Hydrogen/Deuterium Exchange Mass Spectrometry - A Fourth Dimension in Structural Virology?

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A review on recent applications of hydrogen/deuterium exchange in structural virology.

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Layman's Summary

Viral diseases cause a lot of harm on society. The recent Covid-19 pandemic has had enormous effects on the day to day life of many people. To combat these diseases, it is important to understand how the viruses causing these diseases function. On an elementary level, viruses are genomes that are encapsulated in a protein, and sometimes lipid, coat. Viruses are incapable of replicating on their own and need host mechanisms to do so. The infection by viruses starts at the cell membrane of the host, where it attaches and fuses with the host membrane, injecting the genome into the host cell. Once the virus is inside of the host cell, it hijacks host mechanisms to produce new proteins and the virus starts to synthesise new genomes. During the infection, the virus needs to stay hidden from the host defence mechanisms and if all of this is successful, the newly synthesised genomes and proteins will assemble into new virus particles.

The mechanisms used by viruses during infection are complex, and not all are well understood. Research in how viruses function is therefore very important. One way to get to better understand viral functions is by studying the tree dimensional structures of their proteins, as the structure of these proteins is highly correlated to the function. This can be done using high magnification and high resolution electron microscopes, for example. This review will provide an overview of recent studies that used hydrogen/deuterium exchange mass spectrometry (HDX-MS) to study the structure and mechanisms of viral proteins. Deuterium is a heavier isotope of hydrogen, and it can replace hydrogens on de protein backbone when the protein is placed in heavy water. The amount of deuterium that is taken up by the amino acids in the protein is determined by the location within the protein, as amino acids that are deep inside of the protein, or that are part of highly structured parts of the protein take up less deuterium.

This makes HDX-MS an excellent tool for researching binding of viral proteins to host proteins or antibodies, as this binding will cover-up parts of the protein, thereby decreasing deuterium uptake. It can also be used to better understand changes in protein dynamics, as parts that become less structured will start to take up more deuterium. Over the last years, HDX-MS has aided in the mapping of antibody binding sites that were not uncovered by other techniques and it has helped in better understanding how Influenza virus hemagglutinin, which is the protein that fuses the viral membrane with the host membrane, gets activated. These are some examples of how HDX-MS has aided in gaining better fundamental knowledge on viral diseases.

Abstract

Combatting viral diseases starts with a solid understanding of the viruses. Protein structures are an important part of this, as these structures are often highly correlated with protein function. Mass spectrometry can be employed to aid in the elucidation of these protein structures. This review will take a look in recent studies using hydrogen/deuterium exchange mass spectrometry (HDX-MS) in the context of structural virology. HDX-MS is a technique that can measure the amount of deuterium uptake in viral proteins under different conditions. Deuteration rates are heavily dependent on the environment of the residue, as reduction of solvent exposure and involvement in hydrogen bonding are key factors in the reduction of deuteration. This review will give examples of studies in which HDX-MS helped with the discovery of binding sites, protein conformations and allosteric effects of binding. Among others, HDX-MS helped in better understanding Influenza hemagglutinin activation, mapping of antibody epitopes on several viruses and better understanding allosteric effects in viral proteins. It will also give an overview of the general HDX-MS workflow and provide strengths and weaknesses of HDX-MS

Contents

Introduction

Infectious diseases have recently taken centre stage with the SARS-CoV-2 pandemic. Over 530 million people have caught the disease since the first case of Covid-19 was registered in December 2019 and of those, over 6.3 million people lost their lives to this disease (WHO, 2022). The efforts to slow down the spread of the virus and keep healthcare systems afloat often included heavy lockdowns, social distancing and curfews. This coronavirus is only one example of a viral outbreak with a huge societal impact. The arthropod-borne Zika virus emerged in 2015 and managed to spread to 86 countries. The Zika virus is suggested to cause microcephaly in unborn infants and there currently is no vaccine nor treatment available (WHO, 2016). Ebolavirus outbreaks in West and Central Afrika have reported case fatality rates between 25 and 90 percent (WHO, 2021). This limited number of examples illustrate that viral diseases are a continuous risk for society and it is important to develop suitable treatments for these diseases.

The first step in developing treatments against viral diseases is understanding how these viruses function. On an elementary level, most viruses are a genome packed by a coat which contains proteins and sometimes lipids. These viruses cannot reproduce on their own and need to hijack the molecular machinery of a host to replicate. Viruses carry proteins that facilitate cell entry and genes for their replication machinery (Alberts et al., 2015). Viruses have high mutation rates, which help them evade the immune system. These high mutation rates are caused by the lack of proofreading capabilities in many viral polymerases and the generally lower fidelity of these proteases (Sanjuán & Domingo-Calap, 2016). These mutations are relevant to therapeutics, as is seen with SARS-CoV-2 variants that could evade the acquired immune response from previous infections and cause reinfection (Ettaboina et al., 2021).

Structural tools to understand viral mechanisms

One important aspect in understanding viral proteins is understanding their molecular structures, as the structure of a protein is often highly correlated to its function (Hegyi & Gerstein, 1999). Protein crystallography has been a dominant technique to study protein structures for a long time, with this technique, protein crystals are grown from purified proteins under the right conditions and these crystals can be shot with high intensity X-ray beams. The crystal lattice causes the X-rays to scatter in a characteristic pattern which can be deconvoluted to reveal the three dimensional structure of the protein (Smyth & Martin, 2013). Another technique that has become increasingly popular to study protein structures is cryogenic electron microscopy (Cryo-EM), in which the short wavelengths of electrons in combination with relatively recent developments in equipment allows for sub-nanometre resolution imaging of protein samples (Egelman, 2016; Vénien-Bryan et al., 2017). The main advantage of Cryo-EM is that proteins do not need to be grown into crystals, which is often the main bottleneck in protein crystallography workflow.

Hydrogen/Deuterium Exchange Mass Spectrometry

Mass spectrometry is a technique that can aid in understanding viral structures and mechanisms. Precise mass spectrometers are able to precisely determine the masses of larger viral complexes or even in-tact viral particles, allowing researchers to determine the individual contents of these complexes (Leney & Heck, 2017). This equipment is capable of detecting subtle differences in masses of proteins, this includes the exchange of hydrogens with the deuterium isotope in the solvent. This means that proteins can be labelled in deuterated solvent and then the deuterium uptake could be measured using mass spectrometers, this is called hydrogen/deuterium exchange mass spectrometry (HDX-MS) (Englander & Kallenbach, 1983).

Within a protein, all hydrogens can theoretically be exchanged for deuterium, but only the hydrogens positioned at the backbone amide provide a favourable exchange rate that is practical for experimental design. Within these amides, the exchange rates vary between milliseconds and several hours. The rate of exchange is related to protein dynamics and structure. Dynamic parts of a protein are often involved in less hydrogen bonding and more exposed to the solvent, both of which will increase the rate of deuterium uptake. Secondary structure elements, α-helices and β-sheets have their amides involved in stable hydrogen bonds, resulting in drastically lower exchange rates in these amides (James et al., 2021).

Another factor that has a drastic impact on the exchange rate is the pH of the environment of the protein. The exchange can occur both via an acid catalysed or base catalysed pathway (Coales, Sook, et al., 2010). Temperature also has a significant effect on the exchange rate, as exchange rates increase tenfold with every 22 degree increase in temperature. On top of this, other functional groups with the protein, such as the C_2 carbon in histidine residues, also become subject to exchange at higher temperatures (Miyagi & Nakazawa, 2008). On top of pH and temperature, ionic strength, pressure, and solvent make up also play a role in determining the total exchange rate.

General HDX-MS workflow

There are a couple of factors that need to be considered when setting up an HDX-MS experiment. One of the first things that come to mind is the time scale of the exchange. Since the exchange rates can be in the millisecond range, but also in the range of hours. It is important to capture the timeframe of the relevant exchanges (Bai et al., 1993; Mirdita et al., 2021). Another important consideration is the selection of proper controls. A non-deuterated sample is often an important starting point for setting the controls in an HDX-MS experiment and sometimes it is also useful to have a maximumly deuterated control. Full deuteration can be reached when heating a sample to 75 degrees for 10 hours or by incubating a sample for 12 to 24 hours in urea or guanidine (James et al., 2021).

Once the parameters of the experiment have been set, it is time to perform the experiment. The protein gets diluted in a solution that has a high deuterated water content (>90%) and is then incubated at the determined experimental time. After the incubation, the pH is dropped to 2.5, which is the pH at which the exchange rate is at its minimum (Walters et al., 2012). This is also called the quenching condition. At this point, a race against the clock begins, as the exchange cannot be fully stopped. This means that it is possible that deuterated proteins exchange back to hydrogen. This back-exchange is important to consider when performing an HDX-MS experiment, as it can result in an overall decrease in resolution and variability within the protein, as less exposed amides are less likely to undergo back-exchange. The earlier mentioned maximally deuterated sample can be used as a measure for the total back-exchange.

Most proteins will be unstable at the low pH that is required to quench the exchange, but some proteins will need to be denatured, for example by using guanidium, before further processing. Cysteine bonds call also be problematic for further processing and these bonds might therefore need to be reduced. This can be done by adding tris carboxyethyl phosphine (TCEP) to the sample or by the use of an electrochemical reduction cell (ERC) (Kraj et al., 2013). These electrochemical reduction cell can be used online with other mass spectrometry equipment, reducing sample handling and is also faster than reduction using TCEP.

