An overview of hydrogen deuterium exchange mass spectrometry (HDX-MS) in drug discovery

Glenn R. Masson, Meredith L. Jenkins & John E. Burke

To cite this article: Glenn R. Masson, Meredith L. Jenkins & John E. Burke (2017) An overview of hydrogen deuterium exchange mass spectrometry (HDX-MS) in drug discovery, Expert Opinion on Drug Discovery, 12:10, 981-994, DOI: 10.1080/17460441.2017.1363734

To link to this article: https://doi.org/10.1080/17460441.2017.1363734

Accepted author version posted online: 03 Aug 2017.
Published online: 17 Aug 2017.

Submit your article to this journal

Article views: 750

View related articles

View Crossmark data

Citing articles: 2 View citing articles
An overview of hydrogen deuterium exchange mass spectrometry (HDX-MS) in drug discovery

Glenn R. Masson a, Meredith L. Jenkins b and John E. Burke c

Protein and Nucleic Acid Chemistry Division, MRC Laboratory of Molecular Biology, Cambridge, UK; bDepartment of Biochemistry and Microbiology, University of Victoria, Victoria, Canada

ABSTRACT

Introduction: Hydrogen deuterium exchange mass spectrometry (HDX-MS) is a powerful methodology to study protein dynamics, protein folding, protein-protein interactions, and protein small molecule interactions. The development of novel methodologies and technical advancements in mass spectrometers has greatly expanded the accessibility and acceptance of this technique within both academia and industry. Areas covered: This review examines the theoretical basis of how amide exchange occurs, how different mass spectrometer approaches can be used for HDX-MS experiments, as well as the use of HDX-MS in drug development, specifically focusing on how HDX-MS is used to characterize bio-therapeutics, and its use in examining protein-protein and protein small molecule interactions. Expert opinion: HDX-MS has been widely accepted within the pharmaceutical industry for the characterization of bio-therapeutics as well as in the mapping of antibody drug epitopes. However, there is room for this technique to be more widely used in the drug discovery process. This is particularly true in the use of HDX-MS as a complement to other high-resolution structural approaches, as well as in the development of small molecule therapeutics that can target both active-site and allosteric binding sites.

1. Introduction

Hydrogen deuterium exchange mass spectrometry (HDX-MS) is rapidly becoming an important part of the biochemist’s toolkit for exploring protein structure, dynamics, and function. HDX-MS has been applied to study protein folding [1], protein oligomerization [2,3], protein:protein [4], protein:DNA [5], protein:small molecule [6], and protein:membrane [7–9] interactions, as well as being applied to complement orthogonal biophysical techniques such as X-ray crystallography [10,11], nuclear magnetic resonance [12], and electron microscopy [13]. Central to this diverse array of applications is the measurement of deuterium uptake along a protein’s backbone amide groups. The rate of uptake of deuterated solvent of an amide hydrogen is primarily dependent on its involvement in hydrogen bonds present in secondary structure elements [14,15]. The technique is particularly well suited to examine changes in conformation in disordered regions of proteins, as disorder-order transitions will cause extremely large changes in the exchange rates of amide hydrogens [16]. Furthermore, this rate of exchange is also sensitive to changes in protein conformation/solvent accessibility that occur on the addition of binding partners. Thus, through the observation of amide exchange rates under various conditions, such as the presence and absence of a ligand, structural and dynamic properties of the protein and the binding interface can be examined.

The utilization of the hydrogen/deuterium exchange phenomenon to study protein structure is not a new concept, with much of the theoretical basis being determined in the 1950s [17]. Exchange rate measurements were initially conducted using tritium and density gradient columns, and then replaced with deuterium and nuclear magnetic resonance (NMR) experiments in the 1980s and 1990s (for a review on the early history of H/D exchange please consult [18]). NMR is still used in H/D exchange measurements and can provide highly accurate single-residue exchange data; however, there are disadvantages in the amount of protein required and the size of proteins that can be studied. H/D exchange mass spectrometry experiments rely on the coupling of low-temperature ultra-high-pressure liquid chromatography with the high sensitivity and resolution associated with modern mass spectrometry instruments to determine the locations and rates of amide hydrogens deuterium uptake. Additionally, advances in the automation of both sample preparation and software-driven early stage data analysis has allowed for an increase in popularity of HDX-MS studies, especially in the field of protein biopharmaceuticals where it is now routinely employed in epitope determination and quality control (for a review see [19]). Examples of the use of HDX-MS in drug discovery processes are highlighted in Figure 1. HDX-MS has long been described as an ‘emerging’ technology, but its use is now fairly commonplace – it can now be more accurately described...
HDX-MS is also well suited upstream in the drug discovery process as improvements in instrumentation and data analysis have greatly expanded the complexity of samples that can be interrogated by HDX-MS, as well as increasing the spatial resolution at which they can be measured.

HDX-MS is well suited as a tool for determining altered protein dynamics upon binding to both protein and small molecule ligands.

HDX-MS provides major advantages in the rapid determination of both protein and small molecule binding sites on proteins.

HDX-MS is also well suited upstream in the drug discovery process as it can be used for the development of optimal constructs for high resolution structural approaches that can be used for SAR studies.

This box summarizes key points contained in the article.

As an ‘emerged’ technology, this review hopes to highlight the theoretical basis of amide exchange, how technological advancements in mass spectrometry have expanded the capabilities of this technique, and focus on specific examples that demonstrate the power of this technique in drug discovery.

