



## CRITICAL INSIGHT

# Hydrogen Exchange Mass Spectrometry: Are We Out of the Quicksand?

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**Abstract**

Although the use of hydrogen exchange (HX) mass spectrometry (MS) to study proteins and protein conformation is now over 20 years old, the perception lingers that it still has “issues.” Is this method, in fact, still in the quicksand with many remaining obstacles to overcome? We do not think so. This critical insight addresses the “issues” and explores several broad questions including, have the limitations of HX MS been surmounted and has HX MS achieved “indispensable” status in the pantheon of protein structural analysis tools.

**Key words:** Deuterium exchange, HDX, H/D exchange, Protein conformation

## Introduction

Twenty years ago, Katta and Chait [1] showed that mass spectrometry (MS) could be used to monitor hydrogen exchange (HX) and, therefore, protein conformation. Since then, the field has been rapidly growing due to remarkable advances in protein availability, instrumentation, and software. However, despite all the successes that hydrogen exchange mass spectrometry has witnessed over the years, there are still some who doubt that this method can provide anything useful and that it can be routinely applied to the study of protein conformation and dynamics. Is HX MS, in fact, still in the quicksand with many remaining obstacles to overcome? This critical insight is not intended to be a “classic” review but, rather, it is the authors’ collection of opinions, which answer the basic questions (Figure 1): Have the limitations of HX MS been surmounted and has HX MS achieved “indispensable” status in the pantheon of protein structural analysis tools?

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## Is HX MS Useful for Protein Structural Analysis?

Since the early reports of Anfinsen and coworkers [2, 3], it is clear that protein structure is tied to protein function and vice versa; therefore, understanding the structure of proteins provides tremendous insight into how proteins function and what their roles are in living organisms. The well-established structural methods (X-ray crystallography, NMR, and cryoEM) can routinely provide highly detailed structural data for many proteins. For these methods, structural analysis may require large quantities of soluble protein, often at a high concentration. However, some proteins cannot be produced in the quantities that might be required, some proteins may aggregate or precipitate at higher concentrations, or some are, for a variety of reasons, just simply not amenable to crystallization. HX MS is especially suited for analysis of these “difficult and otherwise incompatible” proteins that cannot be studied with methods like crystallography, NMR, and cryoEM. With only a nanomole of relatively dilute protein, details of conformation and conformational changes can be obtained with HX MS [4–10]. A high-resolution structure cannot be obtained from HX MS and atomic coordinates cannot be generated but, following the old adage, something is better than nothing for these proteins that cannot be analyzed by other methods.

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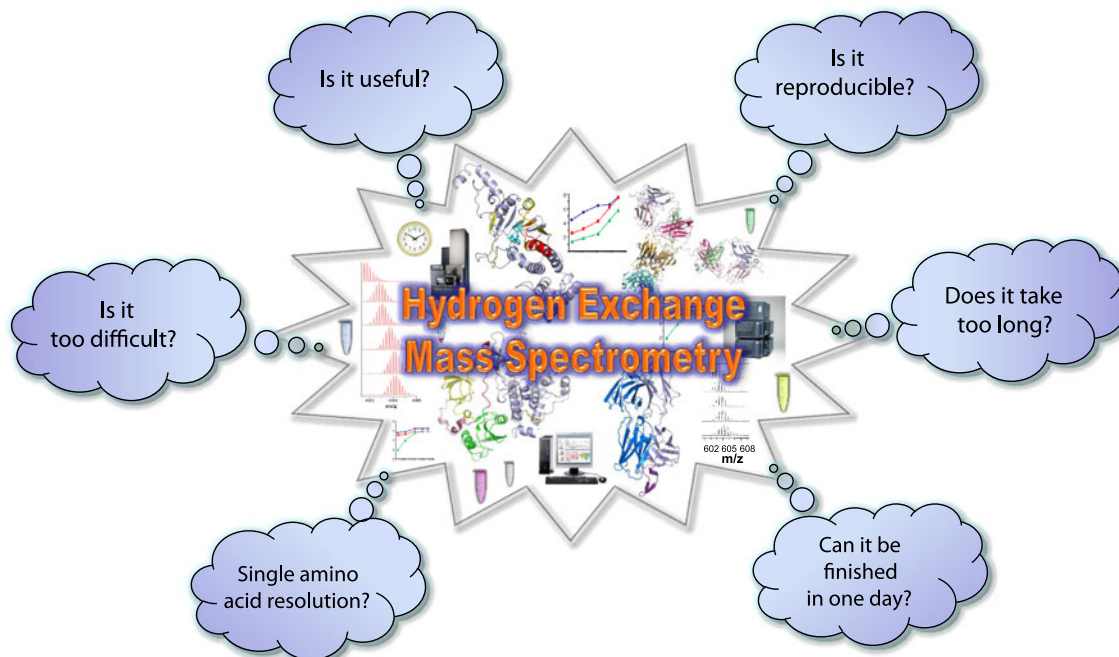


