Investigating the Interaction Between Influenza and Sialic Acid: Making and Breaking the Link

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Abstract Since the early 1940s sialic acid (Sia) has been regarded as the primary receptor for influenza virus. This Sia is usually bound to an adjacent galactose (Gal) in an α2-3 or α2-6 configuration. This led to a concept about an interspecies barrier as avian viruses preferentially bind to Sia α2-3 linked to Gal, whereas human viruses have a preference for the Sia α2-6 linked to Gal and that transmission from one species to another would preferentially occur only in a host species in which both types of Sia were present . The viral haemagglutinin binds to Sia to facilitate cellular entry. To release progeny viral particles the second main component of the influenza viral envelope – neuraminidase, cleaves Sia. The viral-receptor interaction was initially investigated using agglutination of red blood cells and later using lectin histochemistry. Recent techniques investigating the HA-Sia/NA-Sia link have employed the use of glycan arrays and virus-like pseudoparticles with STD-NMR.

1 Influenza Virus and Haemagglutination

In 1941, two publications by George Hirst and McClelland and Hare demonstrated that influenza virus was able to agglutinate red blood cells (reviewed in [1]). Before 1941 the diagnosis of influenza was made by virus neutralization and complement fixation but the in vitro haemagglutination showed that the quantity of virus as well as the presence of antibody could be used as a simple, rapid and less laborious test for influenza virus infection as the haemagglutination reaction could be detected by the naked eyes in minutes. Studies in subsequent years further characterized the

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nature of the haemagglutination – it was temperature dependent but the cell receptors were comparatively heat stable resisting 100°C for 5 min [2, 3]. The haemagglutination reaction was also pH dependent as some strains of influenza did not agglutinate at pH 5.0 though agglutination could continue up to pH = 10 [4–6].

2 The Nature of the Red Blood Cell Receptor

Because of the sensitivity of haemagglutination to temperature and formalin treatments, and its resistance to oxidizing agents, Hirst suggested that the influenza receptors present on red blood cells could be mucoproteins [6]. The finding that agglutination was not blood group dependent (A, B, AB or O) and that treated cells could still agglutinate with appropriate antisera excluded the blood group antigens as the potential link. Gottschalk and Lind in 1949 showed that the ovomucin fraction of egg white contained a haemagglutination inhibitor and further work by Gottschalk on this mucin narrowed this virus inhibiting nature down to a disaccharide – sialic acid bound to galactosamine and that this disaccharide was O-linked [7, 8]. Parallel studies by Burnet and Stone demonstrated that the haemagglutination reaction could be prevented by treatment of red blood cells with enzymes from *Vibrio cholerae* [9]. This so-called receptor destroying enzyme (RDE) was later shown by Gottschalk to be a sialidase or neuraminidase [10]. (Though the terms neuraminidase and sialidase are used interchangeably most researchers tend to use the term sialidase rather than neuraminidase, but the latter term is commonly used for influenza virus to be consistent with the H and N subtype classification.) The predominant glycoprotein present on red blood cells is glycophorin which contains 28% Sia by weight and accounts for 80% of the total erythrocyte Sia [11]. As Sias have a negative charge in a physiological condition, they mutually repel each other, and in the case of the high molecular weight filamentous glycoprotein mucins that were used by Gottschalk and Klenk, the attached sialic acids give a high viscosity to mucus to act as a lubricant. This negative charge prevents the spontaneous clumping of blood platelets and protects macromolecules, such as intrinsic factor, from being destroyed by proteolytic enzymes [10].

3 Not All Sialic Acid Is the Same

Using red blood cells from different animal species agglutination assays produced conflicting results and this was previously explained by strains of virus, temperature of reaction and species used. For instance, while viruses were able to agglutinate red blood cells from most animal species (man, monkey, guinea pig, dog, ferret, rat, mouse, duck and birds) at both 4°C and 20°C, some viruses would only agglutinate horse, ox, sheep, pig and hamster at 4°C but not 20°C [12]. Later work showed that

though the most common Sia found in mammals was *N*-acetylneuraminic acid (Neu5Ac), some animal species including great apes contained *N*-glycolylneuraminic acid (Neu5Gc) [13]. Neu5Gc is absent from humans due to the lack of 92 base pairs segment of the CMP-*N*-acetylneuraminic acid hydroxylase gene responsible for its synthesis and some virus strains preferentially bind to Neu5Ac than Neu5Gc which could explain the previously reported differences in haemagglutination.

