. Matter 30, 064006 https://doi.org/10.1088/1361-648x/aaa43b

Kuner, L., Pan, J. and Liu, Y.-H. (2011) Hydrogen exchange mass spectrometry for studying protein structure and dynamics. Chem. Soc. Rev. 40, 1224–1234 https://doi.org/10.1039/C0CSR0113A

Brown, K.A. and Wilson, D.J. (2017) Bottom-up hydrogen deuterium exchange mass spectrometry: data analysis and interpretation. Analyst 142, 2874–2886 https://doi.org/10.1039/C7AN00620D