Hydrogen/Deuterium Exchange for the Analysis of Carbohydrates

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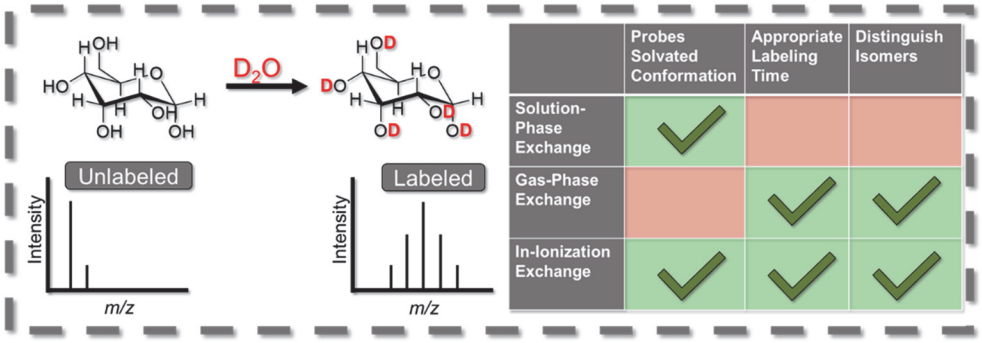
Abstract:

Carbohydrates and glycans are integral to many biological processes, including cell-cell recognition and energy storage. However, carbohydrates are often difficult to analyze due to the high degree of isomerism present. One method being developed to distinguish these isomeric species is hydrogen/deuterium exchange-mass spectrometry (HDX-MS). In HDX-MS, carbohydrates are exposed to a deuterated reagent and the functional groups with labile hydrogen atoms, including hydroxyls and amides, exchange with the 1 amu heavier isotope, deuterium. These labels can then be detected by MS, which monitors the mass increase with the addition of D-labels. The observed rate of exchange is dependent on the exchanging functional group, the accessibility of the exchanging functional group, and the presence of hydrogen bonds. Herein, we discuss how HDX has been applied in the solution-phase, gas-phase, and during MS ionization to label carbohydrates and glycans. Additionally, we compare differences in the conformations that are labeled, the labeling timeframes, and applications of each of these methods. Finally, we comment on future opportunities for development and use of HDX-MS to analyze glycans and glycoconjugates.



Keywords:

Carbohydrate, Mass spectrometry, Hydrogen/deuterium exchange, Electrospray, Ionization, Glycan

1. Introduction

Carbohydrates are critical in biology, including serving as energy storage and structural support, mediating cell-to-cell recognition and host-pathogen interactions, and indicating disease progression [1, 2]. In addition, carbohydrates function as biomarkers for disease states such as cancer, liver disease, and cardiovascular disease [3, 4]. With this wide breadth of utility, there is a need to characterize carbohydrate structures so that they can be correlated with these diverse biological functions. However, carbohydrates are difficult to analyze with traditional analytical techniques, largely due to the extent of isomerism and structural heterogeneity [5]. Carbohydrate isomers exist due to (1) monosaccharide epimers in which hydroxyls differ based on their equatorial or axial orientations (Fig. 1A), (2) the location of glycosidic bonds between

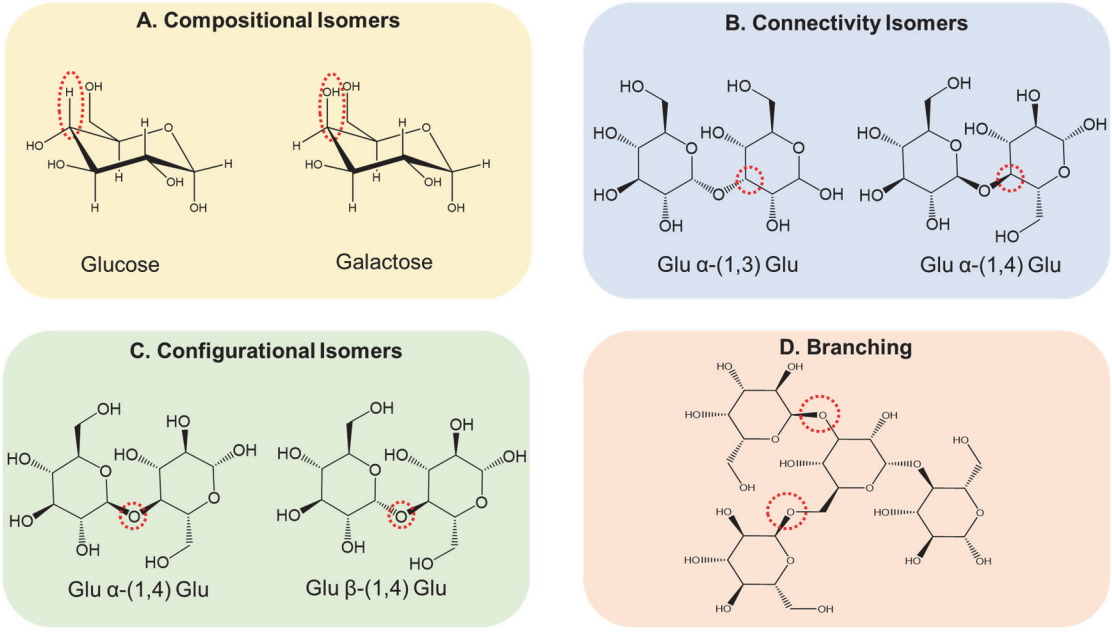


Figure 1. Isomerism within carbohydrates includes (A) composition, (B) connectivity, (C) configuration, and (D) branching.

monosaccharide units (Fig. 1B), and (3) the stereochemistry of these linkages (Fig. 1C). Glycans also form nonlinear, branched structures, further complicating analyses (Fig. 1D). Finally, compared to other biopolymers (*e.g.* DNA, RNA, proteins), glycans are synthesized by enzymatic reactions rather than from a biological template. This yields heterogeneous glycan structures based on (1) the activities and localization of enzymes and (2) the concentration of monosaccharides at the time of glycan synthesis and processing. This glycan heterogeneity increases the difficulty of acquiring sufficient quantities of pure sample for analysis [6].

With the important biological roles and complicated structures of carbohydrates, several analytical techniques have been utilized for their characterization, including lectin microarrays [7], X-ray crystallography [8], nuclear magnetic resonance spectroscopy (NMR) [9, 10], and mass spectrometry (MS) [11]. Lectin microarrays are prepared by immobilizing carbohydrate-binding proteins, called lectins, which recognize specific features in glycan structures, such as epimers, linkages, *etc*. [7, 12]. A glycan sample of interest is modified with a fluorescent label and is incubated with the lectin array. Glycans with specific motifs are captured by different lectins. These captured glycans can then be quantified using fluorimetry. The structural information determined by a lectin microarray is limited by the specific lectins present in the array [7, 12-14]. In addition, lectin arrays provide snapshots of the carbohydrate motifs present in the sample rather than yielding complete glycan structures.

Compared to lectin microarrays, X-ray crystallography, NMR, and MS yield complete biomolecular structures. Yet, X-ray crystallography, while effective for probing low-energy states of both DNA and proteins, has had limited success as an analytical tool for carbohydrates due to the innate flexibility of glycosidic bonds, which prevents crystal formation [8, 15, 16].

Both one-dimensional (1D)- and two-dimensional (2D)-NMR have been applied to characterize carbohydrate structures [8, 9, 17, 18]. 1D-NMR, which includes experiments such as proton, Carbon-13, and Nitrogen-15 NMR, can be used to determine the monomer composition of carbohydrates. Yet, due to the similar functional groups present throughout carbohydrates, 1D-NMR spectra can quickly become difficult to interpret due to overlapping peaks. 2D-NMR provides data defined by two frequency axes, examining coupling between homonuclear or heteronuclear species that is caused by atoms either being in close spatial proximity or chemically bonded. 2D-NMR can separate overlapping peaks observed in 1D-NMR and can enable characterization of the connectivity and configuration between monosaccharides [19]. Heteronuclear single-quantum correlation (HSQC) spectroscopy, a type of 2D-NMR where two different nuclei (typically a proton and another nucleus, *e.g.*, ^{13}C or ^{15}N) are detected and correlated, has been used successfully in carbohydrate analysis. Unfortunately, the use of 2D-NMR is often limited due to the low natural abundance of ^{13}C and ^{15}N in glycans, which are required for generating these spectra [20]. Additionally, both 1D- and 2D-NMR require large quantities of high purity sample, often in the range of milligrams, which can be inhibitive for the analysis of heterogeneous biological glycans.

Mass spectrometry (MS) has become a prominent technique for analysis of carbohydrates because small sample sizes can be used and the instrument can tolerate structural heterogeneity, particularly when the structures have different masses. In MS, analytes are analyzed as gaseous ions and the mass-to-charge ratio (*m/*z) is used to distinguish different analytes. Carbohydrates are regularly detected in positive-ion mode when adducted to a metal ion or in negative-ion mode when deprotonated [21]. Though MS can distinguish samples with different masses, the

technique lacks the ability to differentiate isomers with different monosaccharide epimers, connectivity, or configurations (Fig. 1).