4 The Second Interaction of Influenza with Red Blood Cells: Elution

The initial studies of Hirst on haemagglutination showed that adsorbed virus could be eluted from red blood cells at 37°C without significant loss but that the cells from which the virus had been eluted could not be agglutinated by fresh virus, indicating that the interaction of the virus with the red blood cell had structurally modified them so that the same reaction could not occur. This lack of re-agglutination was proposed to be due to enzymatic destruction of the receptor [2]. Klenk subsequently incubated influenza virus with urinary mucin to crystallize neuraminic acid. Initially, it was thought that the sialidase activity was combined with the haemagglutinin, but in 1961 Howe et al. were able to show that the HA was distinct from the NA and that they were distinct independent entities of the virus particle. This work was followed later by Laver who in 1963 was able to separate neuraminidase from influenza virus (reviewed in [9]). The role of neuraminidase in the viral replication cycle was investigated by Noll et al. who found that no NA was present within the first 4 h of infection, but it increased from 6 h onwards and this correlated with release of virus [14]. The NA was thus considered essential for viral particles being released from the cell.

5 Different Viruses, Different Agglutination and Elution

Though influenza was suspected to be a diverse agent it was not until 1940 that Francis and Magill, working independently were able to demonstrate two new strains of influenza that were antigenically different from strains of influenza A virus (reviewed in [15]). Studies by Paulson and colleagues in the late 1970s and 1980s using ortho- and para-myxoviruses exploited the RDE nature of *V. cholerae* to desialylate red blood cells and then resialylate them using different sialyltransferases (ST) – enzymes that attach Sia to the terminal galactose. They found that depending on the ST used they could change the haemagglutinating ability of the native virus and that even though glycophorin (which had mainly *O*-linked glycosylation) was involved, *N*-linked glycans that composed a minor component

of the red cell membrane could play a part. Indeed in their studies using resialylation a relatively small incorporation (10%) of Sia into a specific band of the erythrocyte membrane protein (Band 3) was able to restore agglutination of influenza to normal levels [11]. Furthermore, viruses that were antigenically similar (A/RI/5$^-$/57(H2N2) and A/RI/5$^+$/57(H2N2)) could have different binding properties. These studies demonstrated that it was not just the Sia that was involved in the virus binding and haemagglutination, but the link of the Sia to the adjacent galactose that was important. Furthermore, depending on the receptor binding properties, the viral neuraminidase would have different eluting effects, thus suggesting that the link between the Sia and galactose would influence the effectiveness of the viral NA. Jameson and Levine in 1953 also demonstrated that certain strains of influenza were unable to hydrolyse porcine submaxillary mucin which contains 90% Neu5Gc suggesting that the viral neuraminidase could distinguish between Neu5Ac and Neu5Gc [13].

6 The Addition of Sialic Acids to Galactose Requires Different Sialyltransferases

As indicated previously, Sia are added to the terminal sugar of glycoproteins and glycolipids by enzymes called sialyltransferases (ST) and normally once Sia are added they cannot be further extended [16]. The three common sugar residues that can have Sias linked to them are galactose (Gal), *N*-acetylglucosamine (GlcNAc), *N*-acetylgalactosamine (GalNAc) or another Sia. When Sia is added to a Gal it can either be bound to the hydroxyl group attached to carbon-3 (3-OH) of Gal to form an α2-3 glycosidic linkage, or to the 6-OH to form an α2-6 glycosidic linkage. When Sia is added to another Sia it is usually in a α2-8 linkage to form dimers, oligomers or longer homopolymers of Sia. The synthesis of the Sia terminated glycans is carried out by a family of 20 ST that are highly conserved from mouse to man [17]. For example, those that transfer Sia in an α2-6 linkage to Gal are ST6Gal transferases and those that transfer Sia in an α2-3 linkage are ST3Gal transferases. In humans, there are two ST6Gal transferases (ST6Gal I and II) both of which act on type II glycan chains (Galß1-4GlcNAc-R). The ST3Gal family is more complex with six members (ST3Gal I-VI). ST3Gal III, IV and VI sialylate type II glycan chains (Galß1-4GlcNAc-R), and ST3Gal I and ST3Gal II have a preference for type III glycan chains present on *O*-glycans (Galß1-3GalNAc-R), ST3Gal V acts on lactosyl-ceramide glycolipids (Galß1-4GlcNAc-Cer). Thus even though the STs use a common donor, CMP-Neu5Ac, the ST within the same family can differentiate between disaccharide acceptors with a ß1-3 link and a ß1-4 linkage and this has recently been attributed to single amino acid changes in the ST [17]. In *O*-linked glycans, the 2 main 6-linked STs transfer Sia to a GalNAc rather than Gal. The expression of ST is dynamic and can be upregulated by disease states in tumour