Sample digestion into peptides can be beneficial, as this will allow for the determination of deuteration levels on the peptide level, rather than on the protein level. Conventional mass spectrometry proteases, such as trypsin, are inactive at quench pH levels. Pepsin is a protease which is active at quench pH (Cravello et al., 2003).

This protease is, together with its relatives, a non-specific protease. This means that it can cleave at multiple sites, unlike trypsin. While this results in a more complex sample mixture, it also provides more overlap in sequences. This overlap data can result in higher sequence coverage. The cleaving can be performed online with other equipment for reduced sample handling and increased speed (Busby et al., 2007).

The denatured and digested proteins are put on chromatography columns, this is often performed online with desalting. In order to keep the back exchange to a minimum, the chromatography is performed close to the freezing point and often using shorter timespan regimes, all to keep the back-exchange to a minimum. Ionization is mostly performed using electrospray ionisation (ESI), matrix assisted laser desorption ionisation (MALDI) has some advantages when measuring samples in parallel. Essentially all ion sources can be optimized for HDX-MS, and are often operated at lower temperature to keep the back-exchange to a minimum (Coales, Tomasso, et al., 2010). As for mass detectors, any instrument with sufficient resolution can be used. Most studies use quadrupole time-of-flight (Q-TOF) or Fourier transform (FT)-based equipment. Although both types have their specific advantages and disadvantages. For peptide identification, the limiting factor is often the co-elution of different peptide that occur due to the shorter chromatography regimes used in HDX-MS. Ion mobility mass spectrometers also separate particles based on their shape and size, and can therefore aid in resolving otherwise overlapping peptides (Cryar et al., 2017). Orbitrap equipment that is capable of faster tandem mass spectrometry scans are also very useful for identifying peptides in these more complex samples (Burns et al., 2013).

Viral proteins are especially challenging to work with, as those proteins are often heavily glycosylated and many viral proteins are also membrane proteins. Glycosylation is an especially difficult post-translational modification (PTM) to deal with, as it is large and very heterogeneous. Glycans often result in less sequence coverage, as they interfere with digestion, chromatography, detection and identification. Prior knowledge of these PTMs is very important for their identification and can aid in improving sequence coverage (Liu et al., 2019). Proteoglycans are also relatively hydrophilic, resulting in a large shift in retention time and making it difficult to determine their elution times. Heterogeneity is again a problem here, as different glycan sidechains will elute at different times.

Sometimes, it can be better to deal with glycans before chromatography. This can be done by using samples from systems where proteins are not glycosylated or by removal of the glycan by endoglycanases. The latter requires endoglycanases that are active at quenching pH, however. An example of these enzymes are PNGase A (Jensen et al., 2016). Another endoglycanase suitable for HDX-MS workflows is PNGase H^+, which has an optimal pH of 2.6 and can be used in online setups (Guo et al., 2020).

For membrane proteins, it is important to consider the solubilization technique, as this can affect the dynamics of the protein, and thus the deuterium uptake. Lipid nano discs are an important tool for membrane proteins in HDX-MS, as they have the most resemblance of the native membrane by keeping the lipids from the membrane (Hebling et al., 2010). Lipids can then also be removed using zirconium oxide depletion, which is also possible in an online format (Anderson et al., 2018).

Recent applications of HDX-MS in the field of structural virology

This review will provide an overview of recently published studies that employed hydrogen/deuterium mass spectrometry in context of structural virology and explore what HDX-MS brings in addition to other structural techniques. There will be a main fucus on viral entry, but the review will also shed a light on viral replication, hijacking of host mechanisms and assembly of newly synthesised virions.

Viral entry and attachment

HDX-MS on Influenza virus hemagglutinin

Viral infection starts at the cell surface, were viral proteins attach to a receptor on the host cell membrane. One protein that is responsible for binding to the host cell receptor and establishing fusion between the viral and host membranes is Influenza virus hemagglutinin (HA). Influenza viruses are most responsible for the seasonal flu. HA consists out of two sub-domains, the HA1 subunit, which contains the receptor binding domain (RBD) and the HA2 subunit, which contains the fusion machinery. HA undergoes major structural changes during attachment and entry, which have been studied with HDX-MS.

The conformational changes in HA during infection were triggered by lowering the pH to 4.9 for three hours, and it was confirmed that the observed changes in deuterium uptake were similar for both virion attached HA and the soluble ectodomain of HA (Garcia et al., 2015). This observation means that the purified ectodomain of HA could be used as a suitable model for the triggering of the virion attached HA. The interesting aspect of this study is that it looked at proteins at a lower pH, which heavily impacts the exchange rates. In order to still get accurate deuteration data, the pH was brought back up to pH 7.5 during the deuterium exchange. The data from the HDX-MS correlates with structural changes observed in crystallography studies of Influenza HA.

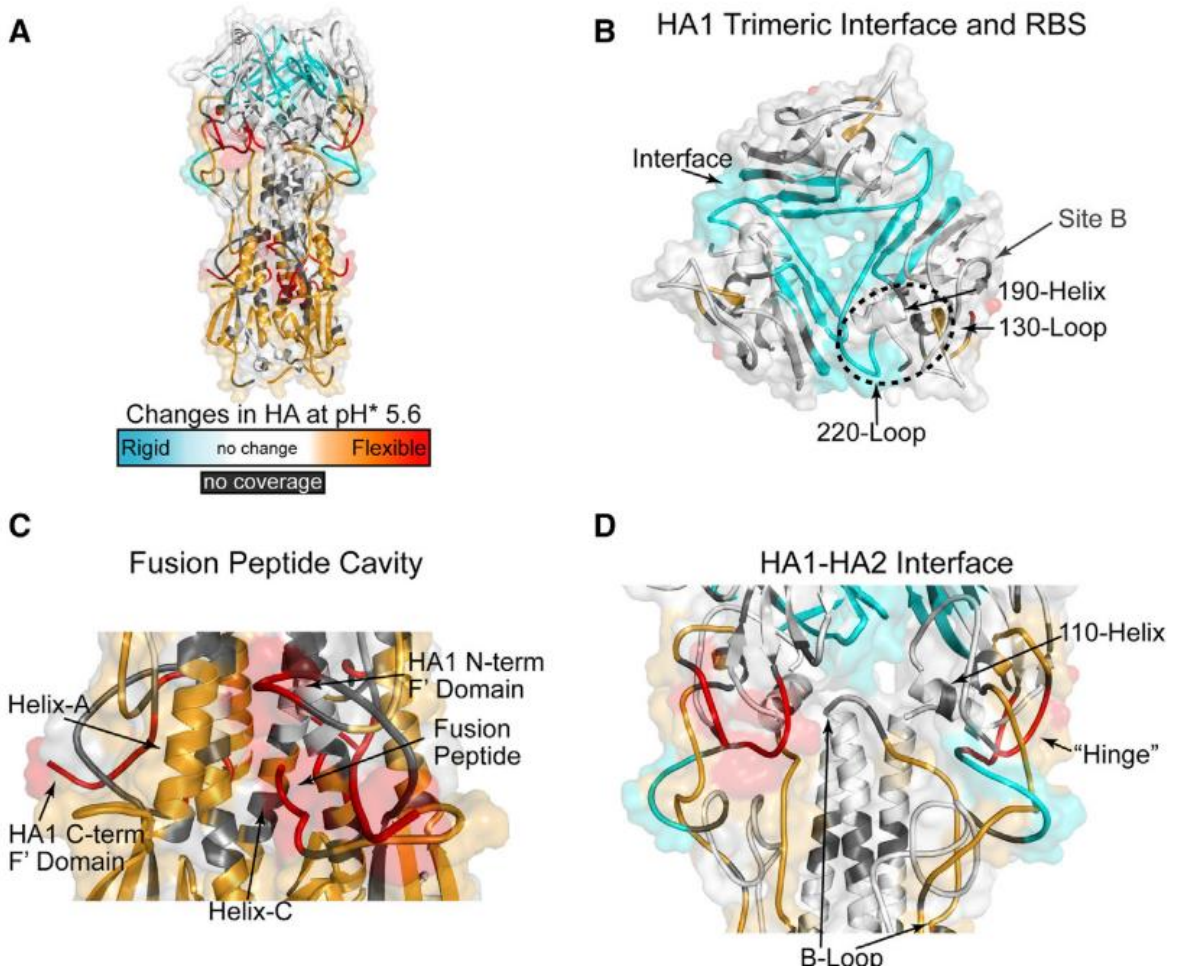

Figure 1: Changes in deuteration on the soluble hemagglutinin structure from the paper from Garcia et al., 2015. (A) Overview of deuteration over the entire hemagglutinin structure, lower deuterium uptake is indicated by blue colours, while increases in deuterations are indicated by orange and red. (B) Deuteration changes on the top view of the globular head domain with the RBD. (C) Close-up of the fusion machinery cavity. (D) Enlarged view of the HA1-HA2 interface including the hinge part of the protein

Native PAGE and small angle X-ray scattering were later used to conform that the triggering of HA starts at pH 5.5 and gets completed at pH 5.0, and follow-up experiments with HDX-MS were performed. This part of the study focused on the changes in deuteration, and thus dynamics, of the HA. In this case, it is not possible to bring the pH back up for the exchange, as this will revert subtle changes in dynamics caused by the lower pH level. For this reason, the incubation times for the exchange were adjusted to compensate for the change in the pH of the solution, as described in older studies (Bai et al., 1993). The PPPI tetrapeptide was used as in internal control for the intrinsic exchange rate of the solution (Z. Zhang et al., 2012). This peptide has a slow intrinsic exchange rate and can be used to determine the effect of the pH change and serve as a control to see if the change in incubation time compensates the change in pH level.