1.1. Theoretical underpinnings of amide deuterium exchange

Backbone amides engaged in secondary structure exchange with solvent via the following mechanism (see Equation 1).

\[
\text{N-H}_\text{cl} \xrightarrow{k_{\text{op}}} \text{N-H}_\text{op} \xrightarrow{k_{\text{ch}}} \text{N-D}_\text{op},
\]

where the equilibrium between ‘open’ and ‘closed’ forms of the backbone amide is determined by the rate constant \(k_{\text{op}}\) for the opening of the amide hydrogen bond and \(k_{\text{ch}}\) the rate constant for the closing of the amide hydrogen bond. In this scheme, solvent exchange can only occur while the amide group is in the ‘open’ conformation – when there is a localized minor unfolding event. The rate of solvent exchange that occurs while the amide group is in the open conformation, \(k_{\text{ch}}\), is governed by the steric and electrostatic effects of surrounding residues, the residue itself, the pH (or pD) of the solution, and the temperature [28]. This is also frequently called the intrinsic rate constant (frequently referred to as \(k_1\) or \(k_{\text{int}}\)). Overall, the observed exchange rate under the steady state approximation is given by Equation 2.

\[
k_{\text{obs}} = k_{\text{op}}k_{\text{ch}} / (k_{\text{op}} + k_{\text{cl}} + k_{\text{ch}})
\]

This scheme assumes that the protein is exposed to 100% D\(_2\)O, and only the deuteration exchange reaction proceeds, that is, there is no H\(_2\)O present, and no incorporated deuterium could be replaced with a proton (known as back-exchange). The intrinsic rate constant for the amide hydrogen of any residue can be theoretically calculated for a specific pH and temperature, as the parameters governing this exchange have been carefully measured experimentally [28]. Within a folded protein the observed rate of hydrogen deuterium exchange of an amide (\(k_{\text{obs}}\)) can be decreased relative to the intrinsic rate (\(k_{\text{ch}}\)) by \(10^{10}\), and this decrease in exchange can be represented as a protection factor \(P\), which is the ratio of the intrinsic rate \(k_{\text{ch}}\) over the experimentally measured exchange rate \(k_{\text{ex}}\).

**Figure 1.** Utility of HDX-MS in drug discovery. (a) HDX-MS to probe the dynamics of protein-ligand interactions. The difference in HDX-MS induced upon the interaction of the Retinoic Acid Receptor gamma (ROR\(\gamma\)) with an inverse agonist, T0901315 is shown. Three peptides (399–410 in green, 365–373 in violet, 276–287 in yellow) all exhibited alterations in H/D exchange on binding to the compound (shown in pink, stick form) [20,21]. (b) HDX-MS to inform construct design for high resolution structural approaches [22,23]. HDX-MS exchange rates for a full length PI4K\(\beta\)I\(\beta\) construct (HDX incorporation shown in red on the uptake graph) was used to identify disordered regions, which were subsequently removed to create an optimized crystal construct (HDX incorporation shown in blue). This construct maintained the same overall fold, and allowed for the high resolution structure determination of PI4KB. (c) HDX-MS for characterizing biopharmaceuticals. HDX-MS was used to determine the structural deformations of Interferon \(\beta\) 1a (IFN) that occur due to oxidation of various cysteine and methionine residues (shown in green). Five peptides, shown in orange, exhibited a large increase in HDX, most likely caused by large-scale unfolding [24,25]. (d) HDX-MS for use in Epitope Mapping. HDX-MS was used in conjunction with a variety of other techniques, to determine the epitope of Interleukin-23 [26,27].
Amide exchange MS profiles have been classified into two different regimes depending on the relative rates between $k_{ch}$ and $k_c$. In EX2 kinetics, where $k_{ch} \ll k_c$, the amide bond must undergo numerous unfolding events before the amide will exchange with solvent. EX2 kinetics can be identified from the characteristic gradual increase in a peptide’s isotopic distribution as the heavier deuterium is incorporated into backbone amides. Alternatively, EX1 kinetics, less common under native conditions, occurs when $k_d \ll k_{dp}$, leading to a situation where the opening and closing rates are slow compared to the intrinsic exchange rate. This leads to every opening event leading to exchange with deuterium. Peptides exhibiting EX1 kinetics produce a signature bimodal isotopic distribution that can present a challenge to data analysis of deuterium incorporation [29].

While the equations governing H/D exchange rates with solvent have been known for decades, the exact molecular underpinnings determining why amide hydrogens exchange at a given rate are still not exactly clear. The most commonly cited reasons for why amide hydrogens are protected from exchange is due to their involvement in secondary structure, as well as their protection from bulk solvent [14,15]. However, it must be taken into account that these same studies also identified a number of amide hydrogens in unstructured regions that still showed significant protection factors. One of the contributing factors of decreased exchange rates for these exposed regions was the presence of structured water molecules [14]. However, this is unlikely to fully explain why these regions are protected as experiments using ubiquitin as a model protein found that exposed unstructured regions showed large differences in protection factors, with no clear relationship to nearby structured water networks [30]. Initial attempts have been made to examine the open amide state as described in equation 1 using long (~1 ms) molecular dynamic simulations, with potential open amide state intermediates being identified as very short-lived (~100 ps) species [31]. In summary, there is still much we do not understand concerning why amide hydrogens in a protein will be protected from exchange, and caution must be applied in the use of overly simplistic models that define protection from exchange as only being driven by either involvement in secondary structure or accessibility to solvent.

### 1.2. HDX-MS methodology

A HDX-MS experiment can be split into numerous discrete steps. This section will introduce these different methodological steps, and specifically highlight the most frequently used approaches. The important steps in any HDX approach are: (1) deuterium incorporation, (2) spatial localization of deuterium incorporation, and (3) data analysis of deuterium incorporation. Readers are advised to consult excellent recent reviews for more comprehensive methodological details on best HDX practice [32–34].