cells, inflammation, following steroid treatment and tumour necrosis factor α stimulation [18–21].

7 Binding Affinity for Sia Can Be Affected by Many Components of the Glycan

When Rogers and Paulson desialylated human red blood cells and re-sialylated them they took advantage of the different affinities of the ST and thus using ST6Gal, ST6GalNAc and ST3Gal were able to create red blood cells with different Sia linkages. They found that human and swine influenza viruses preferentially agglutinated red blood cells with a terminal Siaα2-6Gal and avian viruses preferentially agglutinated Siaα2-3Gal expressing cells [22]. While there was preference for a α2-3 or α2-6 binding in most of the viruses, in some there was still agglutination of the other receptor suggesting that the binding preference may not be absolute. Furthermore, within the Siaα2-3 binding group, the inner configuration of the oligosaccharide also affected binding – all the viruses that agglutinated red blood cells with Siaa2-3Galß1-3**Gal**NAc would agglutinate when the GalNAc was changed to a **Glc**NAc. However, the converse was not universally true as only half (two out of four) of the viruses that bound Siaa2-3Galß1-**4/3Glc**NAc would tolerate a change to Siaa2-3Galß1-**3Gal**NAc. In particular, an H1N1 virus (A/PR8/34) could tolerate a change from a Siaa2-3ß1-**3Gal**NAc to Siaa2-3ß1-**4Glc**NAc but H3N2 viruses lost binding when GlcNAc was changed to GalNAc – indicating that the viruses had different affinities for additional glycan structural features – not just the linkage position of the terminal Sia [23].

The importance of the subterminal sugars has been explored more using Sia linked polymers and glycan arrays developed by the Consortium for Functional Glycomics that allow for a large range of glycans to be investigated [24]. In the case of avian H5N1 viruses, Gambaryan and colleagues have found that chicken and duck H5N1 viruses differ in their affinity for the subterminal and subsequent residues of Siaα2-3 oligosaccharides – duck viruses bound type I glycan chains (Galß1-**3Glc**NAc) and type III glycan chains (Galß1-**3Gal**NAc), but chicken viruses preferred a type II core (Galß1-**4Glc**NAc), especially when it was sulfated on the 6 position of the GlcNAc [25]. This sulfation is important as sulfotransferases responsible for the generation of such high affinity sulfated sialyloligosaccharide receptor are expressed in human airway cells [26]. Fucosylation at the 3 position of the GlcNAc to generate the SiaLe^X structure (NeuAcα2-3Galß1-4(Fucα1-3) GlcNAc) also allowed preferential binding of some gull and H7N1 viruses [23]. These changes in the subterminal residues appear to have more effect on Siaα2-6 than Siaα2-3 glycans owing to their postulated binding interactions with haemagglutinin. The Siaα2-3 configuration is more rigid and appears to bind in the same way with less concern for the underlying HA configuration [27]. Siaα2-6Gal configurations, however, result in different relationships with the underlying

HA and the affinity varies quite considerably depending on the amino acid structure of the HA [28]. A potential explanation of these variations in HA specificity could be due to changes in the glycosylation sites of the HA molecule itself [29], resulting in changes to protein folding and conformation in the haemagglutinin or possible masking of the receptor binding site, and this glycosylation change has been demonstrated by Klenk in 2002 [30] and Zhang in 2004 [31].