The results from this HDX-MS experiment show that there was a slight increase in the deuteration up until pH 5.6 and a more drastic increase at pH 5.5, indicating the trigger point of hemagglutinin. The largest increases in deuterium uptake were found to be in the fusion machinery itself, as well as downstream in the fusion helices and the hinge regions, the data from this is shown in *figure 1*. The RBD and the globular head domain showed these drastic changes in deuteration. This suggests that there are limited refolding events in the fusion domain during endosomal acidification.

This experiment was sometime later followed up by a pulse-labelling experiment using whole Influenza virions (Benhaim et al., 2020). The goal of this study was to identify intermediate states of the HA protein. In the pulse labelling, the virions would experience a short pulse at pH 8 at which the exchange could occur. This pulse will label the exposed amides on the HA within 20 milliseconds. While some changes in HA folding might be reverted by the short pulse at pH 8, this should not be a concern, as the duration is too short to fully revert these changes. Within this experiment, a fast exchanging intermediate was observed after 5 seconds at the triggering pH level. Another intermediate, with lower exchange rates in the A-helix was detected at a minute after the pH drop. The latter intermediate was less populous and could be detected by the use of bimodal fitting on the spectra, these spectra are displayed in *figure 2*.

These observations lead to the model in which an extended helix goes through the helical bundle. The previously accepted model of hemagglutinin activation was the HA1 RBD functioned as a clamp around the HA2 fusion machinery. This new model, based on HDX-MS, suggests that the HA2 subdomain undergoes the first changes. These observation were later also confirmed on HA ectodomains and Cryo-EM data on HA close to the trigger point show multiple conformations of hemagglutinin at lower pH levels, further supporting the importance of pH based triggering of HA refolding.

Hosts can defend against viral infections in a plethora of ways, generation of specific antibodies is an effective mechanism to prevent reinfection with the same virus. Avian H7N9 Influenza virus causes rare outbreaks with pandemic potential. Antibody binding was studies with serum from H7 Influenza infection survivors and the observation was made that serum from these patients was cross reactive with both type I and type II HA, in contrast to the control group that did not undergo an H7 infection (Gilchuk et al., 2021). The group II antibodies were able to bind a variety of Influenza HA types, specifically H3, H4, H14 and H15 on top of H7.

These cross-reactive antibodies were further investigated and it was found that five out of these seven antibodies compete for the binding site. HDX-MS and negative stain electron microscopy was used to determine the exact binding sites of these antibodies. All five of these competitively biding antibodies resulted in a decrease in deuteration near the RBD, which, together with the observed competition with sialic acid, suggests that these antibodies prevent H7 Influenza infection by blocking the binding to the

receptor. The non-competitive antibody binding sites were mapped close to the receptor binding site, where it likely restricts HA motion or affects the trimeric structure of the HA spike.

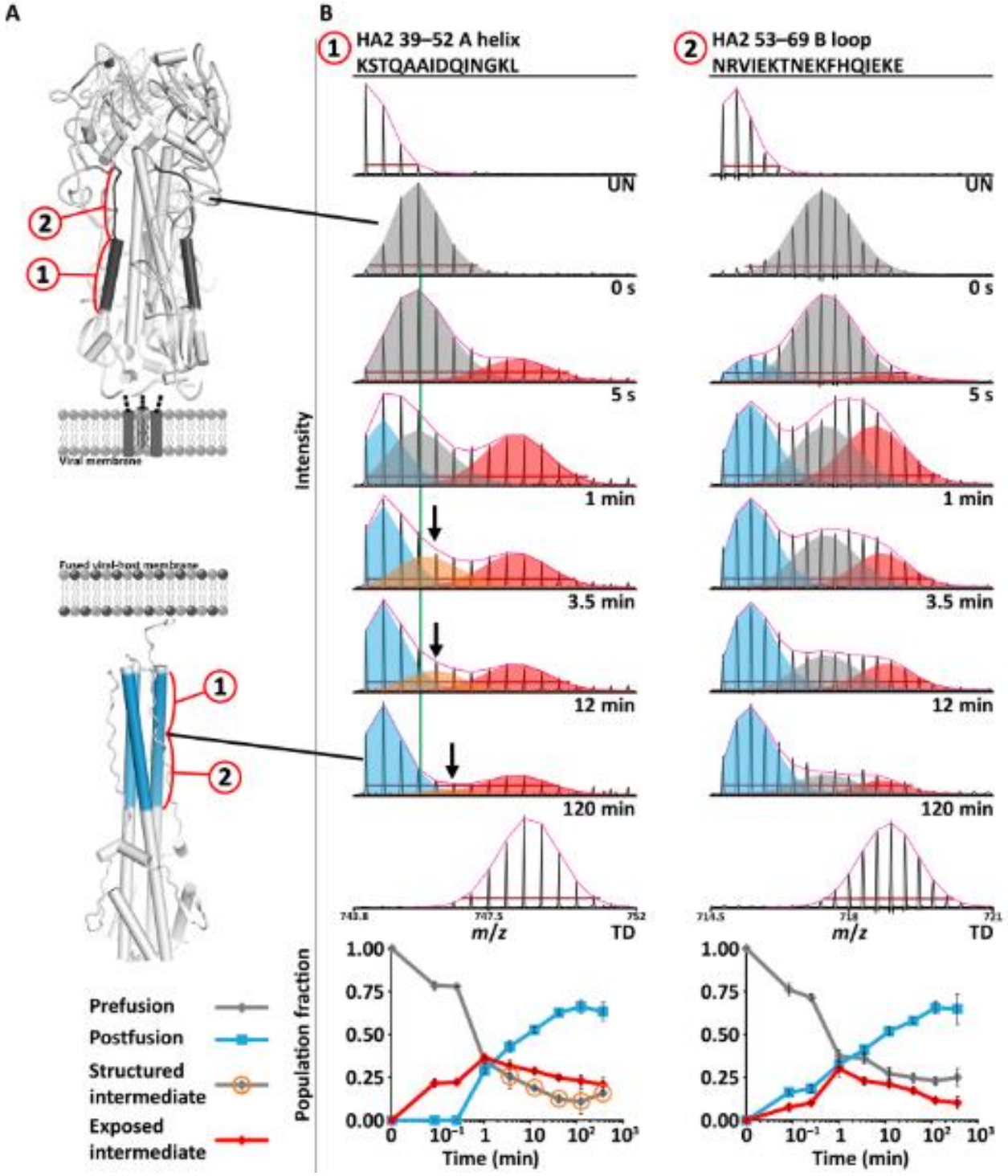

Figure 2: Pulse labelling results form the Benhaim et al., 2020 paper. (A) Overview of the hemagglutinin structure with the peptides shown in panel B indicated by numbers 1 and 2 in both the pre-fusion (top) and post-fusion (bottom) conformations. (B) Overview of the mas spectra at different time intervals with the non-deuterated (UN) and maximally deuterated (TD) controls. The first intermediate which showed higher deuteration is indicated in red, the second intermediate in orange. The post fusion conformation is indicated in blue. On the bottom of panel B, the populations of each conformation is shown on a time graph with logarithmic scale.

Determination of occluded OC43 Coronavirus antibody binding sites

As already seen with anti-Influenza H7 HA antibodies, HDX-MS is a powerful technique to study antibody epitopes due to the fact that the binding protects the epitope from exchange. The OC43 coronavirus is a one of the pathogens causing the common cold. It is well-known for its ability to evade herd immunity, but the exact mechanism to this was not well understood. A study that combined HDX-MS with Cryo-EM investigated the antibody effectiveness against the OC43 Coronavirus (Wang et al., 2022).

The study started by investigating 49 H2L2 antibodies, which are antibodies generated in mice, that target the OC43 glycoprotein S. The OC43 glycoprotein S is the spike protein, and the spike consists out of two subunits, S1, which contains the RBD, and S2, which contains the fusion peptide. The S1 subunit consists out of four domains, $S1_{A\text{-}D}$. The $S1_A$ domain contains the RBD for the 9-O-acetylated sialic acid. Binding assays with these antibodies and the spike protein revealed that almost all of the antibodies target the S1 subunit, with one exception that bound the S2 subunit. Two $S1_A$ and seven $S1_B$ targeting antibodies were humanized by cloning the variable region into human antibodies. Biolayer interferometry using these antibodies found that there are five groups of antibodies that have competition within the groups, which can be seen in *figure 3*.

Cryo-EM follow-ups on the BLI experiments show that antibody 46C12, which is a $S1_A$ targeting antibody, binds the RBD in a very similar fashion to sialic acid, supporting a competitive inhibition mechanism. This was further supported for both this antibody and the other $S1_A$ targeting antibody using ELISA. Mutagenic removal of a salt bridge effectively abolished all binding to the OC43 spike, confirming this mechanism. For the $S1_B$ targeting antibodies, it was found that there was only limited competition between groups in the BLI experiments, and the antibodies binding sites could be mapped for only some of the antibodies using Cryo-EM. This could mean that the binding sites are hidden prior to initial refolding of the spike protein.