Tandem mass spectrometry (MS/MS) involves fragmentation of gas-phase ions, followed by *m/z* detection of the resulting fragments [22-24]. These fragments can result from bond cleavage at glycosidic bonds or across monosaccharide rings, referred to as cross-ring fragments [25, 26]. Fragments at glycosidic bonds provide information about the monosaccharide sequence, while cross-ring cleavages can be utilized to define regioisomers. Thus, MS/MS can yield structural information on carbohydrate composition and linkage; however, sufficient fragmentation must occur to determine these structural features. Collision induced dissociation (CID), the most commercially available fragmentation technique, generates predominately glycosidic-bond cleavages rather than cross-ring cleavages. Furthermore, MS/MS cannot be used to assign bond stereochemistry.

Ion mobility (IM)-MS has been applied to separate isomeric glycans by regiochemistry, stereochemistry, and monosaccharide composition [27-29]. In IM-MS, separation is achieved by moving packets of ions through a cell filled with a neutral gas. When a potential is applied, ions with different three-dimensional conformations experience differences in drag force as they migrate through the neutral gas molecules, resulting in different mobilities and separation of isomers [30, 31]. Some carbohydrates and glycans have been resolved as metal-ion adducts using IM-MS [32]. However, high resolving-power ion-mobility instrumentation is often required and selecting metal adducts to resolve carbohydrate isomers is not intuitive [33]. Additionally, IM-MS provides information on the conformations of gaseous carbohydrate ions, which have different structures in the gas phase due to more intramolecular hydrogen bonding and coordination with metal ions compared to when carbohydrates are solvated [33].

Hydrogen/deuterium exchange (HDX)-MS is a technique that samples biomolecules in biologically relevant, solvated states [34]. HDX-MS has been used to analyze the structures, dynamics, and binding interactions of proteins [35, 36]. With this utility for studying proteins, there is a strong desire to adapt the technique to characterize the structures and dynamics of carbohydrates. In addition, HDX-MS functions as a labeling technique in metabolomic workflows to identify the number of labile hydrogens within a molecule, which can assist in differentiating isomers [37]. Here, we examine recent developments in using HDX-MS for the analysis of carbohydrates.

2. Hydrogen/Deuterium Exchange

H/D exchange (HDX) has been used as a labeling technique for almost 70 years, since LinderstrØm-Lang first described the technique in 1954 [38-41]. In HDX experiments, analytes are incubated in deuterated solvent for various amounts of time, allowing for deuterium in the solvent to exchange with labile hydrogens in the analyte [42-44]. Detection is required to assess the extent of labeling, with D being 1.0062 amu heavier than H [34]. HDX was first applied to proteins. Following incubation of the protein in deuterated solvent for timescales between minutes and days [34, 39, 44], the extent of labeling was analyzed by measuring the densities of labeled proteins using an oil gradient, where more exchange caused an increase in protein density [40, 41]. These gradients were later replaced by NMR because H and D have different nuclear spins. The advent and widespread accessibility of 2D-NMR allowed for time-resolved, site-specific H/D exchange information – including rate and location of exchange — to be gathered [45]. This is accomplished utilizing HSQC NMR, which enables the localization of exchange to specific sites, with hydrogen signals disappearing over time as H is exchanged with D. The site-specific localization also allows the measurement of exchange rates at each site.

However, NMR detection following HDX still requires highly concentrated, pure protein samples and as the protein size increases (greater than 25 KDa), it becomes more challenging to resolve the NMR signals for each site in the protein. In the early 1990's the advent and popularization of electrospray ionization (ESI) enabled HDX detection by MS. In comparison to NMR, MS allowed for analysis of larger analytes at lower concentrations and with lower sample purity [46-48].

During HDX labeling, hydroxyls, amines, carboxylic acids, thiols, and amides readily exchange [34]. Amides exchange on the order of milliseconds to seconds at near physiological conditions, but within a natively folded protein, they typically exchange on the order of seconds to days[49, 50]. The other listed functional groups exchange on a timescale of microseconds to milliseconds [51, 52]. Solution-phase exchange can be catalyzed by H_3O^+, OH^-, and H_2O, to a lesser extent. Therefore, the rate of exchange is pH dependent. As shown in Figure 2, the exchange rate for each functional group is minimized at a different pH due to differences in these acid- and base-catalyzed mechanisms [49, 51, 53].

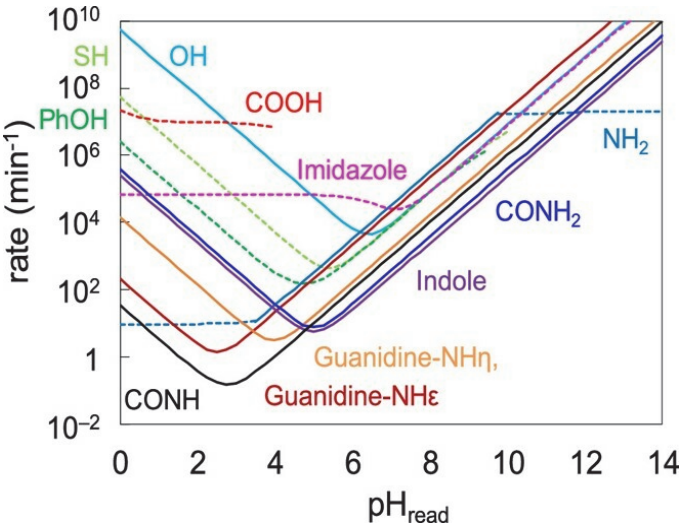


Figure 2. Rate of H/D exchange as a function of pH at 25 ºC for various functional groups. Solid lines represent observed values and dashed lines represent calculated values. Reprinted with

Solution-phase HDX experiments of proteins can have varying workflows depending on the experimental goal. Typically, the protein is labeled, the exchange reaction is quenched, D labels are detected, and the data is analyzed by comparing two different protein states (apo- vs. holo-, wildtype vs. mutant, *etc.*). The protein is first incubated in deuterated solvent for a set amount of time. During this step, the experimental conditions are kept constant, including the solution pH and temperature, both to keep the sample in a biologically relevant conformation as well as to control experimental parameters that effect HDX labeling rates [34]. After labeling, the exchange reaction is quenched. In the case of protein HDX, where amides are the labeling site of interest, the reaction can be quenched by diluting the sample with non-deuterated solvent and lowering the pH from near physiological (7.4), for labeling, to pH 2.5. To further minimize exchange of backbone amides, the temperature is also reduced to near 0 ˚C [34, 38, 49, 54-58]. The difference in exchange rates for different functional groups at pH 2.5 causes the faster exchanging functional groups to lose D labels and back exchange to H during the quench step, while the majority of the D labels at the backbone amides are maintained.

Once labeling has been achieved, MS analysis can occur. In global HDX, D-uptake for the entire protein is detected. In ‘bottom-up’ experiments, the protein is subjected to a rapid digestion using an acidic protease (*e.g.* pepsin) followed by a rapid (<10 minutes) liquid chromatography (LC) separation [59]. Analysis of peptides assists in localizing exchange to smaller regions of the protein sequence. The protein (either whole or digested) is ionized,

primarily using ESI, and the mass of the protein or peptides are detected by MS [59-61]. More D labels on the analyte increases the measured *m/z* of the ions. In addition, peptides can be further analyzed by applying electron transfer dissociation (ETD), a tandem MS (MS/MS) technique, where the positively charged analyte ion is exposed to a negatively charged radical anion (typically fluoranthene), causing the transfer of an electron to the peptide ion and fragmentation [62]. These fragment ions allow for higher resolution in localizing D-uptake along the protein sequence [63]. Following MS, the data is processed through software packages that calculate D-uptake and enable visualization of the locations of exchange along the protein. These visualizations often compare two or more protein samples to identify similarities and/or differences in D-uptake.

The primary value of HDX for protein analysis is that the technique monitors conformational dynamics, which are related to the biological structure and function of the protein [34, 42, 64, 65]. Protein secondary, tertiary, and quaternary structure result from hydrogen bonding networks. In particular, secondary structures are the result of intramolecular hydrogen bonds formed by backbone amides. These hydrogen bonds are in equilibrium between open and closed ("formed") states. Because exchange occurs at "open" hydrogen bonds that are solvent accessible, structure associated with hydrogen bonds significantly lowers the apparent rate of exchange at backbone amides. Thus, when looking at D labeling of backbone amides, areas with lower extents of D-uptake are often more structured. Therefore, when collecting HDX data over several different labeling timepoints, the conformation and dynamics of a protein can be effectively probed.

Protein HDX experiments are conducted either as continuous labeling or pulse labeling experiments. In continuous labeling, an analyte of interest is introduced to deuterated solvent and

assessed after a range of exchange times, varying from minutes to days [42, 66]. Proteins are often analyzed in two forms (apo- versus holo-, wildtype versus mutant, with versus without post-translational modifications, oligomer versus monomer, *etc.*). Therefore, differences in exchange at multiple timepoints are used to determine how the conformations and dynamics of the two protein states differ. In contrast, in pulsed labeling, one analyte undergoes a change in conformation (*e.g.* protein folding or unfolding) [42, 66, 67]. As the process is ongoing in a nondeuterated environment, aliquots of the sample are periodically taken and incubated for a consistent amount of time in deuterating solvent, followed by quench, and analysis. This method allows for a labelled "snapshot" of the analyte during a structural change to assess the dynamics and conformation of the intermediate folding states.