#### 1.2.1. Deuterium incorporation

Deuterium labeling can be carried out as an on-exchange reaction, where deuterium is incorporated through incubation of the protein in a deuterated buffer, or as an off-exchange experiment where loss of deuterium is measured from a fully deuterated protein when it is diluted in a non-deuterated buffer [35]. Most experiments are carried out using an on-exchange labeling approach, as complications can exist in the generation of a fully deuterated protein required for the off-exchange approach, particularly for large and unstable protein complexes. Proper execution of deuterium labeling requires the examination of multiple time-points of deuterium incorporation, so differences in the kinetics of H/D exchange for amides with varying exchange rates can be measured. This is particularly important for users solely using automated sample handling systems, as these frequently underestimate very short deuterium incorporation time points, and this can lead to an underestimation of changes in protein dynamics in areas of weak/transient secondary structure. This can be addressed by varying both the pH, the temperature, and the length of deuterium exposure, which enables the measurement of amide exchange rates that range over 8 orders of magnitude [36,37]. However, caution must be applied when altering pH as this requires that the protein be stable across the pH ranges examined. The use of other biophysical measurements (differential scanning fluorimetry, multi angle light scattering, etc.) is suggested to verify that protein conformation is stable over the desired pH range.

The protein, typically in a high nanomolar to low micromolar concentration, is labeled in an aqueous semi-‘native’ environment both in the presence and absence of any binding partners. Dependent on the mass spectrometer used in the analysis, roughly 5–100 pmol of sample are required. Specialized nano-spray systems have allowed for the use of sub-picomole levels of protein per sample [38]. Of important note is that HDX measurements are dependent on the occupancy of the protein of interest with the binding partner, and therefore careful consideration must be given in the relative concentrations of the protein of interest and the binding partner to maximize occupancy. D$_2$O exposure is carried out for a variety of time points ranging typically from seconds to hours, although this can be extended to days for extremely stable proteins. The use of pulsed label and stop flow apparatus is particularly useful for the analysis of extremely rapidly exchanging amide hydrogens with rates on the millisecond timescale [39,40]. Amides with extremely rapid exchange rates can also be labeled in the gas phase of the mass spectrometer, with a 0.1–10 ms labeling pulse of deuterium using ND$_3$ in the ion guide of the MS [41]. Carrying out experiments at very short deuterium incorporation time courses is exceptionally important in the analysis of intrinsically disordered proteins and is necessary to properly identify disordered regions that can be removed for optimization of constructs for other structural approaches.

#### 1.2.2. Spatial localization of deuterium incorporation

Different techniques to spatially resolve H/D exchange information are summarized in Figure 2. The simplest HDX-MS experiment is carried out by examining global differences in H/D exchange. It is carried out by directly injecting the intact protein onto an LC-MS system, and the overall mass of the entire protein is measured. The protein of interest can be incubated with different ligands (proteins, inhibitors, etc.), and any differences in H/D exchange can be visualized. This provides no information on the location of the altered deuterium exchange, and any conditions where there are an equal amount of increases and
decreases in exchange in multiple different regions will show no net change in global deuterium incorporation.

Spatial localization of amide exchange rates using HDX-MS can be carried out using both top-down and bottom-up approaches. In the bottom-up approach, deuterium incorporation is localized in solution using protease-generated peptide fragments, and in the top-down approach deuterium incorporation is localized within the mass spectrometer through the fragmentation of the protein. These techniques can be combined in a so-called middle-down approach where the location of deuterium incorporation within protease-generated peptides can be

---

**Figure 2.** The various experimental approaches for HDX-MS. All HDX-MS (with the exception of gas-phase HDX-MS) starts with an aqueous labelling step, where a protein of interest is exposed to deuterium for a pre-determined amount of time, and then quenched. This protein can then either be injected onto a fluidics system and digested by an acid-functional protease (the 'bottom-up' approach), or injected onto the mass spectrometer for an intact mass measurement. This intact protein may then be partially fragmented whilst inside the mass spectrometer to yield either intact domains or single peptides for comparison between states (the Top-Down approach). The bottom-up approach may be furthered by either the a) mathematical determination of individual exchange rates (to achieve pseudo amino-acid resolution) or b) targeted ETD fragmentation of individual peptides (middle-down approaches). Data adapted from [42].
further refined through fragmentation within the mass spectrometer [43].

1.2.3. Bottom-up HDX-MS approaches

Due to the simple requirements for MS infrastructure and robust experimental workflows, most published HDX-MS experiments have been performed using a bottom-up approach. The key steps in a bottom-up H/D exchange experiment downstream of labeling are: (1) Quenching, (2) Digestion, (3) Separation, and (4) Mass analysis. Top-down experiments exclude the digestion step, as fragmentation of the protein occurs in the mass analysis step.

After deuterium labeling has occurred, the exchange reaction can be quenched. Quenching the exchange reaction can be achieved by simultaneously reducing the temperature to 0°C and the pH to 2.5. The exchange of amide hydrogens is an acid and base catalyzed process with a global minimum at a pH of ~2.5–2.8, thus the addition of a quench buffer minimizes back-exchange of amide hydrogens in all subsequent steps. All steps from this point on must be performed as rapidly as possible at low temperatures, and held at pH 2.5, to prevent back-exchange. Generally, while quenching the exchange reaction the labeled protein is also denatured through the inclusion of a denaturant (e.g. guanidinium chloride) and an acid-functional reductant (e.g. TCEP) in the quench solution. TCEP is particularly important for proteins containing multiple disulfide bonds [9,44], as peptides containing linked cysteines present a challenge for peptide identification. Denaturing and reducing the protein (either through the use of an acid functional reductant or an electrochemical cell [45]), facilitates the proteolysis of the protein using an acid-functional protease either in solution or through an immobilized protease column within a fluidics system. The primary protease used for these experiments is pepsin, with other acid functionalized proteases such as fungal XIII, XVI, and nepenthesin also being used [46–49]. The proteolysis reaction may be conducted at slightly elevated temperatures (~10–20°C) to increase digestion efficiency for proteins that are resistant to protease digestion. Caution must be taken when digesting at higher temperatures, as this can lead to increased back-exchange rates with protiated solvent. The use of pepsin coupled to small particles that operate at high back pressures (~10,000 psi) also leads to enhanced protease efficiency [50,51].