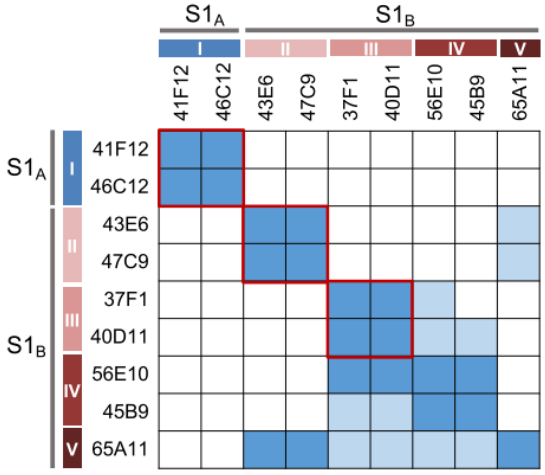

Figure 3: Overview of the competition between different antibodies as shown in the Wang et al. 2022 paper. Heavy competition (75% or greater inhibition) is shown in dark blue, and moderate competition (between 40 and 75%) in light blue. Less than 40% inhibition is not coloured.

The study used HDX-MS on monomeric S1 subunits in order to identify these occluded binding sites. Fab fragments of the antibodies were added to these S1 subunits and the deuteration was determined in presence and absence of the fabs. There was found to be no overlap in binding sites, meaning that the limited competition observed between the groups is caused by steric clashes that occur after binding. These binding sites were found to be hidden towards the inside of the trimeric spike protein and only get exposed after initial activation of the spike protein. It is therefore likely that the inhibition by the antibodies is caused by immobilizing the spike protein after activation and thereby blocking fusion of the viral membrane with the host membrane.

The paper also shed some light on the effect of mutations on antibody effectiveness, showing that mutants can abolish antibody binding completely. The anti OC43 antibody binding sites were shown to be poorly conserved in beta coronaviruses, and thus evolving quickly to avoid these neutralizing antibodies. This study shows how mass spectrometry plays a key role in uncovering

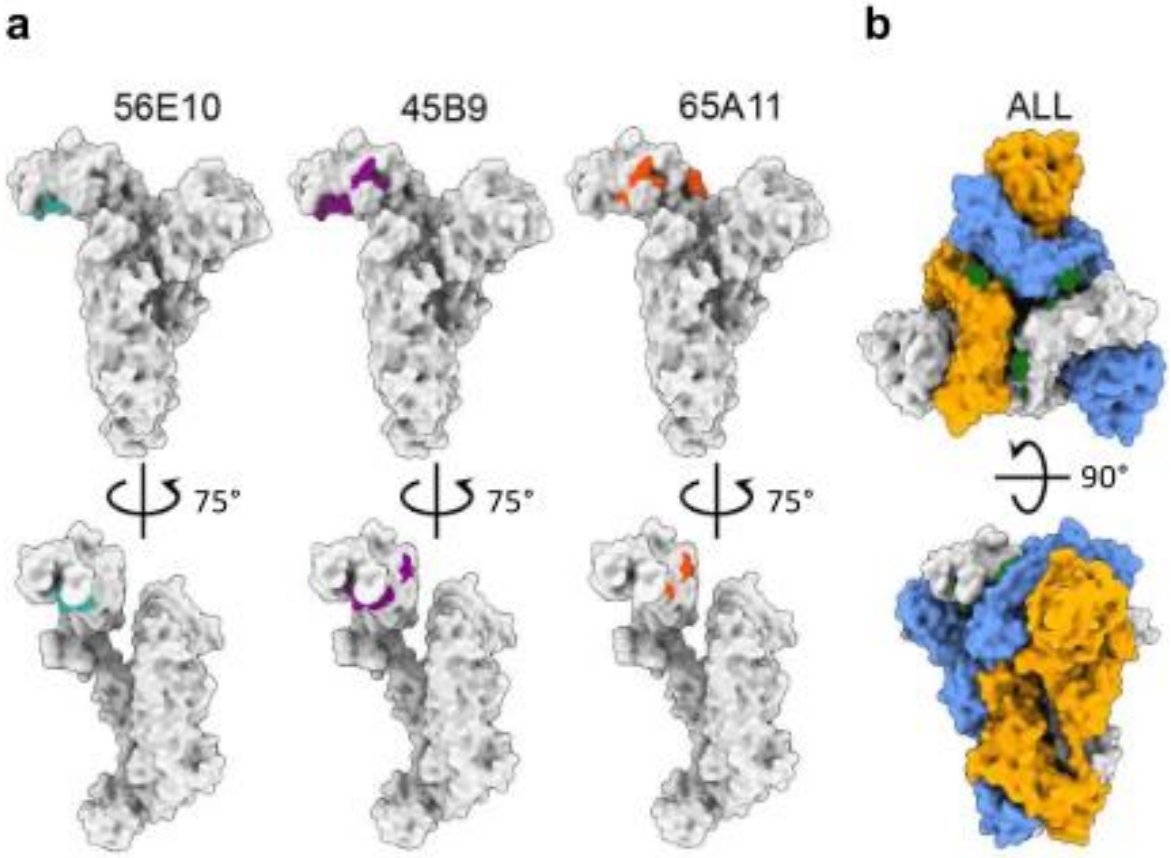

Figure 4: Overview of neutralizing epitopes which are shown in the paper from Wang et al., 2022. (A) Individual overview of each binding site on S1 monomers for each antibody. (B) The three neutralizing epitopes shown on trimeric S1. The S1 protomers are indicted in grey, yellow and blue and the neutralizing epitopes are displayed in green.

molecular details that are otherwise occluded in other structural studies, such as electron microscopy in this study. It also shows that prior information about the protein of interest is crucial, as the initial extension of the spike protein after activation is key to interpret the HDX-MS results.

Zika and Dengue virus cross-neutralizing antibody neutralizes via a different mechanism

The arthropod-borne flaviviruses are enveloped viruses that have their membranes covered by dimeric envelope proteins (protein E). This E protein arranges in rafts containing three dimers of the E protein. Two of these dimers are bordering the 5-fold and 3-fold symmetry axes, while the last dimer has a 2-fold axis in the middle, as is shown in *figure 5*. For one of these flaviviruses, Dengue virus (DENV), HDX-MS experiments have shown that the E protein changes conformation when the temperature is increased from the mosquito body temperature of 28 degrees, to the human body temperature of 37 degrees (Fibriansah et al., 2013; X. Zhang et al., 2013). In this conformation change, the E protein gets extended. It was later revealed that temperature at which this transition occurs differs between Dengue virus serotypes (Lim, Chandramohan, Lim, Bag, et al., 2017). With serotype 2 (DENV2), the transition was completed at 37 degrees, while serotype 1 (DENV1) only underwent this transition at 40 degrees.

The effect of this temperature-triggered extension on antibody effectiveness has been studied for DENV2 (Lim, Chandramohan, Lim, Crowe, et al., 2017). The binding affinity of the 2D22 fab was tested for both the mosquito and human-borne conformation. The HDX-MS results showed that the affinity for the 2D22 fab was higher for the 37 degree conformation. This is an interesting observation, as temperature is a determining factor for the hydrogen/deuterium exchange rate. The difference in temperatures

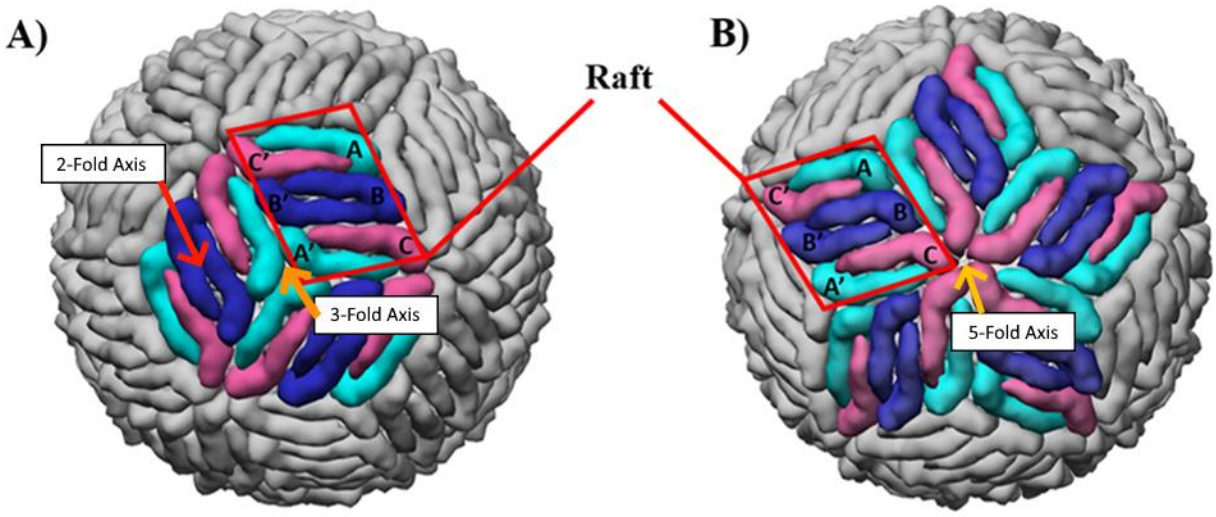

Figure 5: Dengue virus E protein raft structure adapted from Pindi et al., 2020. Centred on the three 3-fold axis (A) and 5-fold axis (B). Individual rafts are indicated by the red diamonds. The 5 and 3-fold axes are indicated by yellow and orange arrows respectively. A 2fold axis is indicated on panel A with a red arrow.

between the samples should lead to a theoretical 2.3 times higher exchange rate in the higher temperature sample. However, the difference in exchange rates is only a minor contribution and is heavily outweighed by the change in exchange rates after binding of the antibody. It was also found that the binding of the heavy chain is independent of the temperature, while the binding of the light chain is increased at the higher temperature. The model that is presented in the paper suggests that the 2D22 antibody interferes with the expansion and disassembly of the Dengue virus.