8 Lectins Also Act as Agglutinating Agents

Since the affinity of viruses for a cell may be dependent on the Sia–Gal linkage it is natural that methods should be sought to determine the status of this linkage in the respiratory tract. Viruses have been previously shown to be agglutinating agents and lectins have also demonstrated the same function. Lectins are proteins or glycoproteins that bind to defined carbohydrate structures [32], and these can be of animal, microbial or plant origins [33]. As lectins bind sugars typically found on cell surfaces, they can agglutinate cells to which they interact (Fig. 1). It was the

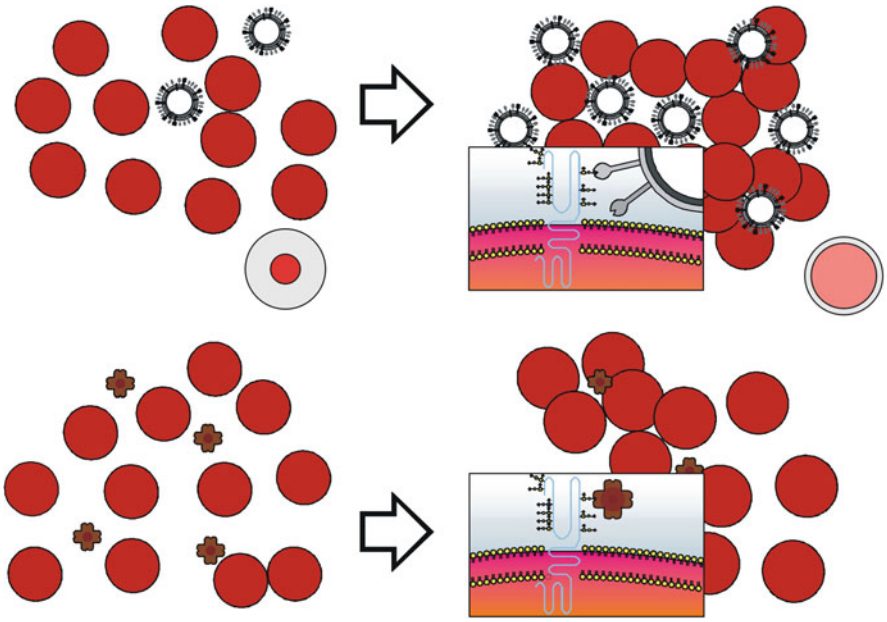

Fig. 1 Hemagglutination due to influenza virus or lectins is due to similar mechanisms. (**a**) The HA of influenza virus recognizes sialic acid terminated oligosaccharides on the surface of red blood cells (*red circles*) leading to agglutination with clumping of the red blood cells. (**b**) Lectins (in this case *Maackia amurensis* brown cross) also recognize the sialic acids present in the oligosaccharides and this leads to agglutination. In the inset, red blood cell glycophorin containing Siaα2-3Gal terminated oligosaccharides is shown

agglutinating activity of these substances that led William Clouser Boyd to use the term lectins for their ability to pick out or choose (Latin *legere*) individual blood group specificity affecting red blood cells of some species of an individual and not others of the same species. For instance, lima bean would agglutinate blood group A but not blood group O or B. In 1961, lectins were identified in seeds from the tree *Maackia amurensis* [34]. Further studies identified two types of *M. amurensis* lectin – one which was haemagglutinating (MAH) and one which was mitogenic (MAM) [35]. The mitogenic MAM was subsequently designated as the *M. amurensis* leukoagglutinin (MAL) due to its ability to agglutinate a mouse lymphoma cell line [36]. MAL was further reported to be specific for Siaα2-3Gal [37]. A lectin from the elderberry plant – *Sambucus nigra* agglutinin (SNA) – was also identified with specificity for the Siaα2-6Gal linkage [38]. Since 1983 the use of *M. amurensis* agglutinin (MAA) and SNA has been used by researchers to determine whether cells express α2-3 or α2-6 linked Sia on the surface. Glycan array data performed by the Consortium for Functional Glycomics has shed more light on the specificity of these lectins for α2-3 and α2-6 linked Sia – the highest binding affinity for MAL-II is for the *O*-linked disialylated Thomsen–Friedenreich (TF) antigen while MAL-I binds to Sia with GlcNAc linked to Gal but also to non-Sia terminated glycans.