The deuterium-labeled peptides are then separated on a chilled HPLC/UPLC system. This system is kept as cool as possible to limit the loss of the deuterium signal through back-exchange. Separation has to occur as fast as possible, and the use of UPLC backpressures (>15,000 psi) allows for excellent peptide separation in 5–20 min [52]. The use of different buffer systems allows for the use of subzero temperatures during peptide separation, further reducing back-exchange levels [53,54]. Robotic liquid handling systems are available that automate the labeling, digestion, and separation steps; however, caution must be applied in the use of this approach depending on the stability of the protein under study. Peptides are then injected into the mass spectrometry instrument and ionized, typically using an electrospray ionization source.

An important note on the methodology of bottom-up deuterium exchange experiments is that users need to pay close attention to back-exchange that occurs during the proteolysis and LC separation steps. For proteins that require longer digestion and reduction times before injection on the fluidics system, considerations about spurious non physiological on exchange that occurs in the quench buffer also need to be taken into account. Some of the different parameters that affect back-exchange have been measured [55], and careful analysis of the back-exchange levels of any given system is suggested through the generation of fully deuterated protein samples (for more discussion of these considerations consult [33]). However, for experiments examining only the relative hydrogen exchange between conditions, the generation of fully deuterated samples is not necessary, although it is important to understand that all H/D exchange incorporation curves will be a relative measurement of deuterium incorporation. One of the most common mistakes new users experience is not properly addressing peptide carryover, which can massively distort H/D exchange profiles [56,57].

The isotopic profile of the charged deuterated peptides are then determined using the mass spectrometer. The exchange rates of individual peptides are then compared in the absence or presence of a binding partner. Information on protein conformation is contained both in the mass centroid of the deuterated peptide, as well as in the shape of the isotopic profile. Isotope profiles are useful in determining EX1 and EX2 kinetics [29], and can also be useful in determining residue-specific exchange information for regions with multiple peptides [58]. Through comparing the profiles of identical peptides that were deuterated under differing conditions – with and without binding partners, for example – it is possible to identify peptides that have altered deuteration rates, and thus determine the location of structural changes that accompany binding events.

Data analysis and interpretation are significant steps in HDX-MS, particularly with larger proteins that may produce thousands of peptides. The discussion of this topic is outside the scope of this review, and readers are recommended to consult excellent recent reviews on different H/D exchange software analysis packages [32,59]. Although software has automated much of the data analysis, the manual inspection of all peptides and their deuterium incorporation is still essential to minimize any complications from overlapping peptides/noisy data.

1.2.4. Top down HDX-MS approaches

Some inherent difficulties with the bottom-up approach are that the spatial localization of any change in exchange rate is dependent on the length of protease generated peptide fragments, as well as the required generation of a number of overlapping peptide fragments. Fragmentation within the mass spectrometer, specifically the use of electron transfer dissociation (ETD) [60–62] or electron capture dissociation (ECD) [63,64], can be used to generate localized spatial information. Collision-induced dissociation (CID) is not compatible with HDX-MS experiments due to the propensity for CID to randomize the
location of the amide hydrogens/deuteriums in the resulting fragments, a phenomenon known as scrambling [65,66]. ETD and ECD can be used for MS/MS experiments, but in order to minimize scrambling it is crucial to use standard peptides to determine the instrument parameters which result in minimal scrambling [67]. The top-down approach can be used on intact proteins, but the coverage and spatial localization will be dependent on the generation of the full spectrum of c/z fragment ions. This can be challenging for large (>40 kDa) proteins; however, this can be surmounted through the use of a middle-down method where protease generated peptides are fragmented using either ETD or ECD to gain site specific exchange information [60,61].

1.3. HDX-MS in drug discovery

HDX-MS has been widely used in the academic community since the early 1990s. It has proven itself to be a valuable biophysical tool to study many biological processes, including pioneering studies examining protein–protein and protein–small molecule interactions. A growing area of interest has been in the study of membrane proteins, including both integral membrane proteins [68–72], as well as the coordinated recruitment of peripheral membrane proteins [7–9,42,69,73–83]. The analysis of membrane proteins has become amenable to HDX-MS studies, including the largest family of cell surface receptors (G-protein coupled receptors) [84]. For technical details on the use of HDX-MS for analysis of membrane proteins readers are advised to consult [33]. We will highlight the use of HDX-MS in drug discovery on three areas: the analysis of protein–small molecule interactions, characterization of bio-therapeutics/biosimilars, and epitope mapping of bio-therapeutics. We will also discuss emerging areas of how HDX-MS can be used in drug discovery approaches, including as an aid for the generation of novel constructs for high-resolution structural approaches.

1.3.1. HDX-MS to examine protein–small molecule interactions

Although the administration of protein therapeutics is rapidly expanding, small molecules – typically in the order of less than 800 Da, are still the most common therapeutics on the market. There are a variety of analytical tools that are able to detect protein–ligand interactions –NMR, isothermal titration calorimetry, microscale thermophoresis, and surface plasmon resonance can all be used to detect and characterize protein–ligand interactions. HDX-MS provides an opportunity to not only observe ligand binding and determine the location of a ligand binding site, but also provides an insight into differences in conformational dynamics that accompany binding – information that is crucial in developing novel pharmaceuticals.

The low protein requirements and sensitivity of HDX-MS makes it well suited to probing the dynamics of ligand binding, especially with low-affinity compounds [85] and when working with proteins that are problematic for crystallography, such as those with extensive unstructured regions [86,87]. Additionally, there is the potential for both the automation of sample preparation using fluid handling robotics and, within certain limits, automated data processing [88]. Despite this, the use of HDX-MS is still not widespread in the screening of compounds. This is primarily due to two issues: the resolution of the technique and the interpretation of binding results.

