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HDX-MS Guided Drug Discovery: Small Molecules and Biopharmaceuticals

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Abstract

Hydrogen/deuterium exchange coupled with mass spectrometry (HDX-MS or DXMS) has emerged as an important tool for the development of small molecule therapeutics and biopharmaceuticals. Central to these advances have been improvements to automated HDX-MS platforms and software that allow for the rapid acquisition and processing of experimental data. Correlating the HDX-MS profile of large numbers of ligands with their functional outputs has enabled the development of structure activity relationships (SAR) and delineation of ligand classes based on functional selectivity. HDX-MS has also been applied to address many of the unique challenges posed by the continued emergence of biopharmaceuticals. Here we review the latest applications of HDX-MS to drug discovery, recent advances in technology and software, and provide perspective on future outlook.

Keywords

HDX; HDX-MS; DXMS; Hydrogen-Deuterium Exchange; Drug Discovery; Therapeutics; Small Molecule; SAR; Large Molecule; Biopharmaceutical; Nuclear Receptor; GPCR; PPARG; Functional Selective; Ligand Bias; Biosimilar

HDX-MS Introduction

Hydrogen/deuterium exchange is an acid-base catalyzed reaction used to label proteins and report on the local environments of amide hydrogens. Coupling this approach with modern high-resolution mass spectrometry to monitor deuterium incorporation enables precise and sensitive data collection (<1ug/experiment at low μM concentration), and interrogation of large proteins and complexes [1-3]. Differential HDX-MS analysis of a protein under different conditions (e.g apo vs. holo protein) has emerged as an important tool to probe the effects of chemical modifications, mutations, and binding events on protein stability and conformational dynamics (Figure 1). The development of fully automated HDX platforms with improved software has enabled the rapid collection and near real-time processing of

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data with statistical analysis, a critical advancement for the integration of HDX-MS into drug discovery programs [4-9]. Correlating deuterium incorporation patterns from several small molecule ligands with functional assays has proven to be an effective approach to develop structure activity relationship and delineate functional selectivity between closely related compounds [10-13]. HDX-MS also provides a means to identify allosteric small molecule binding sites [2, 14, 15], which are often challenging to locate but desirable for the development of agents with improved selectivity.

The application of HDX-MS to the development and manufacturing of biological therapeutics reflects the unique challenges that face this class of drugs. HDX-MS has long been used to map the conformational epitopes of antibody-antigen complexes; however recent applications have focused on monitoring protein stability in response to chemical modifications, protein engineering, and alternative manufacturing processes [16]. These issues have emerged along with the rise of biopharmaceuticals and reflect the expanding focus on manufacturing quality control and defining standards for off-patent biosimilars [17]. Several in depth reviews have been published on the fundamentals of HDX-MS and its application to a range of biological systems [18-23]. Here we review the latest applications of HDX-MS to small molecule and biopharmaceutical drug discovery, the state of the art platform and software technologies, and directions for future development.

HDX-MS for Small Molecule Drug Discovery

Differential HDX-MS is a well-suited approach for interrogating the alterations in protein conformation induced by small molecule ligand binding [24]. The pharmacology of ligands have traditionally been categorized as agonists, partial agonists, antagonists, and inverse agonists depending on whether they fully or partially activate, block, or repress a protein's activity. While these classifications are informative, it has become clear that there is significantly more underlying complexity, and ligand classes can be further delineated. A comprehensive review of differential HDX-MS analysis of protein-ligand interactions has previously been published [22]. Here we focus on the most recent applications of HDX-MS to small molecules targeting the nuclear receptor (NR) and G-protein coupled receptor (GPCR) protein families.

Nuclear receptors

NRs are the pharmacological target of ~10% of FDA approved drugs, a consequence of their implication in human disease and tractability for drug discovery [25]. The challenge of pharmacologically targeting NRs is achieving functional selectivity, a strategy to limit adverse effects due to the complex gene networks controlled by these ligand regulated transcription factors [26]. To that end, differential HDX-MS has been applied to characterize the effects of ligand binding on the isolated ligand binding domains (LBDs) of several NRs [11, 12, 27, 28], and was extended to identify intra-domain allosteric effects in a comprehensive analysis of the full length vitamin-D receptor (VDR) bound to DNA [29]. HDX-MS has been applied extensively to drug discovery efforts targeting the nuclear receptor peroxisome proliferator-activated receptor gamma (PPAR γ), initially providing structural insights to delineate the thiazolidinedione and SPPARM (selective PPAR γ modulator) ligand classes of PPAR γ modulators [10, 30, 31]. More recently HDX-MS

guided drug design was used to develop SR1664, a representative molecule from the novel class of Functional Selective PPAR γ Modulators (FSPPARMs) that act as classical antagonists while modulating the obesity-induced phosphorylation of the receptor and a subset of target genes [32]. HDX-MS was also applied recently to support the finding that multiple ligand copies can bind concurrently to the PPAR γ LBD, with the second lower affinity site having functional consequence at physiological concentrations that were previously unappreciated [33]. Together, these studies highlight the power of correlating subtle but statistically significant perturbations in receptor deuterium exchange with a range of functional assays to better define ligand classes, and to help guide improvement of their functional selectivity and ultimately their therapeutic index (Figure 2).