9 Probing the Glycan Distribution Using Mass Spectrometry

An additional tool for determining the glycans present in the respiratory tract is mass spectrometry. In this methodology, glycoproteins and glycolipids are extracted from the respiratory mucosa and then profiled according to mass and known biochemical profiles. Sialidase digestion using α2-3 only or α2-3/2-6 specific sialidases then allows an analysis of the types of linkages present (Fig. 2). The advantage of this technique is that it provides a systematic analysis of the total glycans present and thus a better indication of the potential binding sites than lectin histochemistry. However, as respiratory tissues are a heterogenous population and analysis needs to be correlated with lectin histochemistry findings for better determination of which cells contain which types of Sia-linked glycans.

10 Changes in the HA Receptor Binding Site Affects Influenza Binding

The differential recognition of Siaα2-3Gal or Siaα2-6Gal by influenza viruses of different species was attributed to changes in the amino acid sequence of the haemagglutinin (HA). A change in the amino acid 226 of HA1 protein (produced by cleavage of HA0 into HA1 and HA2) from leucine to glutamine (or rarely methionine) in a H3 influenza virus led to a change in binding preference from

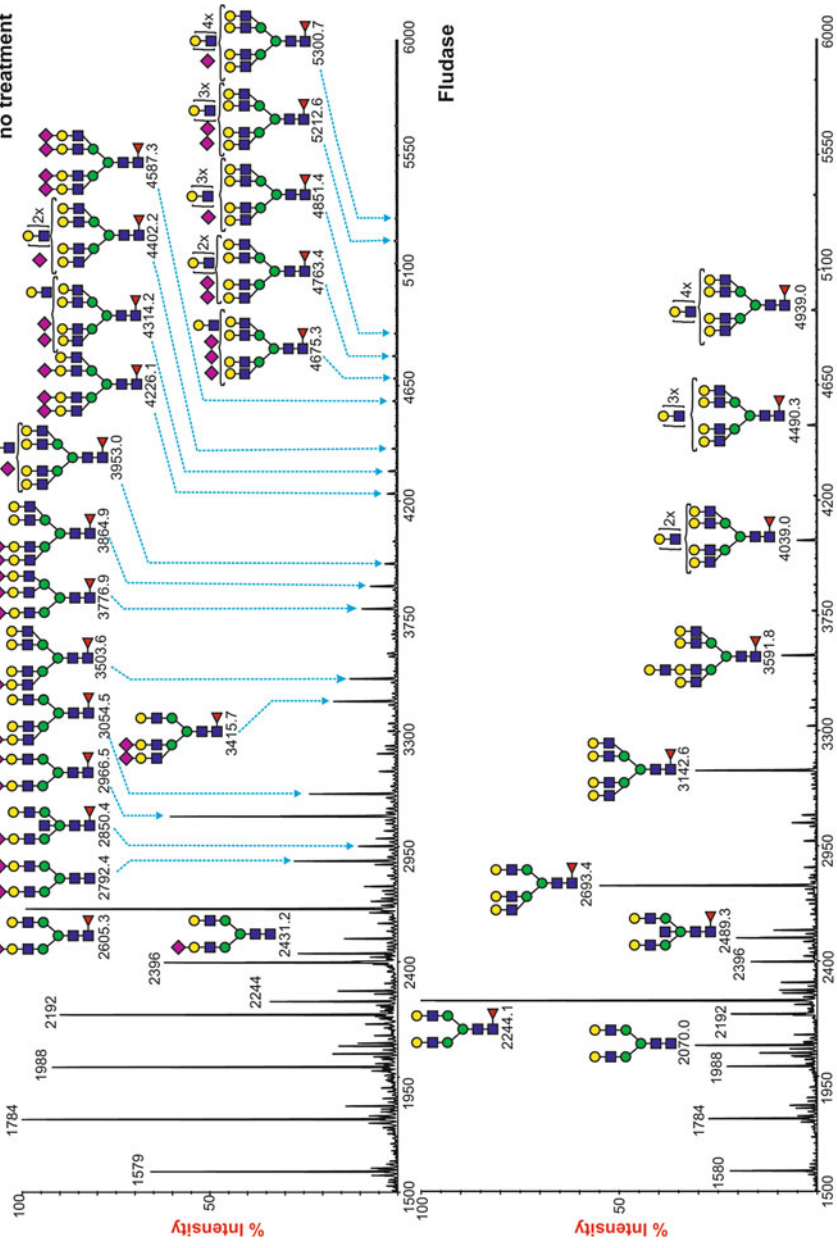

Fig. 2 MALDI-TOF mass spectra of permethylated N-glycans from human ex vivo lung biopsy material before (A) and after (B) neuraminidase (DAS181) treatment. N-glycans were released from the glycoproteins and glycopeptides by peptide:N-glycosidase F digestion and purified. The N-glycans were permethylated (methyl iodide-dimethyl sulfoxide-NaOH procedure) and purified by C_{18} Sep-Pak chromatography. The samples were analysed by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) MS to obtain molecular ion profiles of the glycans, and selected ions were further characterized by tandem MS (MS/MS) analysis [56]

Siaα2-6Gal to Siaα2-3Gal [39]. Conversely, A/Udorn/307/H2 (H3N2) did not replicate in a duck intestine, while an avian reassortment virus A/Mal/NY/6750/78 (H2N2), with seven avian virus genes and a human HA gene with a mutation at 226 and 228, did [40]. Based on these findings, it was proposed that Gln226 correlated with Siaα2-3Gal specificity in avian viruses and Leu226 with Siaα2-6Gal in human viruses of H2 [41] and H3 [39, 42] subtypes. However, these findings do not apply to the H1 [43] or H5 subtype, and human H2 and the avian H6 and H9 bound Siaα2-6 motifs