The interpretation of hydrogen/deuterium exchange data for small molecule binding events requires careful consideration. Amide hydrogen exchange is dependent not only on steric shielding caused by a ligand interacting with the backbone amide groups of a peptide, but also allosteric conformational changes in the surrounding structure and dynamics of the protein [89]. Additionally ligand binding can, possibly counter intuitively, cause both increases in exchange rate and no net change in hydrogen deuterium exchange rate [90] – suggesting that it is an oversimplification to simply designate peptides with the largest decreases in exchange rate as the locus of ligand binding. This problem is compounded by the peptide-level resolution of HDX-MS, as the observed exchange rates are a net change in deuteration for a single peptide, which is the sum of each individual amide’s change in deuteration rate. As recently demonstrated, ligand interactions that result in a mixture of increases and decreases in exchange rate on different amides within the same peptide may ‘cancel out’, making the changes in solvent exchange ‘invisible’ at peptide-level resolution [91]. While peptide-level resolution may be sufficient for many applications, such as protein–protein interactions where there is often a large extended binding surfaces, small molecule-binding events may be the result of very few interactions in noncontiguous amino acids that constitute the binding pocket. This problem is exacerbated in fragment-based lead generation (FBLG) methods, where initial screens may be of compounds as small as 200 Da, and an even smaller binding footprint could be expected. Increasing the resolution of HDX-MS to the single-amino acid level can be achieved through two methods: using tandem mass-spectrometry with ETD/ECD [64], or computationally using overlapping peptides [58,92–94]. The primary means of enhanced coverage for most H/D exchange experiments has been through the use of overlapping peptides. However, there can be difficulties in the use of overlapping peptides for single amide resolution, particularly depending on variable levels of back exchange and the efficiency of protein digestion in producing a sufficient number of overlapping peptides, as well as destructive interference that may occur with FT-MS instruments [95].

However, even with these caveats of data interpretation and analysis, HDX-MS has played a key role in numerous laboratories for the characterization of protein–small molecule interactions. The following is not an exhaustive summary of all protein–small molecule HDX-MS experiments, and selectively highlights a number of studies that verify the usefulness of using HDX-MS as a tool to study protein–small molecule interactions.

The use of HDX-MS is particularly well suited for the discovery of allosteric inhibitors, as well as how inhibitors mediate conformational changes distant from their binding site. HDX-MS carried out on inhibitors of the Hepatitis C Viral RNA dependent RNA polymerase revealed long range
allosteric conformational changes driven by inhibitor binding, revealing the inhibitor mode of action [96]. HDX-MS has revealed how alternative ligand binding sites on the nuclear receptor transcription-factor PPAR-gamma cause conformational changes potentiating the receptor for hyperactivation [97]. HDX-MS on the retinoid X receptor alpha ligand binding domain revealed how it is differentially effected by the presence of co-activating peptides and antineoplastic therapeutic agents [98]. Combined crystallographic and HDX-MS studies on ligand binding of the estrogen receptor alpha [99], provided useful mechanistic insight into differences between the inhibitors, and confirmed crystallographic analysis. HDX-MS was critical for the identification of a novel allosteric inhibitor binding site on antiapoptotic protein MCL-1, providing a novel strategy to target this protein in disease [100]. HDX-MS was used to determine the ligand binding site on IL-17A [101] and revealed that an additional α-helical region of the cytokine that was destabilized upon compound binding, providing a hypothesis to why they were unable to crystallize the ligand-protein complex. Combined crystallographic and HDX-MS analysis of the excitatory amino acid transporter 1 revealed the structure of allosteric inhibitors bound to this membrane protein, and how differences in protein dynamics in the presence of the allosteric inhibitor mediate inhibition determining [102].

HDX-MS can also provide insight into distant structural rearrangements that occur upon ligand binding in fragment screening approaches. A recent study investigated both high-affinity ligands (Kᵩ approx. 20 nM) and low-affinity fragments (500 μM) binding to the N-terminal ATPase domain of Hsp90 using HDX-MS [85], concluding that both the ligands and fragments shared the same interaction loci. Through overlaying HDX-MS data onto structures produced from X-ray crystallographic studies, not only was the primary binding site determined, but orthosteric sites were also identified, along with structurally distal differences in conformational dynamics of the protein which accompanied ligand binding, structural insights that would not have been observed using crystallography alone. Significant differences in exchange were determined between two low-affinity phenolic fragments that were of similar size and affinity – highlighting the possible utility of HDX-MS in structurally screening low-affinity compound series.

HDX-MS has been particularly useful in the targeting of protein kinases using small molecule inhibitors. The binding of both allosteric and active site inhibitors has been examined for the oncogenic Bcr-Abl protein [103], which revealed how allosteric inhibitors modify the conformation of the active site. Further HDX-MS studies revealed novel allosteric communication between the active site and myristate binding site [104] on both WT and the T315I gatekeeper mutant, revealing the advantages of targeting non-active site inhibitor binding sites. Study of the protein kinase Hck and its activation downstream of the HIV-1 viral protein Nef revealed that Nef led to subleaky dynamic conformational changes that activate the kinase, and that the presence of Hck antiretroviral inhibitors reversed these changes [105].

HDX-MS has also shown promise in the design and characterization of small molecule inhibitors for lipid-modifying proteins, as many times these inhibitors can be extremely hydrophobic and unsuitable for crystallographic approaches. Inhibitors of the phospholipase A₂ family of enzymes have been designed using a combined HDX-MS and molecular dynamics approach [6,106–108] that were retractable to other structural methods. HDX-MS analysis of different inhibitors of the monoacyl glycerol lipase critical in cannabinoid signaling revealed distinct stabilization of different conformational ensembles by different active site inhibitors [109]. HDX-MS was critical for the identification and mutation of a novel allosteric inhibitor binding site in phosphatidylinositol 5-phosphate 4-kinase gamma, allowing for the study of the role of this kinase in epithelial cell polarity [110].