G protein-coupled receptors (GPCRs)

GPCRs constitute the largest family of cell surface signaling proteins, relaying extracellular signals into intracellular responses to maintain cellular homeostasis. This transduction is achieved through ligand-induced alterations to the equilibrium of conformational ensembles, and is typically accompanied by allosteric changes to distal regions of the receptor. Activation of GPCRs by endogenous ligands or synthetic pharmacophores drives downstream signaling events that are mediated by G proteins, β -Arrestins, and GPCR kinases (GRKs). Dysregulation of GPCR function is the underlying cause of many human diseases; a primary reason GPCRs are targeted by nearly half of currently approved drugs [34, 35]. Similar to NRs, recent advances have revealed the complexity of GPCR function as more than a binary on/off switch modulated by agonists and antagonists, with modern pharmacology paradigms of allosteric modulation and ligand bias providing strategies to fine-tune receptor signaling [36-39].

HDX-MS has emerged as an important tool for probing the conformational ensembles and allosteric changes of GPCRs, complementing what can be garnered from other structural techniques such as NMR and crystallography. The primary hurdle for the initial application of HDX-MS to interrogate GPCRs was identifying mass spectrometry compatible detergents that would maintain protein solubility. These conditions were first reported in a study profiling the ligand-dependent perturbations to β 2-adrenergic receptor (β 2-AR) in response to the inverse agonist carazolol [40]. This study enabled a more rigorous HDX-MS analysis of β 2-AR in the presence of a range of ligands from full agonist to inverse agonist [41]. Together, these findings illustrate that the intra- and extracellular loop regions of β 2-AR, previously unresolved or truncated in crystal structures, exhibit unique HDX perturbations in response to functionally-distinct ligands and provide a strategy to distinguish novel β 2-AR modulators with desired physiological response. To further study the molecular underpinnings of G protein activation by agonist-bound β 2-AR, HDX-MS was applied to identify structural links between the receptor-binding surface and nucleotide-binding pocket of the G protein, providing a rationale for the mechanism of activation [42]. While this study provided critical insights into G protein signal transduction and function, β 2-AR coverage was not obtained, likely the result of ion suppression due to the poor digestion efficiency of GPCRs relative to soluble proteins. This limitation highlights the technical challenge of improving GPCR digestion efficiency, which may be aided by recent additions to the repertoire of HDX compatible proteases [43, 44], or by applying affinity capture to remove

the cytosolic G proteins prior to proteolytic digestion [45]. An alternative strategy to improve digestion efficiency may be to utilize more native-like nanodiscs to reconstitute β 2-AR in the absence of detergent micelles as recently demonstrated with transmembrane protein Gamma-glutamyl carboxylase (GGCX) [46].

HDX-MS and Biopharmaceuticals

Biopharmaceuticals have had continued success and are projected to account for the majority of newly FDA approved drugs in the near future [47]. Biopharmaceuticals such as monoclonal antibodies, synthetic peptides and recombinant proteins have their own unique development challenges, but can be exquisitely selective relative to small molecules contributing to their generally excellent safety profiles (excluding mechanism based side effects). Previous reviews have focused on the expanding role of HDX-MS to process related aspects of biopharmaceuticals such as manufacturing quality control of recombinant molecules [16], as well as defining 'similarity' guidelines for off-patent biosimilars [48]. Here we present a focused review of recent HDX-MS applications to protein therapeutic discovery.