Another antibody that targets Dengue virus is the C10 antibody. This is an antibody that targets E protein dimers. The C10 antibody is cross-reactive and can also inhibit Zika virus (ZIKV) (Lim et al., 2021). Cryo-EM studies have shown that this antibody binds to the receptor binding site. These viruses are acid-activated and will aggregate at pH 5.0 and below as their membranes start fusing. This does not happen in presence of sufficient C10, however, suggesting that the C10 antibody limits fusion on top of partially blocking receptor binding. For Zika virus, it has been shown that the binding site is also has overlap with the receptor binding site.

HDX-MS experiments determined that binding of the C10 antibody caused decreased deuteration in ZIKV E protein, indicating a decrease in dynamics. This observation was also made on soluble DENV2 E protein. However, DENV2 E protein showed increased deuterium uptake outside of the RBD when C10 was added to in-tact virions. The mass spectra revealed that there were both fast and slow exchanging conformations of the DENV2 E protein in these samples. The C10 antibody itself showed a decrease in deuteration after binding.

The observed increase in deuteration in DENV2 warranted further research. Cryo-EM images of DENV2 E protein and C10 in a 3:1 and 3:2 ratio, it was found that most of the C2 binds to the E protein that is neighbouring the 5-fold and 3-fold symmetry axis. At a 3:3 ratio, it was found that 60 percent of virions showed distorted morphology and limited density was observed at the dimer forming the 2-fold axis. The hypothesis following this observation is that the dimers near the 5-fold and 3-fold symmetry axes are saturated first, as there is higher affinity for the C10 antibody at these epitopes. The last dimer is saturated only at higher C10 ratios and this binding causes distortion in the particles. This effect is likely allosteric, as binding of C10 to DENV2 had been shown to increase deuteration with HDX-MS. This was not observed for ZIKV, indicating that these viruses are inhibited by C10 via a different mechanism.

Receptor binding

Attachment to the cellular receptor is an important step in the viral life cycle. In Nipah virus there are two envelope glycoproteins that are important for the attachment and entry of the virus, these are the attachment protein (G) that binds to the receptor and then activates the fusion protein (F). The receptor of Nipah virus is ephrin B2 and HDX-MS was used to study the effect of the receptor on deuteration of these viral proteins (Wong et al., 2017). This led to the discovery of an allosteric network, in which the receptor binding domain was stabilized and another part of the G protein showed increased deuterium uptake.

HDX-MS was also employed to study the effect of ACE2 binding to the receptor binding domain (RBD) of the SARS coronavirus 2 (Raghuvamsi et al., 2021). It was found that the binding of ACE2 resulted in a decrease of deuterium uptake near the RBD, and also in the stalk region, indicating a stabilization of the stalk region. Increased deuteration was observed in peptides that flanked the S1/S2 cleavage site. This study used a cleavage inactive mutant, but still observed this increase in deuteration, indicating that this site allosterically changes conformation upon ACE2 binding.

Viral genome replication

After viral entry, one of the things that needs to be done in the infection cycle is the replication of the viral genome. Viruses often carry genes for proteins that aid with this replication. One of these proteins is flavivirus non-structural protein 5 (NS5), which is the viral polymerase. Dengue virus polymerase assembles with other components of the replication machinery. Polymerases are U-shaped, like a hand, and their N-terminal domain is called the finger region, the middle part the palm region, and the C-terminal part is the thumb region. A study on the DENV3 NS5 revealed that the thumb region of the protein had increased deuteration, which is in line with crystallographic temperature factors (Zhao et al., 2015). In this case, HDX-MS confirms the higher mobility earlier observed in x-ray crystallography data. On top of this, HDX-MS was used to map the binding site between the methyltransferase domain and the RNA dependent RNA polymerase (RdRp), which turned out to be supported mainly by charged residues on these proteins.

Chikungunya virus (CHIKV) non-structural protein 4 (nsP4) is its RdRp. The protein did not have an experimentally elucidated structure, but it was modelled using computational techniques. HDX-MS of this protein found that the finger domains showed higher deuterium uptake, which is in line with its suggested role in nucleic acid translocation (Chen et al., 2017). This study also provides information on which parts of nsP4 are most stable, which will aid with crystallization attempts of this protein.

Another protein involved in CHIKV replication is non-structural protein 2, which is the helicase. The effect of RNA and ATP analogue ADP-AlF_4 were determined using HDX-MS, and it was found that the binding of just the RNA resulted in a local and minimal decrease in the deuterium uptake (Law et al., 2019). A much larger decrease in deuteration was observed when both the ADP-AlF_4 and RNA were bound to the nsP2. A follow-up experiment which tested non-specific protease effectiveness found that the nsP2 in its RNA and ADP-AlF_4 bound state was most protected against digestion, concluding that it adopts a more stable conformation after binding to these two partners.

HDX-MS can also be used to investigate the interaction between two viral proteins, as is illustrated with Vaccinia virus DNA polymerase. There is a binding site between the A20 subunit of the processivity factor and the E9 catalytic domain that was discovered with HDX-MS (Tarbouriech et al., 2017). It was also discovered that there was a significant decrease in the deuterium uptake in both partners in this interaction, indicating that this interaction stabilized the complex. This paper also identified two key residues that drove this interaction, these were confirmed using mutagenesis.

Cellular Functions

Some of the proteins are not involved in the entry nor the replication of a virus, but carry out functions that enhance the effectivity of other proteins or hijack host pathways. These proteins can be involved in suppressing the innate immune system, or help the virus compartmentalize its replication for higher efficiency, for example. One of these proteins is Hepatitis B virus protein X (HBx), which is responsible for the downregulation of the host DNA damage response pathway (Ramakrishnan et al., 2019). HDX-MS experiments were performed with HBx, in presence and absence of a zinc-chelating agent. It was determined that the deuteration was lower when the zinc-chelating agent was present in the sample, indicating that HBx does bind zinc. Additional experiments determined that HBx does not form internal cysteine bonds, and that the cysteines are indeed involved in zinc binding.

Dengue viruses have non-structural protein 3 (NS3) which gets targeted for degradation by TRIM69 E3 ligase. An HDX-MS study helps with understanding the mechanism at which NS3 gets targeted by TRIM69 (Bagga et al., 2022). The NS3 is often found in complex with non-structural protein 2B (NS2B), but it was found that NS3 alone is sufficient to establish binding with and ubiquitinoylation TRIM69. The interaction was mapped using HDX-MS and it was found that the binding site was close to the NS3-NS2B interface and that the deuteration increased throughout the complex, indicating a destabilization after binding of TRIM69.

Human Immunodeficiency Virus 1 (HIV-1) Nef is another accessory protein. Nef has a multitude of functions, but is most known for interfering with the innate immune response. It does this by binding Src-family kinases, mainly SH2 and SH3. A larger scale study was performed on multiple members of the Nef family (Wales et al., 2016). This gives an overview of the interactions between different forms of Nef and SH2-SH3, and this study provides a framework for studying protein families with HDX-MS.

Another study by the same group on HIV-1 Nef it was found that Nef interacts with the Hck regulatory domains of SH2-SH3 (Moroco et al., 2018). The study used HDX-MS in order to determine the interaction when binding the SH2-SH3 complex and SH3 alone. It was found that binding of these partners results in a decrease in uptake in the αB helix, which is consistent with the binding site that was mapped using protein crystallography. The study also found that Nef was able to dimerize after binding the SH2-SH3 Hck regulatory domain and elimination of this dimerization by mutagenesis did not result in folding defects in Nef.

The Marburg virus is a filovirus that is closely related to the Ebola virus. Marburgvirus protein 24 (mVP24) is involved in the modification of the host antioxidative response and it was discovered that a mutation which eliminates the dimerization of mVP24 increased the affinity for the host protein Keap1 (Johnson et al., 2016). HDX-MS lead to the mapping of the dimerization site, as well as the interaction site with Keap1. The host protein Keap1 is normally regulated by Nrf2, which binds to the same site as mVP24. This means that HDX-MS helped discover the mechanism at which Marburg virus modulates the oxidative response is competition binding between its accessory protein and the host regulator.

Another Marburg virus accessory protein is mVP35, which is involved in antagonising the host innate immune response and is also a polymerase cofactor and a viral chaperone. HDX-MS was used to determine the effect of oligomerization of this protein (Bruhn et al., 2017). This protein is trimeric in Marburg virus and tetrameric in Ebola virus and it is conserved in filoviruses. The study shows that there are more residues that have low exchange rates than were previously used in crystallization experiments using mVP35. This means that the boundaries for the crystallization of this protein are likely further than those used in the x-ray crystallography experiments, indicating that HDX-MS can be employed to optimize x-ray crystallization experiments.