3. HDX for Carbohydrates

3.1. Solution-Phase HDX

Applying solution-phase HDX methods that have been used for proteins to carbohydrates and glycans is appealing because the methodology is robust and well developed. Unfortunately, hydroxyls are the primary labile functional group in carbohydrates with amides, the primary functional group detected in protein HDX experiments, present in only *N*-acetyl hexosamines (HexNAcs) and sialic acids, such as *N*-acetylneuraminic acid.

Guttman *et. al.* demonstrated that glycan moieties within glycoproteins retain D labels in the bottom-up HDX workflow, impacting the analysis of protein structure [68]. In this work, glycans were investigated using a solution-phase HDX method. Glycans were released from fetuin and RNAse B utilizing an endoglycosidase digestion and further treated with exoglycosidases to yield a diverse panel of glycans. The glycans were then isolated and incubated in 96% D_2O for multiple hours before being quenched at ~0 ˚C by 100-fold dilution into aqueous 0.2% formic

acid and immediately injected into the MS for analysis over a 30 minute time course to monitor the loss of D labels. Figure 3A shows the mass shift as a function of time for an *N*-linked glycan core structure, a trisialylated triantennary *N*-linked glycan, and an asialylated triantennary *N*-linked glycan. The *N*-linked glycan core structure had a lower initial mass shift due to the reduced number of HexNAcs, and thus acetamides, compared to the other two structures. The trisialylated triantennary and asialylated triantennary structures had similar mass shifts at the earliest time points and the same numbers of HexNAcs in the structures; though the trisialylated structure had three additional acetamido groups due to the presence of the sialic acids. The mass loss over time for all three structures illustrates the rate of back exchange as D labels are exchanged for H in quench conditions. The trisialylated structure loses D labels at the fastest rate, as indicated by the steepest slope in Figure 3A.

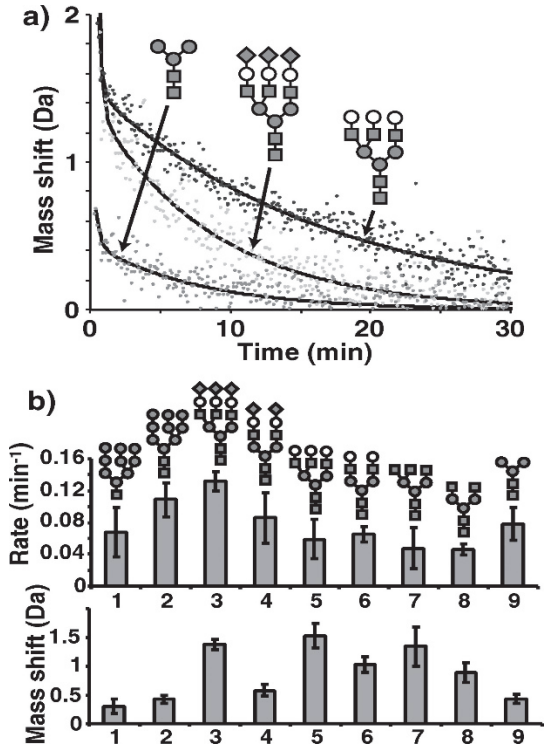


Figure 3. Exchange rates and mass shifts for various glycan chains. (A) Mass shifts as a function of time. (B) Exchange rates (top) and mass shifts (bottom) for various glycans. Grey and white

circles represent mannose and galactose, respectively, squares represent *N*-acetyl glucosamine (GlcNAc), and diamonds represent sialic acid (Neu5Ac). Reprinted with permission from Guttman, M. *et. al.* Tracking Hydrogen/Deuterium Exchange at Glycan Sites in Glycoproteins by Mass Spectrometry *Anal. Chem.* 2011, 83, 7492-7499. Copyright 2011 American Chemical Society.

It was shown that deuterium labels were retained in a variety of glycans, including high mannose, complex, and sialylated structures. Figure 3B (bottom) shows the mass shift associated with exchange for these different glycan structures. For the structure with a single GlcNAc, a mass shift of approximately 0.3 Da was measured. This is significantly less than the 1.0 Da mass shift expected when labeling a structure with a single acetamido group. This mass shift also shows that D is not retained in the multiple hydroxyls throughout the glycan. As the number of GlcNAcs and sialic acids in the glycan structures increase, the mass shift increases (Fig. 3B bottom), though the average uptake is always less than 1.0 Da for each additional acetamido group. In addition, Figure 3B (top) shows that glycans with different levels of sialylation and branching have exchange rates between 0.06 min^{-1} and 0.12 min^{-1} with the sialylated structures having faster rates of exchange then their asialylated counterparts. Compared to backbone amides of peptides, these rates of exchange are 10-fold faster.

This work is significant in showing that D labels can be partially retained within glycans following solution-phase HDX experiments. This exchange must be accounted for during HDX to prevent artifacts when analyzing protein structure and dynamics. Additionally, because solution-phase HDX experiments for proteins have been optimized to label backbone amides to retain D labels during quench and sample work up, the rapid rate of exchange for hydroxyls prevents the use of this workflow for labeling and detecting carbohydrate hydroxyls. Work has

been done to develop a short reaction time mixing apparatus for solution-phase labeling directly prior to ionization for MS [69-72], but these apparatus typically function on a microliters-per-minute flow rate that causes solvent vapor build up in the ionization source and non-solution phase (in-ionization) HDX [73, 74].

The HDX trends for glycans were also observed for glycopeptides. For example, Guttman *et. al.* generated samples by incubating fetuin and alpha-1-acid glycoprotein (AGP) in D_2O, followed by pepsin digestion, and LC separation of the peptides [68]. The results suggested that glycopeptides retained deuterium, likely at acetamido groups within the core of the glycan structure as well as along the antennae, because the D-uptake was higher than that of the peptides without glycans. Huang *et. al.* confirmed that HexNAcs retained D-labels by incorporating ETD into the bottom-up, solution-phase HDX workflow [75]. ETD was used to identify sites of D-uptake in glycopeptides by fragmenting along the peptide backbone [75]. ETD was used because (1) the method preferentially fragments along the backbone of the peptide leaving the glycan moiety intact [60] and (2) electron-based fragmentation minimizes scrambling, or the redistribution of H and D in gas-phase ions [16]. HDX experiments were performed to examine how terminal sialylation of glycans affected the biophysical properties of AGP. Using ETD, amino acid specific D-uptake was resolved, including at the glycosylated asparagine. This allowed for the total deuterium contribution of the glycan to be isolated from any D-labels along the peptide. Glycan deuteration was confirmed to be present in both sialylated and asialylated glycopeptide fragments, meaning that for these experiments, the glycan acetamido contribution to D-uptake must be taken into consideration to prevent these labels from contributing to the analysis of the peptide or protein D-uptake and the associated biological interpretation of the results. Due to both the glycans and the peptide backbone retaining D labels, peptide sequences

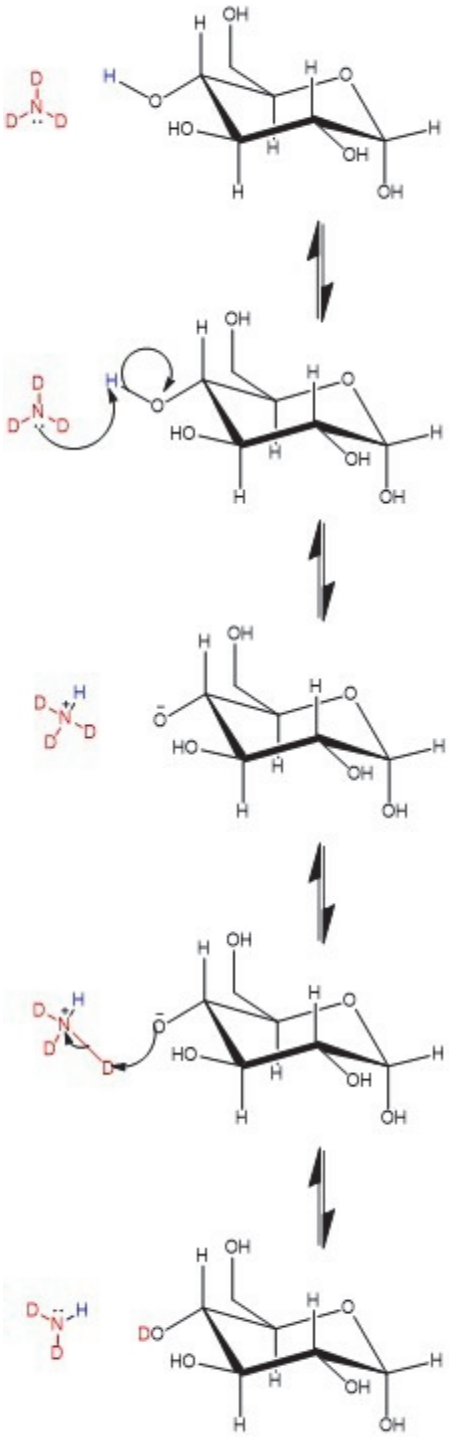
with higher D-uptake could be indicative of the glycan composition rather than the peptide's native structure and solvent accessibility. The presence of heterogeneous glycoforms could also result in variability in D-uptake within a glycopeptide, depending on the glycan present.