1.3.2. HDX-MS to examine bio-therapeutics

The bio-therapeutic market has grown explosively, and has led to a number of challenges in how to characterize these drugs (the following review provides an excellent summary of some of these challenges [111]). One of the major growth areas of bio-therapeutics has been in the development of humanized monoclonal antibodies. With this growth, HDX-MS has taken an important role in mapping epitopes of antibody–antigen interactions. Another major area of growth for HDX-MS has been in the characterization of posttranslational modifications in protein therapeutics, specifically how these posttranslational modifications modify protein conformation, as well as determining similarity guidelines for biosimilar therapeutics. We will highlight a subset of studies that highlight the usefulness of HDX-MS for bio-therapeutic characterization and discovery.

1.3.3. Characterization of bio-therapeutics by HDX-MS

The development of bio-therapeutics has fundamentally changed the drug development landscape. Almost all bio-therapeutics are posttranslationally modified, and there are important aspects of their production that require novel bioanalytical techniques to carry out batch-to-batch quality control. HDX-MS has emerged as an important technique to carry out this analysis. Houde et al. were one of the first to use HDX-MS to probe antibody structure, using both global and local HDX-MS on IgG1 antibodies to localize structural changes which accompanied glycosylation [112]. HDX-MS was also used to examine how posttranslational modifications of IgG1 antibodies, specifically methionine oxidation, fucosylation, and galactosylation, result in changes in protein conformational dynamics, impacting on their ability to binding to the FcγRIIA receptor [113]. The most common posttranslational modification is glycosylation, and this is a particularly challenging modification to deal with, as glycosylation sites are frequently heterogeneous. HDX-MS of glycosylated proteins can be carried out on the modified peptides [114]; however, this can be challenging for very heterogeneous samples. HDX-MS workflows have been developed to analyze these highly heterogeneous samples, specifically using deglycosylating enzymes to remove all PTMs [115]. HDX-MS is also being used as a tool to define how different biosimilars are from the original bio-therapeutic [111]. One of the most common modifications of protein therapeutics is the addition of covalently attached PEG molecules, and HDX-MS has been used
successfully to examine how nonnative PTMs change overall protein conformation [116].

1.3.4. HDX-MS to map epitope binding sites
One of the most frequent uses of HDX-MS has been for use of characterizing protein–protein binding sites. The development of bio-therapeutic drugs has led to a massive demand for the mapping of antibody epitope binding sites on proteins. HDX-MS has proved to be a robust methodology for this approach with many epitope mapping experiments published using this approach, with many more unpublished studies within the pharmaceutical industry. The epitope mapping approach suffers from some of the same disadvantages described in the small molecule section, as changes in deuterium exchange can be driven by both direct interactions, as well as long-range allosteric conformational changes. Even with this caveat, HDX-MS has been proven to be exceptionally well suited to determining antibody–antigen epitopes [26,117–119]. The HDX-MS approach is particularly useful for epitopes that span multiple protein binding sites, as these are particularly challenging to study by other structural approaches [117]. Another major advantage of the HDX-MS approach is that these interactions can be determined within their native environment, which is particularly important for studying antibody interactions with integral membrane proteins [120]. The use of HDX-MS has been particularly useful for the development of novel vaccines, as it allows for the determination of epitope binding sites for neutralizing antibodies [121–127]. HDX-MS has proven itself as a well validated approach for the epitope mapping of antibody–antigen complexes, and the further development of methodologies that allow for a faster, more comprehensive, and higher resolution analysis will only lead to further adaptation in both academia and industrial laboratories.

1.4. Other possible uses of HDX-MS in drug development
HDX-MS is a powerful bioanalytical technique, but it will most likely have the most impact on questions of major biological significance if it is used in a synergistic manner with other biophysical and structural techniques. The most commonly applied approach for HDX-MS experiments is mapping the dynamic differences in amide exchange between conditions on previously determined structures determined by NMR or X-ray crystallography. This is particularly useful in examining conformational changes that are unamenable to high-resolution approaches, especially in examining protein membrane complexes. The recent explosion of Cryo-electron microscopy, and the development of novel infrastructure and data analysis pipelines for high-resolution EM structures, provides an exciting opportunity for synergistic application with HDX-MS. Since Cryo-EM is also able to identify multiple different protein conformations, it is also particularly well suited to be combined with the dynamic information provided by hydrogen exchange. Further, there are many opportunities for HDX-MS to be used as a key tool for optimizing approaches for high-resolution approaches.

HDX-MS was first reported as a potential tool to optimize crystallization constructs in 2004, with the use of short deuterium pulses (10 s) to identify disordered N- and C-termini that could be truncated in further crystallographic constructs [10,128]. The use of this approach has also been applied to the crystallization of large multidomain protein complexes, as an example the complex of phosphatidylinositol 4-kinase IIIβ and its complex with the G protein Rab11, where intrinsically disordered regions were identified and truncated internally as well as at the N- and C-termini [22,23]. This led to constructs that were amenable to high-resolution structures with potent small molecule inhibitors [129]. HDX-MS was used in both construct design and characterization of small molecule inhibitors in a study targeting the NAD+-dependent lysine deacylase human sirtuin 1 (hSIRT1), a therapeutic target implicated in a wide range of diseases related to aging [130]. Unstructured regions in hSIRT1 were determined, which allowed them to identify the boundaries of constructs used in X-ray crystallographic studies. This approach has not obtained widespread acceptance within the crystallographic community, and there is most certainly a potential for growth in this area. This is particularly important for large multidomain multiprotein complexes containing many intrinsically disordered regions.

Another important advance in protein structural biology has been the use of conformational selective nanobodies as tools for crystal generation, and this has been particularly important in high resolution structures of GPCRs. HDX-MS is well suited for the screening of conformational specific nanobodies, particularly in the selection of nanobodies that are most likely to promote crystallization [75]. HDX-MS is also useful in the generation of constructs for NMR structural approaches [12,131]. Finally HDX-MS is well primed to help interpret structures generated by Cryo-EM, as protein–protein interfaces identified by EM can be verified by HDX-MS [13,69], with HDX-MS also playing key roles in defining dynamic processes invisible to both Cryo-EM and X-ray crystallography. The use of HDX-MS as a tool for optimizing and synergizing with high-resolution approaches provides another opportunity to enhance drug discovery efforts. For systems that are recalcitrant to high resolution approaches the combined approach of using HDX-MS with molecular dynamic simulations and computational docking provides an alternative approach to examine protein structure–function relationships. In the absence of high-resolution information, careful interpretation of HDX-MS data is required, with site-directed mutagenesis driven by HDX-MS as a suggested technique to verify structural hypotheses.