HDX-MS can reveal mechanisms that are not fully understood, this is seen with respiratory syncytial virus (RSV) non-structural protein 2 (Ns2). The protein was found to have a unique fold and was found to downregulate interferon response and thus helping the virus evade the native immune response (Pei et al., 2021). HDX-MS played a key role in determining the mechanism of RSV NS2, as it was determined that Ns2 has a highly dynamic N-terminal domain, which is able to bind and inhibit RIG-1 caspase, which leads to downregulation of interferon responses.

Picornaviruses are known to manipulate host PI4KIIIβ, which synthesizes PI4P. This occurs in infections with many positive sense, single stranded RNA viruses. This protein is regulated by host ACBD3, which is a binding partner for Aichi virus protein 3A. HDX-MS was used to find that the N-terminal region of PI4KIIIβ becomes distorted after binding with ACBD3 (McPhail et al., 2017). This N-terminal region is also crucial in the interaction. On top of that, the Aichi virus 3A-ACBD3 interface was also mapped using HDX-MS. The HDX-MS data on ACBD3 was sufficient to design an effective construct for x-ray crystallography, leading to the elucidation of its structure. Further studies on the PI4KIIIβ mechanism reveal that the c10orf76 protein interacts with PI4KIIIβ, upregulating PI4P levels (McPhail et al., 2020). HDX-MS was used to map this interaction and it was found that Aichi virus 3A competes with host giantin, resulting in PI4P upregulation in viral replication organelles and collapse of the Golgi.

Assembly and Budding

The final step in the viral life cycle consists out of the assembly and budding of new virions. In this phase, protein interactions are crucial, as they will keep new virions together. One protein that is important in assembly is the Marburg virus protein 40, which is its matrix protein. A study on this protein tested the deuterium exchange rates in presence or absence of specific lipids in order to better understand the viral budding process. The study found two loops containing cationic amino acids showed a decrease in exchange rate in the presence of anionic lipids and an oligomerisation interface was discovered on the N-terminal side of the Marburg virus matrix protein. Later experiments on lipid binding confirmed the putative electrostatic interaction between the matrix protein and the lipids, showing that the interaction is different from the Ebola virus matrix protein, which interacts mainly due to hydrophobic interactions (Wijesinghe et al., 2017).

HDX-MS also played a role in studying the Hepatitis B virus capsid assembly, as it was found that the woodchuck hepatitis virus has a more stable capsid than the Hepatitis B virus, as deuterium uptake was lower in the woodchuck virus (Patterson et al., 2020). This difference in exchange rate was higher than expected when looking at just sequential differences between the proteins. The study also investigated a mutation of the Hepatitis B capsid protein that is unable to assemble into a mature capsid. It was found that the dimerization was still able to take place, but the other side of the protein had increased deuteration compared to wild type, indicating that this side does no longer get stabilized. This lead to the conclusion that Hepatitis B capsid assembly requires stabilization via an allosteric network.

Some viral matrix proteins change conformation after oligomerisation, as was discovered for the Lassa virus matrix protein, protein Z (Hastie et al., 2016). HDX-MS results found that the exchange of oligomeric protein Z was substantially lower and did not show any sign of a bimodal curve, indicating that there is no interconversion between the two states. This indicates that this complex is stable once it is formed.

During assembly of the Ebolavirus nucleoprotein, it was discovered that the N-terminus was both sufficient and necessary for oligomerisation (Su et al., 2018). Specific mutations on this part of the protein were tested using HDX-MS and it was found that some of these mutations abolish all oligomerisation observable with HDX-MS. The paper suggests that oligomerisation allows for the binding of RNA to the

nucleoprotein. This was supported by FPA binding assays and HDX-MS data, which found that the deuterium uptake decreased in the RNA binding pocket in oligomeric conditions.

Discussion

Since the first experiments three decades ago, hydrogen/deuterium exchange mass spectrometry has become a powerful tool within the field of structural virology. It is very potent in the mapping of interactions, which can be applied on entire protein families, as demonstrated by the study from Wales et al., 2016. It can also aid in mapping interactions that are not found in other structural studies, as is shown by the paper on OC43 coronavirus from Wang et al., in 2022 and the paper on Dengue virus by Lim et al., from 2021.

The mapping of interactions using HDX-MS does not come without limitations. This is firstly due to the often shorter chromatography regimes that are used in order to minimize back exchange. These shorter protocols can make it more difficult to correctly identify peptides, as retention times are often not directly correlated to the usual, longer regimes. On top of that, these shorter protocols give less separation and peptides might co-elude from the column. Libraries from non-deuterated controls can aid with more robust identification and this limitation will be less severe in systems using purified proteins. Another limiting factor is the peptide fragmentation, as throughput needs to be high to keep up with the faster workflow compared to other MS techniques. This means that all ion fragmentation is often used. Orbitrap mass spectrometers that are capable of rapid MS/MS scans will also aid in better identification of peptides, as these faster scans will make it easier to fragment individual peptides.

Comparative studies, as shown on HIV-1 Nef by Wales et al., in 2016 also have some limitations, firstly, the intrinsic exchange rates, both forward and backward, can vary between members of the same protein family. Secondly, the non-specific proteases can result in a different set of peptides for each family member, this will in its turn affect the chromatographic retention time and therefore complicate identification. On top of that, the changes in retention time can result in a varying degree of back-exchange between the family members. Lastly, lower sequence overlap in more distant relatives can make direct comparison especially difficult.

More often than not, HDX-MS is used on purified protein systems, but it can also be used on whole virions as can be appreciated in the study by Benhaim et al., from 2020. This study resulted in the identification of two transient intermediate states of Influenza hemagglutinin during activation during endosomal acidification. This identification can be challenging, as intermediates can be scarcely populated, meaning that they can be less abundant in the mass spectra. On top of that, these more complex protein samples are prone to have lower sequence coverage, as there will be more noise in the spectra. In this study, there was a 93% sequence coverage for HA1, but only 34% for HA2. More robust identification, as discussed above, can aid with increasing sequence coverage. Better separation of proteins can improve identification and coverage, but will on its turn increase back-exchange. Continuous adjustments and improvements of methods and instrumentation will improve the capabilities of HDX-MS on more complex protein systems and application on *in situ* samples will likely be possible in the foreseeable future. This is important, as structural studies are often performed on purified proteins, which are stripped of their broader biological context.

Post-translational modifications are also troublesome for HDX-MS and this is especially true for viral proteins, as viral proteins are often heavily glycosylated. Software is becoming increasingly powerful in the detection of PTMs, but the high heterogeneity of glycans makes it difficult to completely identify glycosylated peptides. Chromatography with glycosylated peptides is also more challenging, as proteoglycans

are more hydrophilic than other peptides. In some cases, this will drastically change retention times, while it makes the peptide incompatible with regular chromatography set-ups in others. A way to deal with this is the removal of the glycan using endoglycanases before chromatography. This results in the glycan no longer being a factor in chromatography, nor in identification. This additional step will result in more back-exchange, however. Improvements in mass spectrometry equipment providing faster fragmentation can help with improving glycan identification. Soft fragmentation techniques, such as electron transfer dissociation are already used in glycoproteomics and are compatible with HDX-MS. This type of fragmentation is preferred for HDX-MS, as it has been shown to give less deuterium scrambling and can therefore be used to pinpoint the deuteration on the residue level, improving the spatial resolution of HDX-MS (Rand et al., 2008).

This review has demonstrated that HDX-MS can provide an additional layer of depth to structural biology. It is especially powerful in the mapping of binding epitopes, in the determination of protein conformations and in identifying allosteric effects. It can aid in the improvement of crystallography attempts, as deuteration levels help setting the borders of structure in a protein complex. On the other hand, structural information is key in the interpretation of HDX-MS data, making these techniques go hand in hand. HDX-MS can also be used to determine interaction sites that are not directly found using other structural biology techniques. Its application on larger protein systems will be an interesting and important aspect to keep track of in the upcoming years, as more biological context is often beneficial.

References

Alberts, B., Johnson, A., Lewis, J., Morgan, D., Raff, M., Roberts, K., & Walter, P. (2015). The Molecular Biology of the Cell. In *The Molecular Biology of the Cell* (6th Editio, pp. 1263–1296). Garland Science.