These studies – along with other works investigating the labeling of a trimmed *N*-glycan and a model oligosaccharide – show that some level of hydrogen exchange can be measured for acetamido groups in carbohydrates following solution-phase labeling [76, 77]. However, the technique is unable to provide detailed structural information due to the limited number of acetamido functional groups in glycans, which are only present in a few biological monosaccharides. Additionally, the rapid rate of exchange for hydroxyls, the predominate labile functional group in glycans, makes it difficult to measure and compare exchange between different carbohydrates due to the inherent back exchange.

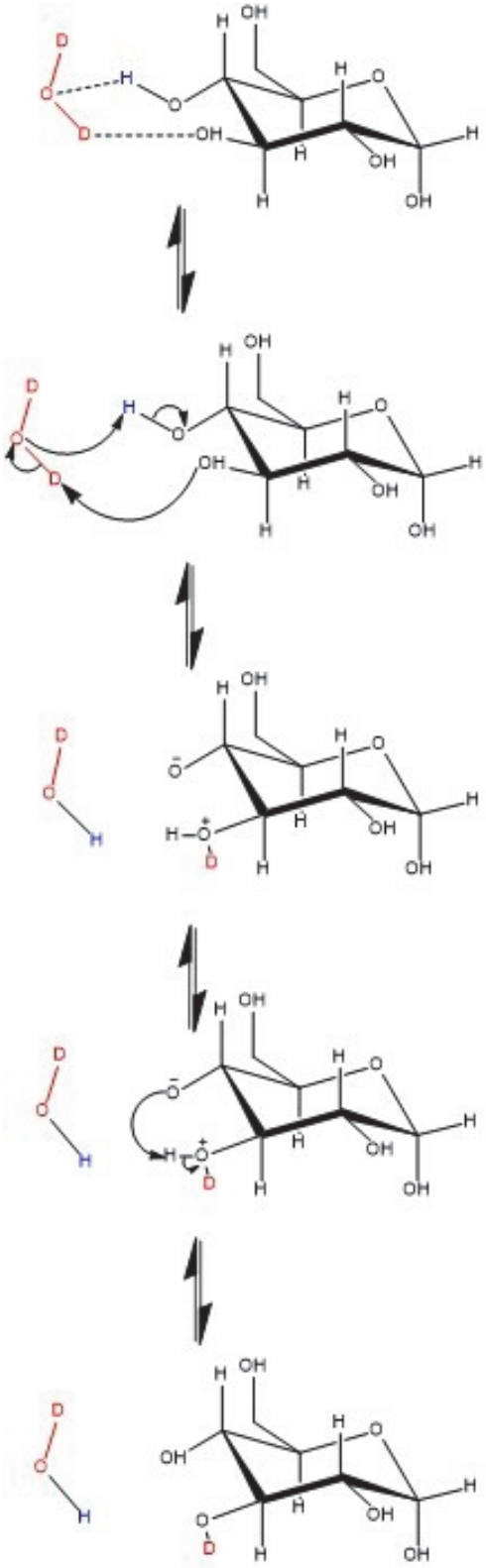
Another use of solution-phase HDX is the analysis of carbohydrate fragmentation pathways in MS/MS [78, 79]. This is accomplished by incubating the carbohydrate in deauterated solvent (*e.g.,* D_2O or deuterated methanol, MeOD) to label all the hydroxyls and spraying the deuterated solvent directly into the MS, minimizing the chance for back exchange. This labeling enables distinction between deuterated hydroxyls and C_α protons. By analyzing the D-uptake in each fragment following tandem MS, mechanistic insight can be attained. By understanding the mechanisms and pathways involved in fragmentation, future fragmentation spectra can be predicted for carbohydrates and glycans. Using deuterium labeling, sodium-adducted carbohydrates were observed to form cross-ring cleavages during CID by a retro-aldol mechanism and lithium-adducted carbohydrates did not experience the loss of C_α protons during fragmentation [79, 80]. Thus, solution-phase HDX can be utilized to generate carbohydrate ions with fully labeled hydroxyls, allowing for mechanistic insight in fragmentation pathways.

3.2. Gas-Phase HDX

Gas-phase HDX differs from solution-phase labeling in several ways, including the driving factors leading to exchange, the chemical mechanisms of exchange, and the potential structural changes associated with the analyte being in the gas phase during labeling. Exchange occurs in the mass spectrometer as a gaseous ion interacts with a deuterating reagent [61, 81-83], with ND_3 and D_2O being the most common deuterating reagents [83]. For gas-phase labeling to occur, analyte ions must collide with the deuterating reagent. Ions are directed through the instrument based on electric or magnetic fields, while gaseous deuterating reagents can be introduced in regions through which the analyte ion travels, such as a drift tube or transfer guide, or regions where the analyte ion can be trapped with the deuterating reagent, such as an ion trap or ion cyclotron resonance cell [74, 82, 84, 85]. The number density of both the analyte ion and deuterating reagent plays a role in the exchange rate because more molecules increase the probability that a collision will occur with the correct molecular orientation to initiate reaction. The probability of collisions can also be increased in trapping cells by increasing the reaction time.



Scheme 1. Salt-bridge mechanism for deuterium exchange.



Scheme 2. Relay mechanism for deuterium exchange.

Gas-phase basicity (GPB) is the Gibb's free energy associated with deprotonation in the gas phase. The GPB of the deuterating reagent is important because HDX is unlikely to occur if the difference in GPB between the substrate and deuterating reagent is greater than 85 kJ/mol [86]. Due to ND_3 having a higher GPB than D_2O, these two reagents enable different mechanisms and rates of exchange in the gas phase [74, 83]. ND_3, as a more basic deuterium donor, typically follows a mechanism where a proton is initially transferred from the substrate to the deuterating reagent (Scheme 1), while less basic deuterium donors, such as D_2O, undergo a mechanism where two functional groups from the substrate interact with the deuterating reagent, leading to a simultaneous transfer of two protons between substrate and deuterating reagent (Scheme 2) [83, 87, 88]. In the case of carbohydrates, with multiple hydroxyls that can readily form intramolecular hydrogen bonds, a one-dimensional Grotthuss mechanism is proposed for less basic deuterating reagents [86]. In this model, the deuterium from the deuterating reagent is donated to one hydroxyl of the substrate that is connected to the hydrogen-bonded system and through a series of proton-hopping events, a proton from a different hydroxyl is transferred back to the reagent [86].

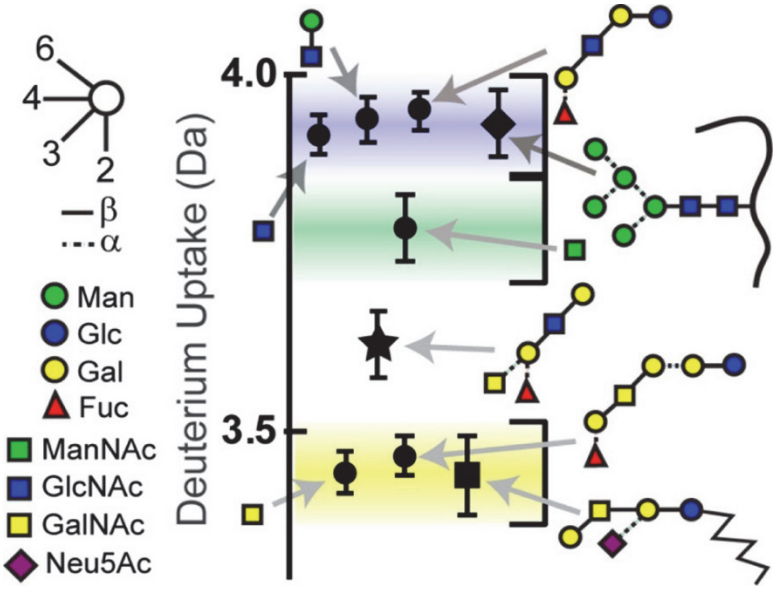


Figure 4. D-uptake after labeling for 9.9 ms with ND_3 for HexNAc oxonium ions (*m/z* 204) fragmented from various precursor oligosaccharides, a glycopeptide (smooth line) and a glycolipid (jagged line) with symbol and linkage legend on left. D-uptake of the 204 *m/z* fragment can distinguish three isomeric HexNAcs. Reprinted with permission from Uppal, S. S. et. al. Gas-Phase Hydrogen/Deuterium Exchange for Distinguishing Isomeric Carbohydrate Ion *Anal. Chem.* 2017, 89, 4737-4742. Copyright 2017 American Chemical Society.