2. Conclusions
HDX-MS has become a widely accepted bioanalytical tool to study protein structure and dynamics. It has obtained wide acceptance in the biotech and pharma industry for the characterization of bio-therapeutics. The continued development of dedicated infrastructure at all stages of the HDX-MS experiment will lead to the ability to examine larger and more complex proteins, at higher spatial resolution. The use of HDX-MS is primed to grow, both as a technique to be used
in tandem with other medium- to high-resolution structural approaches, as well as in the drug development pipeline for screening of both fragments and small molecules. Continued refinement of the theoretical basis for why amides exchange at given rates will allow for a more detailed understanding of the molecular basis for differences in exchange among different protein states. The continued growth of bio-therapeutics provides an exciting application for HDX-MS and will certainly lead to further developments in both infrastructure and data analysis.

3. Expert opinion

Until recently, the majority of HDX-MS publications have examined protein dynamics by applying bottom-up HDX-MS approaches. This was conducted using home-built temperature-controlled fluidics devices, and analytical options were limited. Despite these shortcomings, this particular utilization of HDX-MS had immediate relevance for commercial and industrial scientists working in biopharmaceutical sectors who could immediately apply this to epitope mapping and bio-therapeutic characterization. With an increased interest in HDX-MS from these sectors came further advances in instrumentation, automation, and software that have also greatly benefited academia. In parallel to these improvements has been the concurrent increases in sensitivity and resolution seen in all classes of mass spectrometers.

This has led to HDX-MS experiments that are faster, more accurate, and more reproducible than ever before. With increased automation in both data collection and processing, the amount of time spent on any single experiment has decreased dramatically, and this has allowed for the investigation of larger protein complexes. In addition to this, the possible increases in spatial resolution achievable through the use of middle-down approaches have opened new avenues for research. HDX-MS has been fully utilized in the development of bio-pharmaceuticals and is widely used in various aspects of biopharmaceutical development and manufacture, from epitope mapping to monitoring batch-to-batch variation. However, there are areas where HDX-MS could be more widely applied, particularly as a tool for construct design for high-resolution structural approaches, as well as for more routine use in small-molecule drug development.

HDX-MS is uniquely well placed as a tool for medium-throughput small molecule binding studies in tandem with other biophysical approaches. HDX-MS places very few constraints on the protein being investigated, intrinsically disordered segments are as amenable to analysis as folded protein domains. Membrane proteins can be screened for compound binding effects while held within a lipid or detergent membrane – more accurately mimicking their in vivo environment. The complexity of protein systems able to be studied, as well as the ability to probe protein dynamics, will allow for a much deeper level of investigation into the molecular basis of how small molecules act as agonists, partial agonists, antagonists, and inverse agonists on important therapeutic targets. The small amounts of protein required for HDX-MS (when compared to NMR or X-ray crystallography), when paired with the ability to automate sample preparation, readily allows for the screening of small molecules. Furthermore, given the sensitivity of H/D exchange, and its capacity to tolerate high concentrations of compound present in the labeling experiment, HDX-MS is well suited to screen weakly binding compounds, or early stage fragments. Importantly, the time required for HDX-MS experiments is on the order of days compared to weeks or months in other structural approaches. HDX-MS could also be more widely adapted as a strategy to generate constructs amenable for high-resolution approaches, especially for protein complexes with extensive intrinsically disordered regions. A synergistic application of H/D exchange as a tool for optimization of other structural approaches combined with its ability to probe conformations unamenable to other approaches will greatly improve the ability to answer complex biological questions.

However, despite the increase in the HDX-MS user base, and despite the numerous possible advantages to screening small compounds with HDX-MS, HDX-MS is not yet routinely used in small-molecule drug discovery. This may be due to the fact that many of the same questions that dogged HDX-MS data 10 or 20 years ago still remain, and are exacerbated when working with small molecules. What is a ‘significant’ shift in exchange rate? Why do certain binding events counterintuitively result in increases in amide exchange rates? How can we distinguish between changes in amide exchange that are due to direct ligand interactions, and the allosteric shifts in distant parts of protein structure? How can H/D exchange information be better integrated with high-resolution structural information? Can HDX-MS experiments be streamlined and standardized into a repeatable approach where multiple labs examining the same sample will obtain the same result?

Many of these issues are surmountable, but they require more fundamental research. The application of the underlying theoretical framework surrounding amide-exchange has advanced little from Linderstrøm-Lang – we still work routinely at peptide-level resolution and expect exchange rates to rigidly follow overly simplistic amide exchange models. Higher resolution mapping of amide exchange rates for more and more proteins combined with high-resolution structural information, may help bring some clarity to these questions, with increasingly detailed models for the theoretical basis of amide exchange can be rigorously tested. Extensive work has been done in the use of a variety of computational methods to predict amide exchange rates of a given protein structure, and compare these to experimentally determined rates. However, there are still limitations for our understanding of why amide hydrogens exchange with solvent at a given rate. Further computational and theoretical work combined with rigorous experimental analysis will be essential to elucidate why amides exchange at a given rate.

Broadly, many current commercial software analysis packages attempt to brute-force peptides into EX2 kinetic profiles and carry out all analysis based on mass centroids, largely failing to take into account these nuances. Other software packages are engaging with more detailed spectral analysis however to discern deuterium incorporation patterns across peptides, and further
development in this approach will be useful to fully capitalize on all of the information present in HDX mass spectra.