even though they contained Gln226. A change from Glu190 to Asp has been considered important in changing from Siaα2-3 to Siaα2-6 binding, which did not appear in human H3 isolates until 1992, when they lost the ability to agglutinate chicken red blood cells but were still able to agglutinate human red blood cells. This presence of Glu190 may have been an artefact of passage of isolates in chicken eggs, but recent isolates do not show acquisition of Siaα2-3 binding or the Asp190Glu change when adapted to growth in eggs [28] and have also shown differences in affinity for 2–6 linked glycans from strain to strain in the past 8 years [44].

11 NMR: A Physical Approach to a Biological Situation

All the above mentioned data clearly demonstrate that our understanding on influenza virus interaction with its cellular receptor is much more complex than previously thought, and the limitations of determining the presence of α2-6 versus α2-3 linked glycans by lectin histochemistry needs to be readdressed. Among the new avenues of investigation, a recent technology based on physical measurement – nuclear magnetic resonance (NMR), has enabled a more precise interaction of the HA with the sialylated ligands down to the atomic level.

In 1938, Rabi observed for the first time that when a magnetic nucleus, such as hydrogen ^{1}H, carbon ^{13}C, nitrogen ^{15}N, was placed in a magnetic field and an electromagnetic pulse was applied, it caused the nucleus to absorb energy from the pulse and radiate this energy back out at a specific resonance frequency which depended on the environment of the atom [45]. Ever since, this phenomenon of NMR was used on routine basis for the determination of chemical structure of molecules. For example, as each ^{1}H in a molecule is in a different environment, each of them will have its own "signature." Perturbation of this environment, for example through interactions with another molecule, could be detected in strictly controlled conditions offering a new way of looking at ligand–protein interactions.

Although several NMR protocols have been created to that purpose, one extremely powerful method, called Saturation Transfer Difference NMR (STD-NMR), although known for many years, was developed by Mayer and Meyer [46] for the characterization of ligand binding to large proteins and its application for the screening of libraries of compounds.

As illustrated in Fig. 3, the principle of this method is relatively simple. In macromolecules such as proteins, all the protons are interrelated to each other