Differences in gas-phase HDX enabled three isomeric monosaccharides to be distinguished [82]. Gas-phase HDX of carbohydrates has primarily labeled oxonium ions, which are generated by utilizing low-energy CID to fragment protonated precursor ions, causing the loss of water. Using this protocol, standards of *N*-acetyl glucosamine (GlcNAc), *N*-acetyl galactosamine (GalNAc), and *N*-acetyl mannosamine (ManNAc) were fragmented to form oxonium ions. Then, these ions were passed through an ion mobility cell in which the inert gas was replaced with a deuterating reagent (ND_3) to initiate gas-phase HDX. Figure 4 shows the D-labeling of oxonium ions from three HexNAc monosaccharides generated from different parent molecules. Oxonium ions can also be formed from CID fragmentation of larger polysaccharides. Therefore, Figure 4 compares the D-uptake for HexNAc oxonium ions generated from either monosaccharide standards or fragments of larger polysaccharides and glycoconjugates. Circle, square, diamond, and star markers indicate the HexNAc oxonium ion was isolated from a saccharide system with a single HexNAc, glycolipid, glycopeptide, or polysaccharide containing multiple HexNAcs, respectively. Each HexNAc oxonium ion had different levels of HDX, with GlcNAc and GalNAc having the highest and lowest D-uptake, respectively. These results illustrate that each oxonium HexNAc had a similar level of D-uptake, regardless of the parent

ion. This is seen by comparing the D-uptake for the GlcNAc oxonium ions generated from standards versus ions formed following CID of polysaccharides, a glycopeptide, and a glycolipid. This ability to differentiate monosaccharide units from larger polysaccharides is a novel and important tool to enable full carbohydrate characterization via MS. In addition to these monosaccharide analyses, HDX of oxonium ions of disaccharides and trisaccharides were utilized to distinguish compositional and connective isomer [82, 89, 90]. These studies highlight the potential of gas-phase HDX as a tool to structurally characterize carbohydrates in workflows with MS/MS.

Gas-phase HDX shows potential for structural analysis of carbohydrates; however, there are a number of limitations to the technique. For example, these gas-phase HDX experiments require labeling to occur after CID fragmentation to form oxonium ions but also to minimize scrambling. In CID, a precursor ion collides repeatedly with neutral molecules, causing a slow increase in the internal energy of the precursor ion [91-93]. This increase in energy can provide the driving force for proton and deuteron rearrangement, referred to as scrambling, which changes the observed labeled sites. Thus, CID fragmentation cannot be used following gas-phase HDX to identify specific sites of D labels. In addition, Figure 4 shows that when two different HexNAc monosaccharides, GalNAc and GlcNAc, are present in the precursor carbohydrate, the measured D-uptake is between that of each HexNAc oxonium ion (star icon). Thus, the monosaccharides could not be distinguished. In addition, gas-phase HDX experiments are limited to the carbohydrate conformations that exist in the gas phase. Previous work has shown that compared to solvated carbohydrates, which form intermolecular hydrogen bonds with solvent, gaseous carbohydrates form more intramolecular hydrogen bonds [94, 95]. Furthermore, the hydroxyls of carbohydrates readily coordinate with metal ions. Thus, as carbohydrates

transition to the gas phase, this coordination can limit the inherent flexibility of the molecules [95]. These metal-adducted carbohydrates tend to have higher ion intensities than protonated carbohydrates following ESI and previous work from our lab has shown that these gas-phase, metal-adducted, small carbohydrates do not exchange in the gas phase under typical gas-phase HDX timeframes; thus, preventing the use of gas-phase HDX for the analysis of the more dominate ions formed by ESI [73, 74]. Oganesyan *et. al.* have recently demonstrated that gas-phase HDX can be observed to a minimal extent with larger carbohydrate systems coordinated to a metal ion [96]. These singly charged metal-carbohydrate complexes composed of sodium ions and *N*-linked glycans were exposed to deuterating reagent in the gas phase for 10 seconds to allow excess time for labeling, yet only achieved ~ 25% D-uptake. This 10 second labeling is compared to 10 milliseconds used for full labeling of protonated HexNAcs (Fig. 4). This indicates that only portions of the glycan are able to undergo exchange. Analysis of these labeled complexes indicated that within the gas phase there were several conformations present for gaseous carbohydrate-metal ions, but they are difficult to distinguish using only gas-phase HDX [96].

3.3. In-Ionization HDX

3.3.1. Instrumentation

During in-ionization HDX, exchange occurs simultaneously with analyte desolvation and/or ionization. In-ionization HDX has been performed predominately with electrospray ionization (ESI) [74, 97, 98], but is also being explored with capillary vibrating sharp-edge spray ionization (cVSSI) [99-101] and conductive polymer spray ionization (CPSI) [102], a form of desorptive electrospray ionization (DESI). In these techniques, it is possible for exchange to occur both while the analyte is solvated in a droplet and while the analyte is a gaseous ion prior to entering

the vacuum regions of the instrument [74, 97]. These ionization processes are typically on the order of microseconds to 10's of milliseconds, which is consistent with the exchange rate of hydroxyls, amines, thiols, and carboxylic acids [51, 103].

ESI is one of the primary methods for ionization of carbohydrates [21, 104]. During ESI, the analyte is dissolved in solvent (typically water with methanol or acetonitrile) and is pushed through a capillary. A potential is applied to the capillary, resulting in the formation of a Taylor cone that ejects small, charged droplets that travel towards the inlet of the MS. As the droplets migrate to the MS inlet, solvent evaporates, decreasing the droplet volume and concentrating the charge in the droplet. At the Rayleigh limit – where the charge density overcomes the solvent surface tension – the droplets undergo coulombic fission, ejecting charge in the form of smaller droplets. Solvent evaporation and coulombic fission repeat until the late stages of ESI, when analytes form gaseous ions by one of multiple presumed mechanisms. ESI has become a valuable ionization technique due to its ability to ionize biomolecules and its soft nature, preventing fragmentation during ionization.

Molecular dynamics simulations have been used to probe the ionization mechanisms of a model trisaccharide and the resulting gas-phase structures of the carbohydrate-metal adducts. These simulations showed that carbohydrates ionize in ESI by the charged residue model (CRM) [95]. CRM involves the analyte being retained in ESI droplets until the solvent fully evaporates, causing residual charges to be deposited on the analyte. In positive-ion mode, metal ions, such as Na^+, adduct to carbohydrates at the end of the ESI process, which allow for detection of the carbohydrate by increasing signal intensity [95]. Analysis of the dihedral angle probability distributions for a sodium-adducted, gaseous trisaccharide shows that the conformational freedom is restricted compared to a solvated trisaccharide, which forms hydrogen bonds with the

surrounding solvent [95]. Gas-phase HDX experiments revealed that exchange was not observed for metal-adducted melezitose; therefore, exchange occurs while the carbohydrate is solvated in the ESI droplets [74]. By only exchanging while solvated, in-ionization HDX of carbohydrates allows for the labeling, detection, and analysis of solvated conformations.

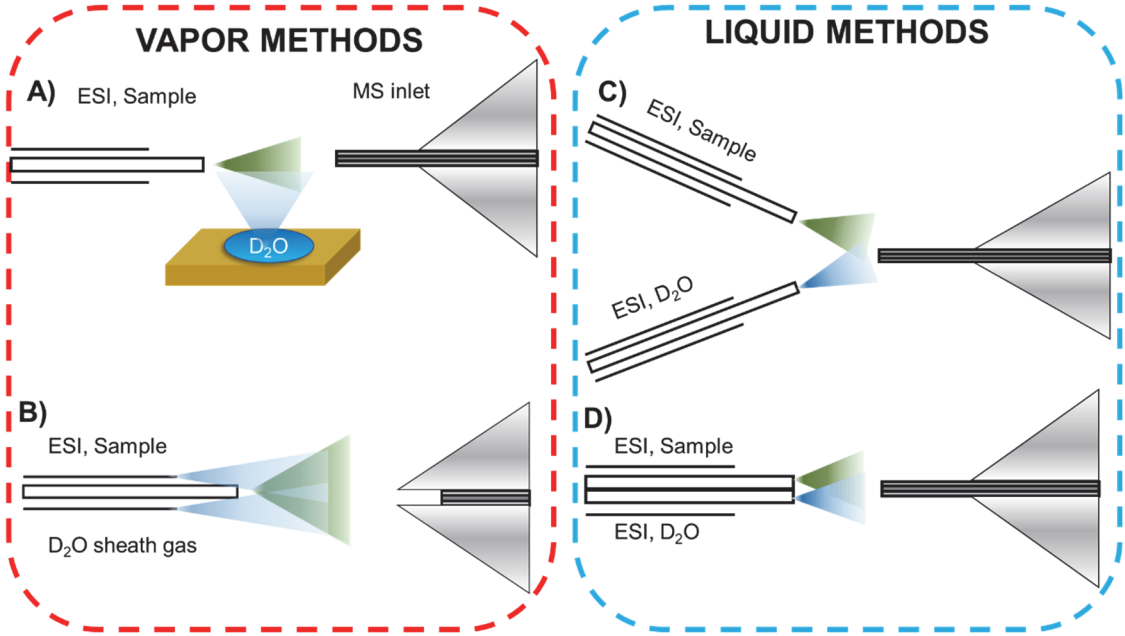


Figure 5. Experimental setups for in-ESI H/D exchange. (A) Free droplet, (B) gaseous vapor, (C) dual spray, and (D) theta-tip methods. All apparatuses can work in the ionization source chamber. Adapted with permission from Kostyukevich, Y. *et. al.* Hydrogen/Deuterium Exchange in Mass Spectrometry *Mass Spectrom. Rev.* 2018, 37, 811-853. Permission conveyed through Copyright Clearance Center, Inc.

