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Hydrogen/Deuterium Exchange Mass Spectrometry for Probing Higher Order Structure of Protein Therapeutics: Methodology and Applications

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Abstract

The higher order structure of protein therapeutics can be interrogated with hydrogen/deuterium exchange mass spectrometry (HDX-MS). HDX-MS is now a widely used tool in the structural characterization of protein therapeutics. In this article, HDX-MS based workflows designed for both protein therapeutic discovery and development processes are presented, focusing on the specific applications of epitope mapping for protein/drug interactions and biopharmaceutical comparability studies. Future trends in the application of HDX-MS to protein therapeutics characterization are also described.

Protein therapeutics have emerged as important therapeutic agents, starting in the early 1980s with the introduction of the first recombinant protein therapeutic — human insulin [1]. Protein therapeutics are now the second largest biopharmaceutical product category after vaccines. Among them, antibody-based drugs are the largest and fastest growing class of protein therapeutics with more than 40 antibodies and antibody derivatives approved for use, 350 more in the entire clinical pipeline including 28 being investigated in late clinical trials [2–4]. Compared to small molecule pharmaceuticals, protein therapeutics have unique characteristics and functions that cannot be mimicked by synthetic small molecules. In solution, under physiological conditions, proteins fold into unique three-dimensional structures that govern their biochemical functions and interactions with targets. It is these three-dimensional structures, therefore, that are in part subsequently responsible for drug efficacy. Consequently, analytical tools that offer information about protein higher order structure are of key importance in biopharmaceutical industries [5–8].

Hydrogen/deuterium exchange (HDX) represents a widely used technique for exploring protein conformation in solution [9,10]. This method relies on the exchange of the protein backbone amide hydrogens with deuterium in solution. The backbone amide hydrogens involved in weak hydrogen bonds or located at the surface of the protein may exchange rapidly while those buried in the interior or those involved in stabilizing hydrogen bonds exchange more slowly. By measuring HDX rates of backbone amide hydrogens, one can obtain information on protein dynamics and conformation. Hvidt and Linderstrom-Lang characterized hydrogen exchange in the mid-1950s, first with insulin, and showed that slower amide hydrogen exchange rates reflect the presence of hydrogen bonding [11,12]. The detection of the deuterium exchanged into the protein can be made with several analytical techniques including Nuclear Magnetic Resonance (NMR) spectroscopy [13] and Fourier Transform Infrared (FTIR) spectroscopy [14]. The exchange can also be readily measured by mass spectrometry (MS) as deuterium has a larger mass than hydrogen. The first experiment coupling electrospray ionization mass spectrometry (ESI-MS) with HDX was carried out by Katta and Chait in 1991 to probe protein conformational changes by MS [15]. The method was then paired with enzymatic digestion, based on earlier work of Rosa and Richards [16], enabling localization of structural changes to short peptic peptides [17]. Although the spatial resolution provided by peptide level HDX-MS may not be as high as other structural techniques such as NMR or X-ray crystallography, HDX-MS is not limited by either the protein size, or the crystallization properties which can be limiting factors for NMR or X-ray techniques. In addition, HDX-MS experiments can provide useful information with a small amount of sample (picomole quantities), can be used to study proteins that are difficult to purify or crystallize, can reveal protein conformational dynamics on a wide time scale and can be used to study complex protein systems [18,19]. Over the past two decades, the HDX-MS technique has evolved with advances in both HDX methodology and MS instrumentation (hardware and software), and emerged as an essential tool for the study of higher order structure of protein therapeutics in solution [20,21]. Applications of HDX-MS in the biopharmaceutical industry range from protein therapeutics discovery to development, including the study of protein conformation, conformational dynamics upon modifications, folding/refolding, protein-ligand/protein-protein interactions and protein aggregation [22–27]. In this review, we discuss general HDX-MS experimental approaches and current applications of the technique in (i) probing antigen/antibody binding interfaces (epitope mapping) in drug discovery and (ii) comparability studies of antibody drugs in product development.