HDX-MS has the potential to play a fundamental role in the future development of therapeutics, both bio-therapeutics and small molecules, and it is particularly well suited to act as complementary technique to other biophysical and structural techniques. The most likely place for hydrogen deuterium exchange to make a major impact is as a powerful tool alongside other structural techniques, as the combination of protein structure and dynamics will allow us to answer many fundamental questions about how proteins function. The technique’s strengths are numerous: an aqueous measurement that is highly sensitive, requiring only minor amounts of protein, utilizes only an isotope as a label, can operate in the presence of lipids, small molecules, and nucleic acids, can use semi-automated sample preparation and data collection, and has the potential for single amino acid resolution on mega-Dalton protein complexes. HDX-MS has far more potential for exploitation in drug discovery, but further research into the theoretical underpinnings of why amides exchange with solvent, as well as further developments in the MS infrastructure and data analysis will be essential for its continued progress.

**Funding**

JE Burke is supported by an open operating grant from the Institute of Infection and Immunity (Grant No. FRN 142393) of the Canadian Institutes of Health Research and a discovery grant from the Natural Sciences and Engineering Research Council of Canada (NSERC-2014-05218). GR Masson is funded by the Medical Research Council (MC_U105184308) and St Catharine’s College, Cambridge.

**Declaration of interest**

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

**ORCID**

Glenn R. Masson [http://orcid.org/0000-0002-1386-4719](http://orcid.org/0000-0002-1386-4719)
John E. Burke [http://orcid.org/0000-0001-7904-9859](http://orcid.org/0000-0001-7904-9859)

**References**

Papers of special note have been highlighted as either of interest (−) or of considerable interest (+) to readers.


* This article provides an excellent summary of how HDX-MS can be used for the design of crystallisation constructs through an optimised generation of N and C terminal deletions.


* This article provides in depth examination for why amide hydrogens in proteins exchange with solvent.


* This article provides in depth examination for why amide hydrogens in proteins exchange with solvent.

This paper is an essential resource for all laboratories carrying out HDX-MS to generate constructs for high resolution structural approaches, particularly for large multidomain, multiprotein complexes.

This article demonstrates the power of HDX-MS to generate constructs for high resolution structural approaches, particularly for large multidomain, multiprotein complexes.

This paper is an essential resource for all laboratories carrying out HDX-MS experiments, as it allows for the calculation of the intrinsic exchange rate for any amide hydrogen depending on conditions and primary sequence.

This is an excellent article that addresses some of the limitations that still exist in modeling why amide hydrogens exchange, fast photochemical oxidation of proteins mass spectrometry, and alanine shave mutagenesis. Anal Chem. American Chemical Society. 2015 Sep;87:8880–8888.

This is an excellent article that addresses some of the limitations that still exist in modeling why amide hydrogens exchange, fast photochemical oxidation of proteins mass spectrometry, and alanine shave mutagenesis. Anal Chem. American Chemical Society. 2015 Sep;87:8880–8888.

This is an excellent article that addresses some of the limitations that still exist in modeling why amide hydrogens exchange, fast photochemical oxidation of proteins mass spectrometry, and alanine shave mutagenesis. Anal Chem. American Chemical Society. 2015 Sep;87:8880–8888.

This is an excellent article that addresses some of the limitations that still exist in modeling why amide hydrogens exchange, fast photochemical oxidation of proteins mass spectrometry, and alanine shave mutagenesis. Anal Chem. American Chemical Society. 2015 Sep;87:8880–8888.

This is an excellent article that addresses some of the limitations that still exist in modeling why amide hydrogens exchange, fast photochemical oxidation of proteins mass spectrometry, and alanine shave mutagenesis. Anal Chem. American Chemical Society. 2015 Sep;87:8880–8888.

This is an excellent article that addresses some of the limitations that still exist in modeling why amide hydrogens exchange, fast photochemical oxidation of proteins mass spectrometry, and alanine shave mutagenesis. Anal Chem. American Chemical Society. 2015 Sep;87:8880–8888.

This is an excellent article that addresses some of the limitations that still exist in modeling why amide hydrogens exchange, fast photochemical oxidation of proteins mass spectrometry, and alanine shave mutagenesis. Anal Chem. American Chemical Society. 2015 Sep;87:8880–8888.

This is an excellent article that addresses some of the limitations that still exist in modeling why amide hydrogens exchange, fast photochemical oxidation of proteins mass spectrometry, and alanine shave mutagenesis. Anal Chem. American Chemical Society. 2015 Sep;87:8880–8888.

This is an excellent article that addresses some of the limitations that still exist in modeling why amide hydrogens exchange, fast photochemical oxidation of proteins mass spectrometry, and alanine shave mutagenesis. Anal Chem. American Chemical Society. 2015 Sep;87:8880–8888.


**This is an outstanding paper showing the power of combining cryo-electron microscopy with HDX-MS to examine challenging protein complexes of important therapeutic relevance.**


**This paper describes the first characterization of a GPCR heterotrimERIC complex using HDX-MS.**


**This is an excellent article that describes the effectiveness of HDX-MS as a screening platform for both low and high affinity inhibitors, and its effectiveness for identifying conformational changes at both orthosteric and allosteric binding sites.**


**This review highlights many of the complications in analysing HDX-MS data for protein small molecule interactions.**


* This paper along with reference 95 describes the usefulness of HDX-MS to probe the dynamic effects of allosteric inhibitors binding to the critical drug target Bcr-Abl.


* This review is an excellent summary of the challenges in characterizing biotherapeutics and outlines the role HDX-MS can play in bio-therapeutic drug development.


* This article demonstrates the power of HDX-MS to probe protein–antibody interactions, particularly for antibodies that bind to multiple epitopes on different proteins.


* This article highlights one of the major advantages of the HDX-MS techniques, as protein–protein interactions, in this case an antibody bond to its target membrane protein, can be interrogated in its native membrane environment.