Several iterations of in-ionization HDX-MS have been described. For ESI, these HDX methods expose the analyte to deuterating reagent after formation of the Taylor cone [73, 74]. In-ESI HDX has been performed by mixing analyte in ESI droplets with either (1) a deuterating vapor or (2) liquid droplets containing a deuterated solvent (Fig. 5).

Deuterated vapor can be introduced into the source in a number of ways. In the free droplet method, a droplet of deuterated reagent (*e.g.*, D_2O) is placed inside the enclosed ESI source (Fig. 5A) [73, 74, 97, 98, 105-108]. The deuterating reagent evaporates, forming a vapor that can interact with analyte molecules in the ESI droplets, allowing for HDX to occur. Previous work has shown that atmospheric water vapor can condense into ESI droplets [109]; therefore, we hypothesize that D_2O vapor condenses into the droplets, initiating solution-phase HDX for carbohydrates. The free droplet method is easy to implement. However, the D_2O droplet must evaporate to generate vapor. The partial pressure of D_2O increases until a consistent level of D_2O vapor is achieved in the source and this is only maintained until the liquid D_2O droplet in the source completely evaporates. Additionally, experimental factors that affect the rate of the D_2O droplet evaporation can also affect the repeatability. For example, vaporization is temperature dependent; thus, the source temperature and D_2O droplet location in relation to the heated MS inlet need to be controlled [36, 73, 74, 95, 105].

Alternatively, deuterated vapor (ND_3 or D_2O) can be introduced into the ionization source (Fig. 5B) [81]. Similar to the free droplet method, the deuterating reagent vapor interacts with solvated analyte in ESI droplets. The deuterating reagent, often introduced as a mixture with an inert carrier gas (such as N_2), can be directed into the source using one of the multiple gas inlets in commercial ESI sources. There are three distinct gas ports that can be adapted for this technique: sheath, auxiliary, and curtain gas. Each of these gases facilitate ESI droplet desolvation and increase the efficiency of ion production and transportion to the MS inlet. Sheath gas is coaxial to the capillary forming the Taylor cone and emitting ESI droplets; thus, the sheath gas surrounds the formed ESI droplets [110]. Auxiliary gas is also coaxial to the sprayed droplets, but surrounds the sheath gas [110]. Curtain gas flows in the opposite direction of the

sprayed droplets and collides with the formed ESI droplets prior to entering the MS inlet [110]. Of these three gases, sheath gas has the most contact with the ESI droplets and thus has the highest chance of initiating HDX labeling, followed by the curtain gas, and lastly the auxiliary gas [81].

To initiate more exchange, the amount of deuterating reagent vapor can be increased by (1) increasing the partial pressure of the deuterating reagent within the sheath, auxiliary, or curtain gas, (2) increasing the gas flow rate, or (3) increasing the gas temperature. By increasing the percentage of deuterating reagent in the gas, the magnitude of HDX can be increased because there is more deuterating reagent present to condense into the ESI droplets and label the analytes [81]. Similarly, by altering the flow rate of the gas, it is possible to alter the extent of HDX, though the relationship to increase or decrease HDX is less clear due to competing effects [81]. By increasing the gas flow rate, the amount of deuterating vapor in the source increases, which would be expected to increase the magnitude of HDX. However, increasing the gas flow rates also decreases the lifetime of the ESI droplets due to increased solvent evaporation, which decreases the time in which the analyte is solvated and can undergo HDX. Finally, exchange is affected by the temperature of the gas, which also has competing effects on the effect of HDX. As temperature increases, the rate of exchange increases according to the Arrhenius equation, leading to faster exchange. However, the rate of ESI droplet desolvation increases as well, leading to reduced time for HDX to occur [81, 111]. Each of these factors alter the total observed HDX. Most mass spectrometers have a clearly defined interface for coupling these gases to the source; thus, the gas can be changed to introduce a mixture containing deuterating reagent. Similar to the free droplet method, when implementing this method, the buildup of deuterated vapor over time must be accounted for to ensure consistent measurements. If the ratio of

deuterated and non-deuterated vapor changes within the source, different extents of HDX would be observed [73].

Analyte molecules in ESI droplets can be mixed with liquid droplets of deuterating reagent during ESI. When these droplets collide, mixing and HDX reactions are initiated. In dual spray systems, analyte droplets and droplets of deuterating solvent are formed from two spray channels (Fig. 5C) [103, 112-114]. The geometry of the two spray channels can be changed so that the spray plumes are orthogonal, partially offset, or coaxial [115, 116]. When orthogonal, the two spray channels are 180^{o} from each other, with each 90^{o} from the inlet to the mass spectrometer [117]. When partially offset both spray channels are offset 30^{o} from the inlet to the mass spectrometer, with a total of 60^{o} between the spray channels [118]. Finally, in the coaxial orientation, both spray channels are parallel to each other. This can either be achieved through a sheath liquid being incorporated with coaxial ionization, where the sheath solution surrounds the analyte solution or through theta-tip emitters where one capillary is split into two distinct channels [35, 112, 115, 116, 119, 120]. Theta-tip emitters (Fig. 5D) are unique in the fact that two Taylor cones are formed next to each other, which enables almost immediate droplet mixing [112]. Dual-spray, in-source HDX allows for direct control of parameters that influence HDX, due to the ability to change flowrates independently for both the analyte and deuterating reagent. However, this technique is limited by the requirement of using either a unique commercial ionization source (such as the Water's LockSpray Exact Mass Ionization Source with the baffle removed) or the development of a custom built ionization source.

In addition to these traditional ESI techniques, multi-cVSSI and CPSI have also been used to analyze carbohydrates. Multi-cVSSI functions similar to dual spray ESI with the exception of ionization being caused by a piezoelectric transducer that vibrates a glass microscope slide with

an attached capillary [99-101]. With cVSSI, droplets are formed due to vibrations, though some applications of cVSSI also involve application of a voltage. Similar to ESI, the droplets desolvate as they move towards the MS inlet. For CPSI, a sample is spotted and dried near the tip of a triangular section of polymer, polymethyl methacrylate (PMMA). Then, an eluent solvent is added and a voltage is applied (4.5 kV) resulting in the formation of droplets. CPSI does not rely on previously diluting the analyte in solution, and thus is an effective ionization technique for analyzing minute sample sizes. For HDX-CPSI-MS, the added solvent contains a mixture of methanol and D_2O initiating labeling with the addition of solvent [102].

3.3.2. Applications

In-ionization HDX has primarily been used to distinguish isomeric carbohydrates and metabolites, with isomers being characterized by different magnitudes of HDX or different HDX peak profiles [100, 102, 121]. Metabolomics is an area in which in-ionization HDX has seen numerous applications. The goal of metabolomics is to identify small molecule metabolites from biological samples. However, these studies are challenging because many metabolites have the same molecular weight and chemical formula, but different functional groups. Multiple techniques, including liquid chromatography (LC), ion mobility (IM), and tandem MS, are frequently used to separate and identify these isomeric compounds. Thus, samples have been run both with and without in-ionization HDX to identify the number of labile functional groups present in the molecules. Thus, some HDX platforms have been applied after LC with a binary set-up, enabling samples to enter the MS either with or without in-ionization HDX.

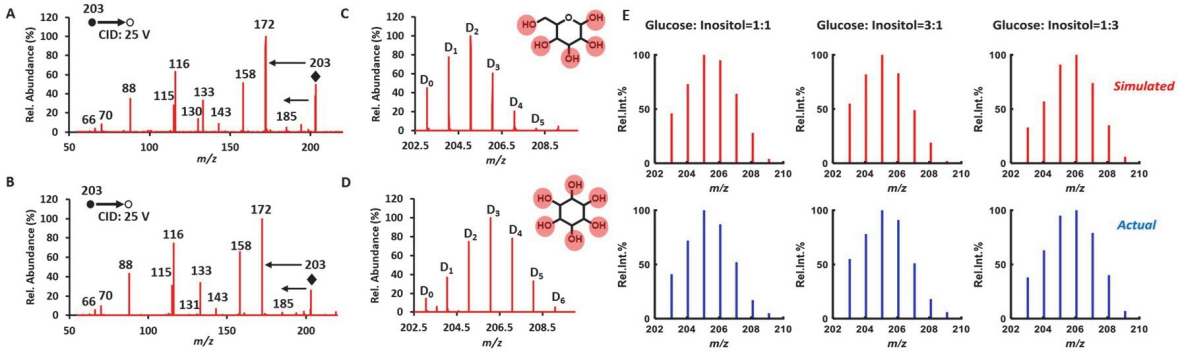


Figure 6. HDX for isomeric ions that also share similar fragmentation patterns. Tandem MS spectra following CID showing similarities for (A) glucose and (B) inositol. Post-HDX spectra for (C) glucose and (D) inositol. (E) HDX–CPSI-MS simulated and actual HDX mass spectra comparing various mixed solutions of glucose and inositol. CID: Collision Induced Dissociation. Reprinted from Song, X. et. al. Distinguishing between Isobaric Ions Using Microdroplet Hydrogen-Deuterium Exchange Mass Spectrometry *Metabolites* 2021, 11, 728. Open Access.