Methodologies

In HDX experiments, protein labeling occurs when labile hydrogens in the protein exchange with deuterium in solution. There are two main approaches to performing protein labeling by HDX: continuous labeling and pulsed labeling [28], as illustrated in Figure 1. In the continuous labeling approach, several aliquots of the protein sample are exposed to the deuterated buffer under conditions that retain its native conformation (i.e., pH 7 and physiological buffer conditions). An aliquot of the protein sample is incubated in deuterated buffer for a certain period of time and then labeling is quenched. This strategy allows the monitoring of deuterium incorporation into the protein structure as a function of time,

providing information on the conformational dynamics of a protein under equilibrium conditions. In the pulsed labeling approach, a protein is first perturbed in some manner, such as by adding a chemical denaturant, forming complexes, changing pH or changing temperature. Then the protein is exposed to deuterated buffer (typically with higher pH, e.g. pH 8–10) for a very brief period of time, typically 10 seconds or less [29,30]. With short pulse time, HDX will only occur at positions that are solvent-exposed and not involved in hydrogen bonds.

Following either labeling approach, the deuterated protein is placed in a low pH (e.g. pH 2.5) buffer at low temperature to reduce the exchange rate and “quench” the reaction during subsequent analysis steps. Quench conditions minimize back exchange in the subsequent analysis steps [17]. MS is used to measure the deuterium incorporation into the protein. Measurements can be performed at the intact protein level to reveal the overall deuterium incorporation, or at the peptide level to reveal localized exchange information. It is important to note that successful HDX-MS experiments require careful design of experimental protocols (see Ref. [21]) and fast, but efficient chromatographic separation at low temperature (i.e. zero degree Celsius) to control the deuterium back exchange [17,31]. For peptide level HDX-MS, quenching is followed by enzymatic digestion (with an acid protease) and fast separation by liquid chromatography (LC). Non-deuterated control experiments are also performed to identify all the peptic peptides that were produced during enzymatic digestion. Pepsin is the most widely used protease in HDX because it is stable and highly active under the acidic quench conditions; pepsin digestion is reproducible under the same digestion conditions [32]. Pepsin digestion produces redundant information through overlapping peptides, helping to refine the location of deuterium in peptide level HDX.

Spatially resolved HDX-MS information can also be obtained by measuring the deuterium levels of overlapping fragment ions created in the mass spectrometer from the deuterated peptic peptides. One of the main drawbacks of HDX gas phase fragmentation is related to hydrogen scrambling, meaning that intra-molecular migration of peptide amide hydrogens can occur, especially in traditional collision induced dissociation (CID) fragmentation [33]. Non-ergodic fragmentation techniques with minimal vibrational excitation such as electron transfer dissociation (ETD) or electron capture dissociation (ECD) are the preferred activation methods used in HDX-MS experiments as they minimize hydrogen scrambling [34,35]. Combining enzymatic digestion and gas phase fragmentation, single-amide resolution for deuterium incorporation measurements has been achieved using model peptide systems [36]. This approach has been applied to the characterization of conformational dynamics of amyloidogenic protein β_2 -microglobulin [37] and the interactions between peroxizome proliferator activated receptor and ligands [38]. It remains to be seen if ETD will become a routine method in HDX because a number of factors – including ETD efficiency, narrow chromatographic peak width in ultra high performance LC (UHPLC), and a general lack of software that considers ETD/HDX – remain obstacles to widespread implementation.