Anderson, K. W., Gallagher, E. S., & Hudgens, J. W. (2018). Automated Removal of Phospholipids from Membrane Proteins for H/D Exchange Mass Spectrometry Workflows. *Analytical Chemistry*, *90*(11), 6409–6412. https://doi.org/10.1021/acs.analchem.8b00429

Bagga, T., Tulsian, N. K., Mok, Y. K., Kini, R. M., & Sivaraman, J. (2022). Mapping of molecular interactions between human E3 ligase TRIM69 and Dengue virus NS3 protease using hydrogen–deuterium exchange mass spectrometry. *Cellular and Molecular Life Sciences*, *79*(5), 1–14. https://doi.org/10.1007/s00018-022-04245-x

Bai, Y., Milne, J. S., Mayne, L., & Englander, S. W. (1993). *Primiry Structure Effects on Peptide Group Hydrogen Exchange*. *1*(1993), 87332. https://doi.org/10.1002/prot.340170110.Primary

Benhaim, M. A., Mangala Prasad, V., Garcia, N. K., Guttman, M., & Lee, K. K. (2020). Structural monitoring of a transient intermediate in the hemagglutinin fusion machinery on influenza virions. *Science Advances*, *6*(18). https://doi.org/10.1126/sciadv.aaz8822

Bruhn, J. F., Kirchdoerfer, R. N., Urata, S. M., Li, S., Tickle, I. J., Bricogne, G., & Saphire, E. O. (2017). Crystal Structure of the Marburg Virus VP35 Oligomerization Domain. *Journal of Virology*, *91*(2). https://doi.org/10.1128/jvi.01085-16

Burns, K. M., Rey, M., Baker, C. A. H., & Schriemer, D. C. (2013). Platform dependencies in bottom-up hydrogen/deuterium exchange mass spectrometry. *Molecular and Cellular Proteomics*, *12*(2), 539–548. https://doi.org/10.1074/mcp.M112.023770

Busby, S. A., Chalmers, M. J., & Griffin, P. R. (2007). Improving digestion efficiency under H/D exchange conditions with activated pepsinogen coupled columns. *International Journal of Mass*

Spectrometry, *259*(1–3), 130–139. https://doi.org/10.1016/j.ijms.2006.08.006

Chen, M. W., Tan, Y. B., Zheng, J., Zhao, Y., Lim, B. T., Cornvik, T., Lescar, J., Ng, L. F. P., & Luo, D. (2017). Chikungunya virus nsP4 RNA-dependent RNA polymerase core domain displays detergent-sensitive primer extension and terminal adenylyltransferase activities. *Antiviral Research*, *143*, 38–47. https://doi.org/10.1016/j.antiviral.2017.04.001

Coales, S. J., Sook, Y. E., Lee, J. E., Ma, A., Morrow, J. A., & Hamuro, Y. (2010). Expansion of time window for mass spectrometric measurement of amide hydrogen/deuterium exchange reactions. *Rapid Communications in Mass Spectrometry*, *24*, 1457–1466. https://doi.org/10.1002/rcm

Coales, S. J., Tomasso, J. C., & Hamuro, Y. (2010). Effects of electrospray capillary temperature on amide hydrogen exchange. *Rapid Communications in Mass Spectrometry*, *24*, 1457–1466. https://doi.org/10.1002/rcm.3512

Cravello, L., Lascoux, D., & Forest, E. (2003). Use of different proteases working in acidic conditions to improve sequence coverage and resolution in hydrogen/deuterium exchange of large proteins. *Rapid Communications in Mass Spectrometry*, *17*(21), 2387–2393. https://doi.org/10.1002/rcm.1207

Cryar, A., Groves, K., & Quaglia, M. (2017). Online Hydrogen-Deuterium Exchange Traveling Wave Ion Mobility Mass Spectrometry (HDX-IM-MS): a Systematic Evaluation. *Journal of the American Society for Mass Spectrometry*, *28*(6), 1192–1202. https://doi.org/10.1007/s13361-017-1633-z

Egelman, E. H. (2016). The Current Revolution in Cryo-EM. *Biophysical Journal*, *110*(5), 1008–1012. https://doi.org/10.1016/j.bpj.2016.02.001

Englander, S. W., & Kallenbach, N. R. (1983). Hydrogen exchange and structural dynamics of proteins and nucleic acids. *Quarterly Reviews of Biophysics*, *16*(4), 521–655. https://doi.org/10.1017/S0033583500005217

Ettaboina, S. K., Nakkala, K., & Laddha, K. S. (2021). A Mini Review on SARS-COVID-19-2 Omicron Variant (B.1.1.529). *SciMedicine Journal*, *3*(4), 399–406. https://doi.org/10.28991/scimedj-2021-0304-10

Fibriansah, G., Ng, T.-S., Kostyuchenko, V. A., Lee, J., Lee, S., Wang, J., & Lok, S.-M. (2013). Structural Changes in Dengue Virus When Exposed to a Temperature of 37°C. *Journal of Virology*, *87*(13), 7585–7592. https://doi.org/10.1128/jvi.00757-13

Garcia, N. K., Guttman, M., Ebner, J. L., & Lee, K. K. (2015). Dynamic changes during acid-induced activation of influenza hemagglutinin. *Structure*, *23*(4), 665–676. https://doi.org/10.1016/j.str.2015.02.006

Gilchuk, I. M., Bangaru, S., Kose, N., Bombardi, R. G., Trivette, A., Li, S., Turner, H. L., Carnahan, R. H., Ward, A. B., & Crowe, J. E. (2021). Human antibody recognition of H7N9 influenza virus HA following natural infection. *JCI Insight*, *6*(19), 1–12. https://doi.org/10.1172/jci.insight.152403

Guo, R. R., Comamala, G., Yang, H. H., Gramlich, M., Du, Y. M., Wang, T., Zeck, A., Rand, K. D., Liu, L., & Voglmeir, J. (2020). Discovery of Highly Active Recombinant PNGase H+ Variants Through the Rational Exploration of Unstudied Acidobacterial Genomes. *Frontiers in Bioengineering and Biotechnology*, *8*(July), 1–7. https://doi.org/10.3389/fbioe.2020.00741

Hastie, K. M., Zandonatti, M., Liu, T., Li, S., Woods, V. L., & Saphire, E. O. (2016). Crystal Structure of the Oligomeric Form of Lassa Virus Matrix Protein Z. *Journal of Virology*, *90*(9), 4556–4562. https://doi.org/10.1128/jvi.02896-15

Hebling, C. M., Morgan, C. R., Stafford, D. W., Jorgenson, J. W., Rand, K. D., & Engen, J. R. (2010). Conformational analysis of membrane proteins in phospholipid bilayer nanodiscs by hydrogen exchange mass spectrometry. *Analytical Chemistry*, *82*(13), 5415–5419. https://doi.org/10.1021/ac100962c

Hegyi, H., & Gerstein, M. (1999). The relationship between protein structure and function: A comprehensive survey with application to the yeast genome. *Journal of Molecular Biology*, *288*(1), 147–164. https://doi.org/10.1006/jmbi.1999.2661

James, E. I., Murphree, T. A., Vorauer, C., Engen, J. R., & Guttman, M. (2021). Advances in Hydrogen/Deuterium Exchange Mass Spectrometry and the Pursuit of Challenging Biological Systems. *Chemical Reviews*. https://doi.org/10.1021/acs.chemrev.1c00279

Jensen, P. F., Comamala, G., Trelle, M. B., Madsen, J. B., Jørgensen, T. J. D., & Rand, K. D. (2016). Removal of n-Linked glycosylations at acidic pH by PNGase a facilitates hydrogen/deuterium exchange mass spectrometry analysis of n-linked glycoproteins. *Analytical Chemistry*, *88*(24), 12479–12488. https://doi.org/10.1021/acs.analchem.6b03951

Johnson, B., Li, J., Adhikari, J., Edwards, M. R., Zhang, H., Schwarz, T., Leung, D. W., Basler, C. F., Gross, M. L., & Amarasinghe, G. K. (2016). Dimerization Controls Marburg Virus VP24-dependent Modulation of Host Antioxidative Stress Responses. *Journal of Molecular Biology*, *428*(17), 3483–3494. https://doi.org/10.1016/j.jmb.2016.07.020

Kraj, A., Brouwer, H. J., Reinhoud, N., & Chervet, J. P. (2013). A novel electrochemical method for efficient reduction of disulfide bonds in peptides and proteins prior to MS detection. *Analytical and Bioanalytical Chemistry*, *405*(29), 9311–9320. https://doi.org/10.1007/s00216-013-7374-3

Law, Y. S., Utt, A., Tan, Y. B., Zheng, J., Wang, S., Chen, M. W., Griffin, P. R., Merits, A., & Luo, D. (2019). Structural insights into RNA recognition by the Chikungunya virus nsP2 helicase. *Proceedings of the National Academy of Sciences of the United States of America*, *116*(19), 9558–9567. https://doi.org/10.1073/pnas.1900656116

Leney, A. C., & Heck, A. J. R. (2017). Native Mass Spectrometry: What is in the Name? *Journal of the American Society for Mass Spectrometry*, *28*(1), 5–13. https://doi.org/10.1007/s13361-016-1545-3

Lim, X. X., Chandramohan, A., Lim, X. Y. E., Bag, N., Sharma, K. K., Wirawan, M., Wohland, T., Lok, S. M., & Anand, G. S. (2017). Conformational changes in intact dengue virus reveal serotype-specific expansion. *Nature Communications*, *8*, 1–13. https://doi.org/10.1038/ncomms14339

Lim, X. X., Chandramohan, A., Lim, X. Y. E., Crowe, J. E., Lok, S. M., & Anand, G. S. (2017). Epitope and Paratope Mapping Reveals Temperature-Dependent Alterations in the Dengue-Antibody Interface. *Structure*, *25*(9), 1391-1402.e3. https://doi.org/10.1016/j.str.2017.07.007

Lim, X. X., Shu, B., Zhang, S., Tan, A. W. K., Ng, T. S., Lim, X. N., Chew, V. S. Y., Shi, J., Screaton, G. R., Lok, S. M., & Anand, G. S. (2021). Human antibody C10 neutralizes by diminishing Zika but enhancing dengue virus dynamics. *Cell*, *184*(25), 6067–6080. https://doi.org/10.1016/j.cell.2021.11.009

Liu, H., Wang, D., Zhang, Q., Zhao, Y., Mamonova, T., Wang, L., Zhang, C., Li, S., Friedman, P. A., & Xiao, K. (2019). Parallel Post-Translational Modification Scanning Enhancing Hydrogen-Deuterium Exchange-Mass Spectrometry Coverage of Key

Structural Regions. *Analytical Chemistry*, *91*(11), 6976–6980. https://doi.org/10.1021/acs.analchem.9b01410

McPhail, J. A., Lyoo, H., Pemberton, J. G., Hoffmann, R. M., Elst, W., Strating, J. R., Jenkins, M. L., Stariha, J. T., Powell, C. J., Boulanger, M. J., Balla, T., Kuppeveld, F. J., & Burke, J. E. (2020). Characterization of the c10orf76-PI4KB complex and its necessity for Golgi PI4P levels and enterovirus replication. *EMBO Reports*, *21*(2), 1–17. https://doi.org/10.15252/embr.201948441

McPhail, J. A., Ottosen, E. H., Jenkins, M. L., & Burke, J. E. (2017). The Molecular Basis of Aichi Virus 3A Protein Activation of Phosphatidylinositol 4 Kinase IIIβ, PI4KB, through ACBD3. *Structure*, *25*(1), 121–131. https://doi.org/10.1016/j.str.2016.11.016

Mirdita, M., Ovchinnikov, S., & Steinegger, M. (2021). ColabFold - Making protein folding accessible to all. *BioRxiv*, 2021.08.15.456425.