Carbohydrates are metabolites with numerous isomers. Inositol and glucose have the same chemical formula and molecular weight; additionally, tandem MS yields similar fragments that cannot be used to distinguish these isomers (Fig. 6A and 6B) [102]. Following CPSI-HDX-MS, the HDX profiles of inositol and glucose were monitored separately. Each analyte was spotted (3 µL at 20 µM carbohydrate) and dried at the tip of a PMMA triangle. Then, a mixture of methanol and D_2O (7:3 v/v) was added to the dried spot, initiating exchange, and 4.5 kV was applied to ionize the samples. Figure 6C and 6D show how these two carbohydrates have different peak profiles following in-CPSI-HDX labeling. Glucose has a D-uptake profile with a maximum at D_2, while inositol has a maximum at D_3. Using these different profiles, simulations were run with the two carbohydrates mixed at different ratios (Fig. 6E). These simulated spectra were in agreement with experimental data collected for mixtures of the two carbohydrates at these ratios,

showing that (1) these isomers can be distinguished by the D-uptake profiles and (2) spectra from mixtures can be deconvoluted.

DeBasatani *et. al.* demonstrated the ability to distinguish between sucrose and platinose in negative-ion mode as well as LacNAc (GlcNAc-Gal) and GlcNAc-Man in positive-ion mode using multi-cVSSI-HDX [99]. For this method, the isomeric carbohydrates produced distinct D-uptake values as well as peak patterns, allowing for them to be distinguished. This indicates that the method works with carbohydrates containing labile hydrogens on more than one type of heteroatom (oxygen and nitrogen), showing that more complicated carbohydrates and glycoconjugates may also be probed with this technique. This labelling technique is able to be toggled on and off by starting or stopping the secondary capillary that is providing the deuterated reagent. This work shows effective on/off D-uptake spectra collection with a duty cycle as low as four seconds, meaning that a total time of four seconds is required to record a non-deuterated sample, a deuterated sample, and to return to non-deuterated conditions. The strength of this on/off HDX method is the ease to which it integrates into a larger overall workflow such as LC-IM-tandem MS adding another valuable layer of distinguishing isomeric compounds that cannot be discerned otherwise [108, 121, 122]. Several works have shown the ability to incorporate in-ionization HDX with LC and MS/MS [99-101, 121, 122].

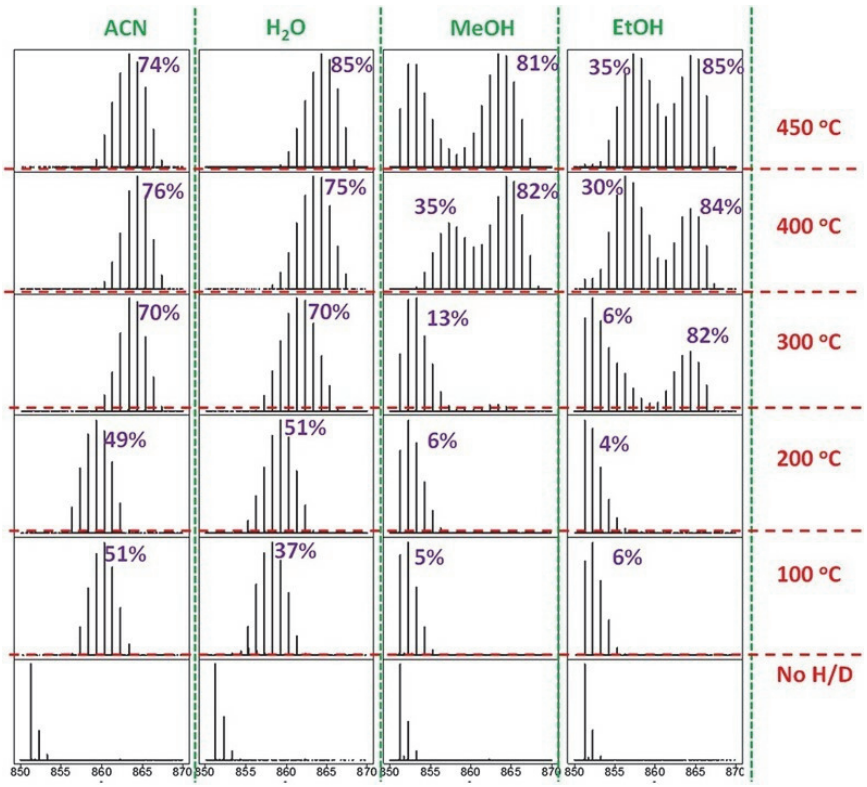


Figure 7. Effect of spray solvent and ion transfer tube temperature on H/D exchange spectra of a sodium-adducted pentasaccharide. Ion transfer tube temperatures ranges from 100 ºC to 450 ºC. Reprinted with permission from Kostyukevich, Y. *et. al.* Conformations of Cationized Linear Oligosaccharides Revealed by FTMS Combined with in-ESI H/D Exchange *J. Mass Spectrom.* 2015, 50, 1150-1156. Permission conveyed through Copyright Clearance Center, Inc.

Certain polysaccharides have been shown to have multiple conformations throughout labeling, depending on the solvent in which they are present. Kostyukevich *et. al.* utilized the ion transfer tube (or desolvation capillary) temperature for regulating HDX [107]. By ranging between 100 ̊C and 450 ̊C, different extents of exchange were observed. In these experiments, they utilized the free droplet method (Fig. 5A) while spraying dextran with potassium or sodium acetate. At low temperatures, minimal exchange was observed for metal-adducted dextran of various lengths. As temperature increased, two distinct distributions of exchange were observed

when dextran was sprayed from methanol or ethanol (Fig. 7). The presence of multiple distributions is indicative of multiple, distinct populations being present within the sample, often representing different conformations [98]. In instances where only acetonitrile or water were present, the metal-adducted oligosaccharide exhibited a single, binomial distribution of peaks following HDX. Bimodal HDX distribution were present when methanol or ethanol were the primary spray solvent and when no acid was added. Different solvents resulting in different distributions indicates that the solvent is playing a role in the analyte conformations that are present [74]. With various solvents having differing polarities and abilities to interact with the hydroxyls present on the carbohydrate analyte, it is hypothesized that these differing solution conditions affect the degree of intra- and inter-molecular hydrogen bonding experienced by the carbohydrate, leading to multiple different conformations being present.

For the applications of in-ionization HDX described above, a single labeling time was used for the carbohydrate analytes. This labeling time was initiated when the ESI droplets containing carbohydrates encountered the deuterating reagent, either in the form of vapor or liquid, and quenched upon analyte desolvation and formation of metal-ion adducts [74]. Our group has shown that small metal-adducted carbohydrate systems do not label in the gas-phase, which we attribute to the charge being localized on the metal, limiting the formation of resonance structures that stabilize the transition states required for gas-phase HDX [74, 83]. Thus, the time for HDX labeling is based on the lifetime of the droplets formed during ionization, *e.g.,* the ESI droplet lifetimes.

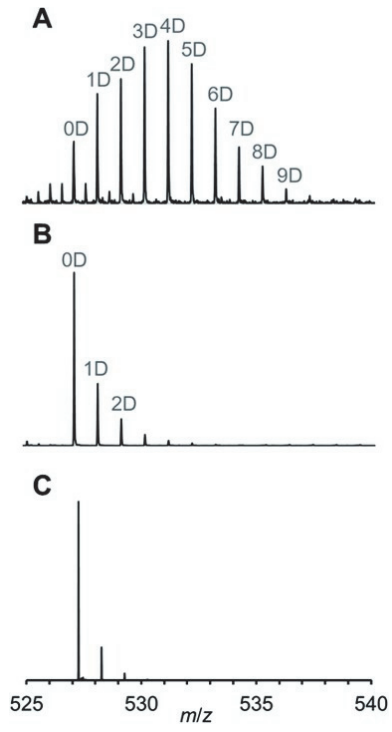


Figure 8. Representative HDX spectra of melezitose when sprayed from theta-capillaries with a (A) 28.22 µm tip, (B) 5.31 µm tip, and (C) 0.50 µm tip showing impact of tip opening on D-uptake. Reprinted with permission from Kim, H. J. *et. al.* Achieving Multiple Hydrogen/Deuterium Exchange Timepoints of Carbohydrate Hydroxyls Using Theta-Electrospray Emitters *Analyst*. 2020, 145, 3056-3063. Copyright 2020 Royal Society of Chemistry.