HDX-MS Applications

Epitope Mapping

Non-covalent binding events, such as protein-protein interactions and protein-ligand interactions, are involved in many drug action mechanisms [39]. Understanding antigen-antibody interactions and identifying the epitopes are of crucial importance in drug discovery for the design of effective protein therapeutics [4,18,40]. The epitope regions also need to be specifically mapped to ensure patentability and protection of distinct intellectual property. X-ray crystallography provides the highest resolution (atomic resolution) for epitope characterization. However, many antigen-antibody crystal complexes are difficult to obtain. NMR spectroscopy is often limited to smaller proteins (<25 kDa). Scanning of synthetic peptide arrays (the affinity selection of short phage-displayed peptides against the monoclonal antibody (mAb) of interest) allows the identification of epitopes, however, erroneous prediction may occur due to the presence of amino acid motifs on the antigen surface similar to those actually present in the mAb-bound region [41]. Conformational epitopes may not be identified by synthetic peptide arrays. Although limited proteolysis by MS can be used to probe epitopes by enzymatically digesting immobilized antigen-antibody complexes [42], this method is laborious due to the requirement of antibody immobilization, and non-robust due to variability of the experimental results generated by different laboratories. Spatially resolved HDX-MS is a good addition to the tools used for analyzing antigen-antibody interactions under physiological conditions. When a protein complex forms, the interface between the binding partners may occlude solvent, hence reducing the exchange rate due to steric exclusion of solvent. However, a number of other factors can complicate the interpretation of exchange into protein complexes, as previously described [18] and below. For example, the stabilization of exchangeable amide hydrogens by the binding interaction could also lead to the reduction of exchange rate.

In order to probe binding sites, HDX experiments are usually performed on both the free antigen as a reference experiment and the antigen-antibody complex. The data generated from the two experiments are compared and regions in the protein that possess different exchange kinetics are highlighted as potential epitopes (Figure 2). More and more epitope mapping studies by HDX-MS are published each year using various labeling approaches [23,25,43–45]. To illustrate the HDX-MS approach for epitope mapping, one example from the continuous labeling approach to defining the protective epitope on factor H binding protein (fHbp) is briefly described below [44].

fHbp is a surface-exposed lipoprotein and an important component of two meningococcal vaccines currently in clinical development [46]. Peptide level HDX-MS was employed [44] to map the interface between fHbp and the bactericidal mAb 12C1 based on the differential rate of deuterium incorporation in fHbp with or without binding with 12C1. Nineteen peptides were identified in the reference experiment, constituting 97% of the fHbp sequence. Seven out of nineteen fHbp peptides revealed reduced HDX rates upon binding with 12C1, with their deuterium uptake curves shown in Figure 3. Four discontinuous segments including three N-terminal and one C-terminal epitope fragments were identified as potential epitopes, and clustered in a distinct region in the 3D structure (Figure 3). All four segments

identified by HDX-MS were present in the final epitope site that was confirmed by X-ray crystallography in a parallel study [44].

Other factors can complicate the interpretation of exchange into protein complexes [18]. In the simplest case, antibody binding does not induce conformational changes to antigen. However, sometimes the binding events induce changes in HDX in regions distant from the epitope [47,48], a phenomenon resulting from allosteric effects. In cases where conformational changes are altered far from the interaction site, the interpretation of HDX data becomes more complex [49]. Binding events may also enhance the thermodynamic stability of a protein, possibly causing the protein complex overall to be more strongly protected from HDX than the free protein [50]. In order to distinguish reduced HDX rates caused by protection in binding surfaces from those caused by conformational changes, a practical approach in the biopharmaceutical industry is to combine HDX-MS results with computational analysis and site-directed mutagenesis, as illustrated in Figure 2. Protein segments identified by HDX-MS with reduced exchange rates upon binding provide invaluable information for guiding subsequent computational docking prediction analysis. Regions that do not show any change in HDX can often be excluded, allowing the restriction of the possible regions involved in the docking study [43]. On the other hand, HDX-MS experimental data can also be used to confirm epitope regions predicted by *in silico* analysis. For example, in a study of Ca^{2+} - independent phospholipase A_2 inhibition by fluoreketone ligands, HDX-MS experiments showed important decrease of deuteration in the inhibitor contact regions predicted by computational analysis [51]. To further confirm the identified epitope, site-directed mutagenesis (single alanine mutation or alanine shaving mutagenesis [52,53]) followed by functional assays can be performed to evaluate if mutations in the antigen's amino acid sequence affect the binding or not. This approach has been demonstrated by many studies, for example by Brier *et al.* to identify the binding region of inhibitors targeting the human mitotic kinesin Eg5 [54]. By conducting mutagenesis on the two regions that showed decrease in deuterium incorporation rates – loop L5/helix alpha2 (region Tyr125-Glu145) and strand beta5/helix alpha3 (region Ile202-Leu227) – the ligand binding site was pinpointed. Combined with computational analysis and targeted mutagenesis, HDX-MS provides an efficient and rapid way to probe epitopes, providing invaluable information to the understanding of the mechanism of actions and structure/function relationship in characterizing novel biopharmaceuticals.