Miyagi, M., & Nakazawa, T. (2008). Determination of pKa values of individual histidine residues in proteins using mass spectrometry. *Analytical Chemistry*, *80*(17), 6481–6487. https://doi.org/10.1021/ac8009643

Moroco, J. A., Alvarado, J. J., Staudt, R. P., Shi, H., Wales, T. E., Smithgall, T. E., & Engen, J. R. (2018). Remodeling of HIV-1 Nef Structure by Src-Family Kinase Binding. *Journal of Molecular Biology*, *430*(3), 310–321. https://doi.org/10.1016/j.jmb.2017.12.008

Patterson, A., Zhao, Z., Waymire, E., Zlotnick, A., & Bothner, B. (2020). Dynamics of Hepatitis B Virus Capsid Protein Dimer Regulate Assembly through an Allosteric Network. *ACS Chemical Biology*, *15*(8), 2273–2280. https://doi.org/10.1021/acschembio.0c00481

Pei, J., Wagner, N. D., Zou, A. J., Chatterjee, S., Borek, D., Cole, A. R., Kim, P. J., Basler, C. F., Otwinowski, Z., Gross, M. L., Amarasinghe, G. K., & Leung, D. W. (2021). Structural basis for IFN antagonism by human respiratory syncytial virus nonstructural protein 2. *Proceedings of the National Academy of Sciences of the United States of America*, *118*(10), 1–12. https://doi.org/10.1073/pnas.2020587118

Raghuvamsi, P. V., Tulsian, N. K., Samsudin, F., Qian, X., Purushotorman, K., Yue, G., Kozma, M. M., Hwa, W. Y., Lescar, J., Bond, P. J., Macary, P. A., & Anand, G. S. (2021). Sars-cov-2 s protein:Ace2 interaction reveals novel allosteric targets. *ELife*, *10*(M), 1–47. https://doi.org/10.7554/eLife.63646

Ramakrishnan, D., Xing, W., Beran, R. K., Chemuru, S., Rohrs, H., Niedziela-Majka, A., Marchand, B., Mehra, U., Zábranský, A., Doležal, M., Hubálek, M., Pichová, I., Gross, M. L., Kwon, H. J., & Fletcher, S. P. (2019). Hepatitis B Virus X Protein Function Requires Zinc Binding. *Journal of Virology*, *93*(16). https://doi.org/10.1128/jvi.00250-19

Rand, K. D., Adams, C. M., Zubarev, R. A., & Jørgensen, T. J. D. (2008). Electron capture dissociation proceeds with a low degree of intramolecular migration of peptide amide hydrogens. *Journal of the American Chemical Society*, *130*(4), 1341–1349. https://doi.org/10.1021/ja076448i

Sanjuán, R., & Domingo-Calap, P. (2016). Mechanisms of viral mutation. *Cellular and Molecular Life Sciences*, *73*(23), 4433–4448. https://doi.org/10.1007/s00018-016-2299-6

Smyth, M. S., & Martin, J. H. J. (2013). X-Ray Crystallography. *Brenner's Encyclopedia of Genetics: Second Edition*, 366–368. https://doi.org/10.1016/B978-0-12-374984-0.01657-0

Su, Z., Wu, C., Shi, L., Luthra, P., Pintilie, G. D., Johnson, B., Porter, J. R., Ge, P., Chen, M., Liu, G., Frederick, T. E., Binning, J. M., Bowman, G. R., Zhou, Z. H., Basler, C. F., Gross, M. L., Leung, D. W., Chiu, W., &

Amarasinghe, G. K. (2018). Electron Cryo-microscopy Structure of Ebola Virus Nucleoprotein Reveals a Mechanism for Nucleocapsid-like Assembly. *Cell*, *172*(5), 966-978.e12. https://doi.org/10.1016/j.cell.2018.02.009

Tarbouriech, N., Ducournau, C., Hutin, S., Mas, P. J., Man, P., Forest, E., Hart, D. J., Peyrefitte, C. N., Burmeister, W. P., & Iseni, F. (2017). The vaccinia virus DNA polymerase structure provides insights into the mode of processivity factor binding. *Nature Communications*, *8*(1), 1–12. https://doi.org/10.1038/s41467-017-01542-z

Vénien-Bryan, C., Li, Z., Vuillard, L., & Boutin, J. A. (2017). Cryo-electron microscopy and X-ray crystallography: Complementary approaches to structural biology and drug discovery. *Acta Crystallographica Section:F Structural Biology Communications*, *73*(4), 174–183. https://doi.org/10.1107/S2053230X17003740

Wales, T. E., Poe, J. A., Emert-Sedlak, L., Morgan, C. R., Smithgall, T. E., & Engen, J. R. (2016). Hydrogen Exchange Mass Spectrometry of Related Proteins with Divergent Sequences: A Comparative Study of HIV-1 Nef Allelic Variants. *Journal of the American Society for Mass Spectrometry*, *27*(6), 1048–1061. https://doi.org/10.1007/s13361-016-1365-5

Walters, B. T., Ricciuti, A., Mayne, L., & Englander, S. W. (2012). Minimizing back exchange in the hydrogen exchange-mass spectrometry experiment. *Journal of the American Society for Mass Spectrometry*, *23*(12), 2132–2139. https://doi.org/10.1007/s13361-012-0476-x

Wang, C., Hesketh, E. L., Shamorkina, T. M., Li, W., Franken, P. J., Drabek, D., Haperen, R. Van, Townend, S., Kuppeveld, F. J. M. Van, Grosveld, F., Ranson, N. A., Snijder, J., Groot, R. J. De, & Hurdiss, D. L. (2022). *Antigenic structure of the human coronavirus OC43 spike reveals exposed and occluded neutralizing epitopes*. 1–15. https://doi.org/10.1038/s41467-022-30658-0

WHO. (2016). *Zika virus disease outbreak 2015-2016*. https://www.who.int/emergencies/situations/zika-virus-outbreak

WHO. (2021). *Ebola virus disease*. 23 Februari 2021. https://www.who.int/news-room/fact-sheets/detail/ebola-virus-disease

WHO. (2022). *WHO Coronavirus (COVID-19) Dashboard*. https://covid19.who.int/

Wijesinghe, K. J., Urata, S., Bhattarai, N., Kooijman, E. E., Gerstman, B. S., Chapagain, P. P., Li, S., & Stahelin, R. V. (2017). Detection of lipid-induced structural changes of the Marburg virus matrix protein VP40 using hydrogen/deuterium exchange-mass spectrometry. *Journal of Biological Chemistry*, *292*(15), 6108–6122. https://doi.org/10.1074/jbc.M116.758300

Wong, J. J. W., Young, T. A., Zhang, J., Liu, S., Leser, G. P., Komives, E. A., Lamb, R. A., Zhou, Z. H., Salafsky, J., & Jardetzky, T. S. (2017). Monomeric ephrinB2 binding induces allosteric changes in Nipah virus G that precede its full activation. *Nature Communications*, *8*(1). https://doi.org/10.1038/s41467-017-00863-3

Zhang, X., Sheng, J., Plevka, P., Kuhn, R. J., Diamond, M. S., & Rossmann, M. G. (2013). Dengue structure differs at the temperatures of its human and mosquito hosts. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(17), 6795–6799. https://doi.org/10.1073/pnas.1304300110

Zhang, Z., Zhang, A., & Xiao, G. (2012). Improved protein hydrogen/deuterium exchange mass spectrometry platform with fully automated data processing. *Analytical Chemistry*, *84*(11), 4942–4949. https://doi.org/10.1021/ac300535r

Zhao, Y., Soh, T. S., Zheng, J., Chan, K. W. K., Phoo, W. W., Lee, C. C., Tay, M. Y. F., Swaminathan, K., Cornvik, T. C., Lim, S. P., Shi, P. Y., Lescar, J., Vasudevan, S. G., & Luo, D. (2015). A Crystal Structure of the Dengue Virus NS5 Protein Reveals a Novel Inter-domain Interface Essential for Protein Flexibility and Virus Replication. *PLoS Pathogens*, *11*(3), 1–27. https://doi.org/10.1371/journal.ppat.1004682