When comparing protein systems, differences in HDX have been observed when analyzing multiple timepoints [123]. Altering droplet lifetime is an effective method for changing the time for HDX for in-source HDX experiments. By sampling multiple timepoints in an HDX experiment, we hypothesize that the dynamics of carbohydrates can be monitored. The most accessible method of controlling droplet lifetimes during the ESI process is to control the initial size of the generated droplets. One technique that has been effectively used to control the initial

droplet size is changing the size of the needle tip used for ESI [35]. By using theta (θ)-tip emitters with tip sizes ranging from 0.5 (±0.04) μm to 20 (±10) μm, different magnitudes of HDX were achieved for melezitose, a trisaccharide (Fig. 8). The tip diameter, or the distance between the middle septum and the outer edge of the channel, was measured for each channel in each tip. For small tip diameters (0.5 μm), the droplet lifetime was approximated to be less than 1 μs and only 0.5 (±0.2) D-uptake was measured for melezitose (Fig. 8C) [35, 112]. For large tip diameters (28.22 μm), droplet lifetimes of ~20 μs resulted in 5(±3) D-uptake (Fig. 8A), while intermediate tip diameters (5.31 μm), with droplet lifetimes of ~6 μs, yielded 1.4(±0.7) D-uptake (Fig. 8B) [35]. By increasing the initial tip diameter, the initial droplet size was increased, increasing the time for HDX and yielding higher magnitudes of HDX. This technique could have many applications because it does not require any alteration of either the solvent composition or any of the parameters that would affect the rate of exchange through the Arrhenius equation, such as temperature.

Internal standards have become important tools in HDX reactions to minimize variability and improve quantitation following labeling [105, 124-126]. The rapid rate of exchange for hydroxyls affects both the forward-exchange reaction (H to D) and the back-exchange reaction (D to H), which can occur from water vapor inside the source. Thus, factors such as humidity can have unintended effects on the observed extent of exchange. Using an internal standard decreases the run-to-run and day-to-day variability [105]. For labeling carbohydrates with in-source HDX, it is common to utilize an analyte with similar properties as the internal standard. Thus, past work has utilized maltoheptaose labeled with Girard's T reagent to provide an internal standard and improve the repeatability of replicate analyses performed on one or multiple days [105].

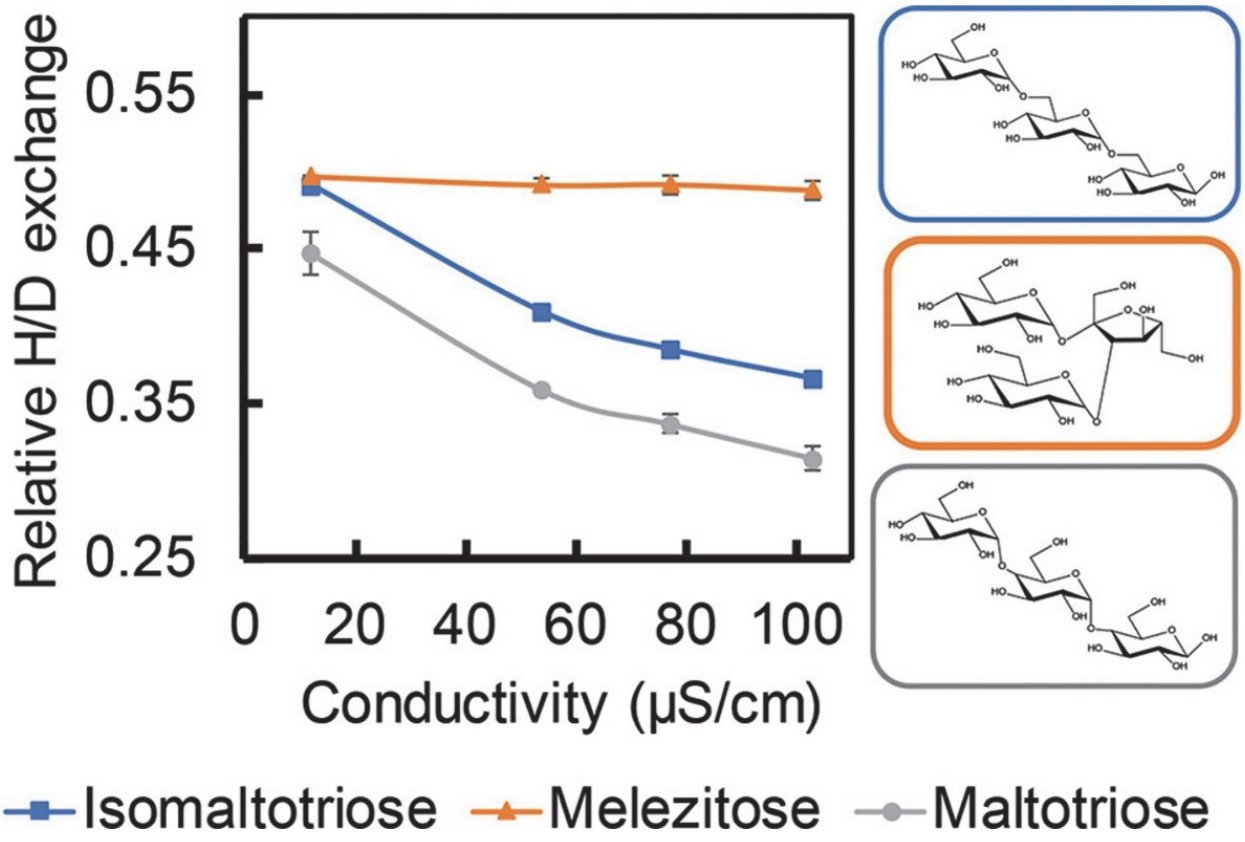


Figure 9. Distinguishing trisaccharides by establishing unique labeling times using varying conductivity for in-ESI H/D exchange with an internal standard. Reprinted with permission from Liyanage, O. T. *et. al*. Distinguishing Carbohydrate Isomers with Rapid Hydrogen/Deuterium Exchange-Mass Spectrometry *J. Am. Soc. Mass Spectrom.* 2021, 32, 1, 152-156. Copyright 2021 American Chemistry Society.

When coupling the internal standard to analyses of carbohydrates, labeled for different lengths of time, carbohydrate isomers could be distinguished (Fig. 9). Changing the spray solvent conductivity in ESI changes the initial size of the ESI droplets [127]. As conductivity increases, the initial droplet sizes decrease, causing the overall lifetime of the droplet to decrease and decreasing the time for HDX [36, 127]. Figure 9 shows the relative H/D exchange of three isomeric trisaccharides [36]. At low conductivity, isomaltotriose and melezitose cannot be

discerned from one another based on the relative H/D exchange that is measured. However, as the solvent conductivity increases, all three trisaccharides are readily distinguished from each other. Distinguishing these carbohydrate isomers by effectively utilizing different timepoints for HDX labeling shows that in-ESI HDX has the benefits of both solution-phase HDX and gas-phase HDX. In-ESI HDX retains the ability to sample HDX timepoints showing changes in D-uptake and characterizing the solvated dynamics of carbohydrates over time similar to solution-phase HDX. Additionally, in-ESI HDX can distinguish small molecule isomers similar to gas-phase HDX. Thus in-ESI HDX provides solution-phase labeling on a timescale typical of gas-phase HDX experiments. This data shows that carbohydrates with different monosaccharide composition or connectivity isomerism could be distinguished by probing the solvated conformations. The characterization of solvated carbohydrates is novel for MS and enables the analysis of biologically relevant structures. This could enable a similar level of characterization and insight to carbohydrates as solution-phase HDX has provided for proteins [34].

4. Conclusions

Hydrogen/deuterium exchange is a valuable tool both for small molecule differentiation and biomolecular studies of dynamics. HDX provides a useful lens to observe, analyze, and differentiate carbohydrates. In traditional, solution-phase HDX, *N*-acetyl hexosamines in glycans can affect the measured D-uptake of glycopeptides and glycoproteins, requiring that this exchange be detected and accounted for to prevent errors in assessing glycoprotein structures both from the presence of a glycan moiety and the heterogeneity caused by varying glycoforms. Gas-phase HDX has been used as an analytical tool to distinguish monosaccharides and disaccharides following formation of oxonium ions, generated from protonated carbohydrates.

However, these methods analyze gas-phase conformations, which are different than solvated carbohydrate conformations. In-ionization HDX is an emerging technique that is showing great promise for the analysis of carbohydrates. This technique can be applied to multiple ionization methods with deuterating reagents being mixed with the sample as either a vapor or liquid to initiate exchange. The methods are valuable because rapidly exchanging functional groups, such as carbohydrate hydroxyls, exchange on the same time frame as ionization. Thus, the structures are labeled while solvated and the exchange reaction is quenched as the ions enter the vacuum regions of the MS. Additionally, these in-ionization reactions have been able to sample carbohydrate dynamics by altering the HDX labeling time, by changing the droplet size and droplet lifetime. From a biomolecular perspective, in-ionization HDX can capture different timepoints of exchange from solution-phase carbohydrates, gathering biologically relevant dynamic and conformational information. Combined, this variety of HDX methods enables the analysis of carbohydrate structures and their dynamics.

In the future, we expect to see this technique be further developed and applied towards carbohydrates and glycans. In-ionization HDX has the potential to become a fundamental block in metabolomic workflows allowing for isomeric and isobaric identification. Further work in assimilating in-ionization HDX with online workflows is needed, especially in the form of software that can pair non-deuterated and deuterated profiles together. In addition, in-ionization HDX shows the potential to analyze biologically relevant conformations of carbohydrates as well as monitor glycans as part of complex biomolecules, including glycoproteins and glycolipids. Thus, HDX is a powerful tool towards analyzing carbohydrates and glycans both structurally and dynamically and can be expected to be further developed to tackle current problems in

distinguishing isomers and assessing the role and specific function of carbohydrates and glycans in biological environments.

5. Acknowledgements

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