Comparability Studies

In the development and commercialization of protein therapeutics, it is important to conduct comparability studies to ensure the consistency of higher order structure and structural dynamics of protein therapeutics [8]. Proteins that are not folded properly are frequently targets for various degradation pathways, are usually prone to aggregation, and may also trigger an immune response by exposing protein segments recognized as foreign by the immune system [55]. Conformational changes in protein therapeutics can be triggered by a variety of factors, such as chemical modification, mutation, surface binding, thermal or pH denaturation caused by differences in manufacturing processes, or during storage. While changes in primary structure can be detected by regular LC/MS or tandem MS (MS/MS) analysis, in some instances conformational changes can occur without a change in protein

primary structure. The investigation of such changes requires techniques that are able to analyze protein higher order structure. In some cases, due to the complexity and limitations of NMR and X-ray crystallography techniques, routine biopharmaceutical analyses of conformation and stability of protein therapeutics typically rely only on classical biophysical methods, such as Circular Dichroism, FTIR spectroscopy, calorimetry and size exclusion chromatography which are less time consuming but only provide limited, global structural information. HDX-MS has emerged as a more informative method for higher order structure characterization, and it is especially suitable for comparability studies detecting more subtle structural differences and offering localized information about protein segments with unexpected protection levels [56–59]. This method is used as an orthogonal measurement along with other biophysical techniques in structural comparability studies for drug development. Two examples are described below, including the assessment of higher order structure and dynamics of a therapeutic protein, Granulocyte Colony Stimulating Factor (G-CSF) upon PEGylation (covalent attachment of PEG, polyethylene glycol) [24] and a comparability study of a full length, glycosylated therapeutic mAb manufactured using different processes.

PEGylation of therapeutic proteins is an effective strategy to improve the pharmacokinetic behavior of proteins [60]. However, it may cause possible conformational changes, steric interferences, and changes in electrostatic-binding properties for certain proteins [61,62]. In a study with G-CSF [24], peptide HDX-MS experiments with a continuous labeling approach were carried out on PEGylated and non-PEGylated forms of G-CSF. Forty-six common peptides were identified in both proteins, constituting linear sequence coverage of 91% of the peptide backbone of G-CSF, with overlapping peptides present in multiple regions. Deuterium uptake levels over a time span of 10 s to 4 h were obtained for each peptide. Small but reproducible differences in deuteration were found in multiple regions of G-CSF, however, there were no detectable differences in deuterium incorporation upon PEGylation for 65% of the G-CSF sequence. A peptic peptide covering residues 19–32 (part of an alpha-helix), which contains multiple amino acids involved in receptor binding, indicated no difference in deuteration and remained low in deuterium uptake for PEGylated and non-PEGylated forms. Overall, no major protein conformational changes occurred based on the extent of the measured HDX differences. This study indicated that the changes observed in HDX, while statistically significant in terms of the method, may not have significant impact on the global conformational properties and biological activity of G-CSF, a finding consistent with other studies showing that G-CSF maintains most of its biological activity after PEGylation [63].

Conformational changes may occur in protein therapeutics as a result of changes in the manufacturing process. Comparability studies with HDX-MS were conducted both at global and peptide level in order to compare mAb samples (same product) from three different manufacturing processes. The HDX-MS experiments were performed on a nano-UHPLC system with Waters HDX technology, equipped with an automated sample handling system for protein labeling (in a continuous labeling approach) and quenching, followed by online pepsin digestion for peptide level analysis [31]. Mass measurements were performed with quadrupole-time of flight (Q-TOF) mass spectrometer. Data were analyzed by DynamX software with additional manual verification of the raw spectra.