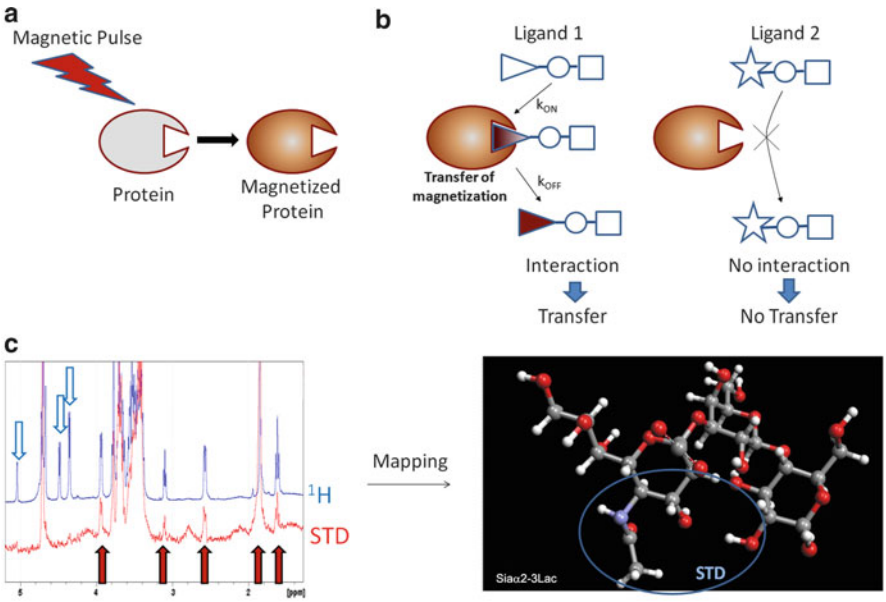

Fig. 3 Principle of interaction mapping by STD-NMR. (**a**) The protein of interest placed in a magnetic field is magnetized. (**b**) If a ligand interacts with that protein, a part of magnetization is transferred to through the interaction to the chemical group in the ligand proportionally (given the proper condition) to the strength and distance to the interaction binding site. (**c**) By comparison between the regular 1H proton spectra (that display all the different type of proton of the molecule) and the STD spectra, one can map the binding of the ligand with the protein

through a matrix of so-called dipole–dipole interactions. Therefore, if a pulse corresponding to one of the protein resonance is applied the magnetization will rapidly spread over the entire protein. As the range of resonance of the macromolecule (receptor) is much wider than of small molecules (ligands) the later one will be unaffected as long as it is in solution. Once the ligand interacts with its receptor, intermolecular transfer of magnetization occurs to the ligand with a graduation: the closer to the interaction the stronger the magnetization. This led these authors to introduce the notion of group epitope mapping [47] and can now provide access on the interaction between a substrate and its receptor with atomic resolution.

Although initially STD-NMR experiments were applied to purified proteins [48, 49] recently full viruses were used when looking at surface proteins. These guaranteed the presentation of the proteins in their native conformation. Nevertheless, this approach is impractical with pathogenic viruses for biosafety reasons. To overcome this problem while maintaining the advantages of the technique, virus-like particles (VLP) were developed, for example, for highly pathogenic avian influenza H5N1 [50].

VLPs are produced by co-transfection of plasmids coding for the core of the virion as one component, and for the surface glycoproteins that will be incorporated

on the virion surface during the budding as other components. As the viral surface glycoproteins are presented in a similar way to native virions, such chimeric pseudoviral particles can be used as mimics of virus for investigations changing the properties of the envelop proteins [51]. For example, Garcia et al. showed it could be used, after introduction of a reporter-gene, for the sero-diagnostic or sero-epidemiological investigation of outbreak of H5N1 in BSL-2 conditions instead of