Therapeutic Antibody Development

HDX-MS has proven a robust contributor in the development of monoclonal antibody therapeutics, as a reliable method for conformational epitope mapping of antibody-antigen interactions in their native solution state [16, 49, 50]. Mapping of conformational epitopes has traditionally been accomplished by alanine scanning mutagenesis, protease-protection experiments, or x-ray crystallography, which can be labor intensive with each approach having unique limitations [50]. Recent studies have utilized improvements in instrumentation to probe antibody-antigen epitopes of larger, more biologically relevant protein complexes [51-56]. Furthering these studies has demonstrated the role of chemical modifications on the global conformation of monoclonal antibodies and the potential impact on therapeutic efficacy and safety [57]. The stability of monoclonal antibodies engineered with alternative domain substitutions has been measured with HDX-MS, highlighting the potential for novel therapeutic motifs and improved pharmacokinetics [58]. The specificity of monoclonal antibodies has been harnessed for targeted delivery of Antibody-Drug conjugates (ADCs) with a particular focus on chemotherapeutic agents in the current pipeline [59]. HDX-MS has been applied to interrogate the higher-order structure of ADCs to evaluate how the process of drug conjugation impacts the conformation and dynamics of the monoclonal antibody [60].

Recombinant Protein Therapeutics

Protein based therapeutic development, which dates back 30 years to the introduction of recombinant insulin, has also benefited from the application of HDX-MS [61]. The oligomeric state and stability of insulin analogs were monitored with HDX-MS in back to back reports, and demonstrated to be predictive of pharmacokinetic properties such as onset and duration [62, 63]. β -glucocerebrosidase (GCase) is an essential metabolic enzyme whose dysfunction due to naturally occurring mutations results in the most prevalent lysosomal storage disorder Type 1 Gaucher's disease. Enzyme replacement therapy consists of

intravenous infusion of recombinant GCase and HDX-MS has been applied to characterize the effect of oxidation on protein stability and dynamics [64]. This series of reports demonstrates the varied applications of HDX-MS to biopharmaceutical discovery and development, an area that is likely to see continued expansion in the coming years.

HDX-MS Technologies and Future Directions

The sophistication of HDX-MS technology has matured considerably over the past decade, evolving from labor-intensive manual bench top experiments to fully automated experimental platforms. Improvements to HDX-MS spatial resolution, from peptide level to single residue have been demonstrated by several groups in recent years with the application of electron-capture and electron-transfer dissociation fragmentation [65-69]. While the requirements for these approaches suggest that they are unlikely to become routine in the near future, combining newly developed HDX-MS compatible proteases may provide a feasible strategy to improve spatial resolution through subtractive analysis. Efforts to advance the detection limits of HDX-MS to sub-second time scales for the purpose of interrogating rapidly exchanging systems have been achieved through the development of micro-fluidic chips [70-72], as well as simulated approaches compatible with automated platforms [73].

The impact that several research groups have had on the field over the past decade has led to instrument manufacturers now selling fully automated HDX-MS systems. This has made HDX-MS more accessible with reduced cost per experiment and significantly higher sample throughput. For drug discovery efforts, the application of HDX-MS to high-throughput screening appears to be a natural progression for which the principles have been described previously [74, 75]. From a broader perspective, HDX-MS will continue to be used in parallel with the repertoire of available structural approaches such as crystallography [10], NMR [31, 76, 77], SAXS [78-80], and Cryo-EM [81-84] to tackle challenging biological questions that require multiple approaches. Protein structural plasticity is directly linked to protein function, and as such HDX will continue to play a significant role in understanding the link between protein structure and activity. These are exciting times for HDX-MS and we expect the field to continue growing, as the barrier for entry has been lowered and the diversity of applications expanded.

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Highlights

- HDX-MS has been applied to develop both small and large molecule therapeutics.
- Recent improvements to experimental platforms and software have increased throughput.
- Correlating HDX-MS with functional assays enables small molecule SAR development.
- HDX-MS can address many of the unique challenges of biopharmaceutical development.

Figure 1. Schematic of a typical HDX-MS workflow

a. A protein sample in the absence or presence of a ligand (shown in magenta) is incubated at 4°C in D_2O containing buffers for various time intervals **b.** After "on-exchange", the protein is denatured and the deuterium uptake is quenched under acidic conditions (pH 2.5) at 0° C followed by proteolytic digestion using an on-line pepsin column **c.** Proteolytic peptides are then separated using a gradient column and subjected to mass determination using a high resolution mass spectrometer **d.** Average deuterium incorporation for each peptide over time is calculated from their mass shifts (top) and the differential HDX data (apo versus ligand bound) is overlaid onto an available three-dimensional structure (bottom). Regions that are differentially protected are color coded according to the HDX WorkBench software scheme.

Figure 2. Correlating HDX-MS with functional output

Analysis of pharmacologically distinct PPAR γ ligands for both cellular transactivation and HDX demonstrates a strong correlation between helix 12 peptide (SLHPLLQEIKDLY) protection and receptor activity. Thus, HDX can be used as a predictive assay to support lead optimization of functional selective PPAR γ modulators (FSPPARMs).