For global HDX-MS analysis, the overall deuterium exchange levels for all three mAb lots were compared. The same experimental setup as described above was employed, but the digestion step was removed. Deuterium uptake levels over a time span of 10 s to 4 h were obtained for each mAb and their relative deuterium uptake curves are shown in Figure 4a. The average relative standard deviation for individual labeling time point was less than 5%, illustrating the high reproducibility of the methodology. No statistically significant differences in measured HDX levels were observed at any labeling time, indicating that there was no global conformational difference among the three lots of mAb samples. The measured average relative standard deviation for the differences between replicates using an automated system and a manual system was estimated to be less than 5%. A positive control experiment was also conducted to compare the deuterium uptake between a mAb sample treated with 1.5 M Guanidine-HCl for 1 h with the same mAb untreated, as shown in Figure 4b. Guanidine-HCl denatured the mAb, therefore, the protein became more unfolded allowing for more deuterium to be incorporated. Consequently, the deuterium uptake for the treated sample was higher than the untreated sample, demonstrating that the sensitivity of the method is high enough to differentiate such global conformational changes.

Peptide level HDX-MS experiments were carried out to ensure the local structural consistency. Common peptic peptides were identified in the three samples and the deuterium uptake was monitored for each of these peptides. Figure 4c exhibits some representative deuterium uptake curves for the four commonly identified peptides among three samples. Such comparison analyses were performed on all the commonly identified peptides, and the deuterium uptake levels in all peptides indicated no significant differences exceeding the error of the measurements among the three samples analyzed. The same comparability studies using the same lots of mAb samples were also conducted at a different laboratory location/site, by a different operator, on a similar type of HDX-MS platform with a different mass spectrometer and no automated sample handling system for protein labeling. No differences in deuterium incorporation between the three antibodies samples derived from three manufacturing processes were observed at either the intact or the peptide level by the second laboratory/site (data not shown). This cross-site comparison study clearly demonstrates HDX-MS as a robust and reproducible method. HDX-MS data can be utilized to establish higher order structure consistency of mAb samples in support of comparability studies [8].

Concluding remarks

As illustrated in this review, HDX-MS is one of the key analytical techniques for epitope mapping in protein therapeutics discovery and for comparability studies in product development. With enhanced performance of MS instrumentation, advances in separation science and software development in data analysis, proteolysis-based HDX-MS experiments can enable the analysis of even larger proteins and protein complexes. Utilizing ETD fragmentation along with peptide level HDX-MS has the potential to enhance the spatial resolution of the technique, although several hurdles remain before it is routine. Coupling HDX-MS with computational modeling and docking studies could further integrate this technique into discovery workflows [64,65]. It is expected that HDX-MS will continue to play an important role in assessing higher order structure of protein therapeutics.

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Figure 1. General scheme for HDX-MS experiments: (a) Continuous labeling, (b) Pulsed labeling, followed by either global level or peptide level analysis. LC/MS: liquid chromatography/mass spectrometry. This figure is derived from Ref [28].

Figure 2.
Scheme for epitope mapping by HDX-MS.

Figure 3.

Example of epitope mapping by HDX-MS. The graphs show the deuterium uptake data over 30 min labeling for fHbp fragments in the absence (red) or presence (blue) of mAb 12C1. Deuterium incorporation was reduced by mAb 12C1 in each fragment shown. The peptides protected by mAb 12C1 in the HDX-MS analysis are highlighted in red on the fHbp structure. Numbered spheres indicate the start/end of each affected fragment. Figure was adapted with permission from Ref. [44]. Copyright (2013) Proceedings of the National Academy of Sciences.

Figure 4.

Comparability studies by HDX-MS. (a) Global level HDX-MS: deuterium uptake curves for intact mAb from three manufacturing processes. (b) Deuterium uptake curves between a Guanidine-HCl (GndCl) treated and untreated mAb samples at intact level; (c) Peptide level HDX-MS: deuterium uptake curves of four representative peptic peptides from mAb manufactured from three different processes: top two peptides are from the light chain (LC) of the mAb, bottom two peptides are from the heavy chain (HC) of the mAb.