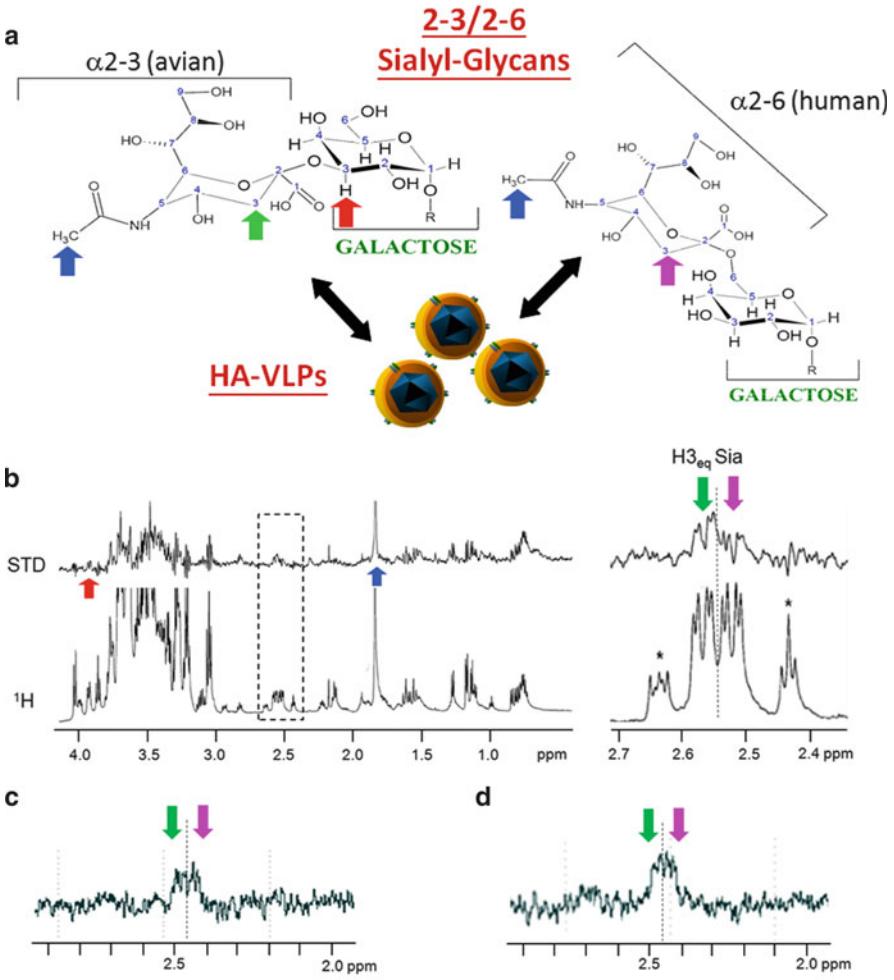

Fig. 4 Application of STD-NMR to the study of influenza receptor. (**a**) Virus-like particles bearing HA proteins (HA-VLP) were analysed by STD-NMR with a mix of Siaα2-3- or Siaα-2-6-sialyllactose (SL). These two ligands differ by the branching of second carbon of sialic acid to next sugar as reflected, among other signals, for example by the $H3_{eq}$ between 2.5 and 2.6 ppm. As expected, avian H5-VLP preferential bind for α2-3SL (**b**). We could also confirm that human seasonal H3 binds equivalently to both α2,3/2,6-SL (c) as do avian H5-VLP with double mutations Q192R/S223N in HA as described by Yamada et al. [57]

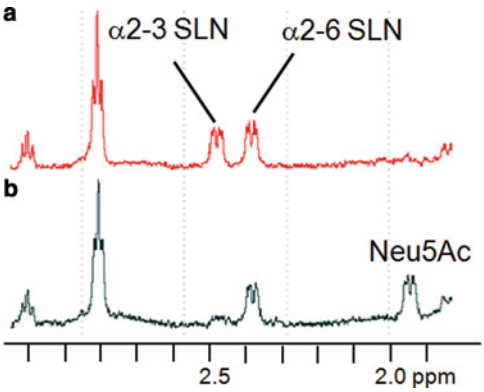

Fig. 5 Application of NMR to the study of neuraminidase sialidase activity. (**a**) Regular proton spectrum of an equimolar mixture of α2-3- or α2-6-sialyllactosamine (SLN). (**b**) After addition of virus-like particles bearing the N1 neuraminidase (N1-VLP) from avian H5N1 virus to the mixture, there is cleavage of sialic acid and the apparition of the end product Neu5Ac with a clear preference for the α2-3SLN substrate

BSL-3 [52, 53]. This is particularly important in countries or region where such high biosafety facility is not available or easily accessible. This assay could also be used in more fundamental research as, for example, to evaluate the cross-subtype neutralizing response [54]. VLP's applications are not restricted to the vial haemagglutinin as previously shown in (Fig. 4). NA-VLPs have used also to monitor the catalytic activity of the neuraminidase as illustrated by Fig. 5 for N1 from H5N1 [55].

These few examples illustrate how physical analytical technologies such as MS or NMR can bring powerful insights on the function of viral proteins and their interactions with host with an increased resolution.

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