Figure 1. Questions to ponder about modern hydrogen exchange mass spectrometry

For proteins that already have high-resolution structures, these structures become even more valuable with the addition of HX information. HX MS does not supersede X-ray crystallography or NMR structural analysis. On the contrary, it is highly complementary to these methods, and HX MS data becomes more powerful when interpreted in the light of three-dimensional structural information, providing substantial evidence about flexibility and solution dynamics, changes in conformation during function, and interaction surfaces (e.g., [11–17]). Protein function is influenced by both protein structure and protein dynamics/flexibility. Monitoring hydrogen exchange (by MS or other means) provides information on protein motion/flexibility [11, 12, 18–23] and the impact of ligand binding on protein dynamics (e.g., [24–29]). For example Kong et al. [27] studied the binding reaction of the HIV-1 gp120 envelope glycoprotein to the CD4 receptor by HX MS and noticed that neither unliganded gp120 nor free CD4 were substantially unstructured, suggesting that most of the diverse conformations that make up the gp120 unliganded state are reasonably ordered. In a study of nucleoside reverse transcriptase inhibitors against HIV [29], HX MS revealed allosteric changes in particular domains of the protein. Tiyanont et al. [30] recently described analysis of the Notch activation switch, revealing that in the auto-inhibited conformation, an important site (S2) is protected against exchange but that conversion to the active state leads to accelerated deuteration in and around the S2 site. HX MS analysis of a Notch transcription complex described the details of conformational changes during protein–protein interactions, flexibility changes during complex formation, and the relative contributions of each member of the complex [31]. Details of the interactions between the

molecular chaperone Hsp90 and its client transfer protein Sti1 [32] recently showed which regions of Hsp90 interact with Sti1 and new details about Hsp90 molecular mechanisms; and the list goes on.... there are many, many more examples of the positive and lasting impact of HX MS measurements on the field of structural biology. Overall, we think HX MS is very useful as a structural analysis tool, especially when combined with other structural techniques.

## Is HX MS Reproducible?

Reproducibility is an important question to address when thinking about HX MS. If an experiment is run today and a replicate experiment in a month or a year, how reproducible are the measurements? What are the error bars one can expect, and can reliable, reproducible results be obtained? Given the large number of variables in an HX MS experiment, many things must be controlled to make the overall experiment a success.

Global reproducibility in HX MS is defined by the combined reproducibility of all the variables within the entire HX MS experiment, beginning to end. The major variables are the protein preparation itself (equilibrium population, concentration, protein pI, and minor components in the purified preparation); the composition of the buffers used to do the labeling; the pH and temperature of labeling and quenching; the digestion conditions (time, pH, temperature, and concentration of protein and enzyme); the LC/MS analysis (time, pH, and temperature); and the mass accuracy of the mass spectrometer. In order to obtain the most reproducible results, each of these variables must be understood, controlled, and made as nearly identical as possible.

Some variables (e.g., pH) have large effects on the overall experiment, and others (e.g., mass spectrometer accuracy) have smaller effects. The mass accuracy of the mass spectrometer makes only a minor contribution to the reproducibility of measuring deuterium in a peptide or protein. The majority of modern instruments can easily measure the mass of a peptide with tolerance of  $\pm 0.01$  Da or less. What contributes more to variability in the mass measurement is the fact that what we want to experimentally determine in HX MS is isotopic enrichment, which by its very nature changes the mass. So the variability associated with mass measurements does not come primarily from the mass spectrometer accuracy but from the isotope pattern that is measured. The isotope pattern that is measured is a result of the way in which the isotope was incorporated. Because we always label an equilibrium population of protein molecules that are not all in the identical conformation state (the native state ensemble [33–35]), there will be variability in the HX that occurs into each of the different co-existing conformational forms. We always, therefore, measure the average of this ensemble by finding the center of the isotopic distribution [4, 36]. Perturbing the native state ensemble (with buffer conditions, concentration, pH, and temperature) will change the average deuterium incorporation and, therefore, the mass that is measured. Just a slight variation in labeling pH (even 0.2 units or less) will change the deuterium incorporation rate [37–39] because exchange is a function of both acid and base concentration, and varies about 10-fold per pH unit with a dependence on primary structure [40, 41]. Variability in the quench and LC analysis pH (as well as temperature and time) can alter both the deuterium back-exchange rate and the digestion pattern of the protein analyzed [1, 4]. The better the pH is controlled—with buffers, reproducible pipetting, and transfer time—the more reproducible each of the steps affected by pH will be and, therefore, the higher the global HX MS reproducibility will be.

With the variables just described in mind, how are we doing? Is it possible to simultaneously control all these seemingly complex variables and be reproducible? Anecdotal evidence and experience have indicated that, yes, the measurements can be done with a fair degree of reproducibility. The anecdotal evidence has recently been supported by detailed measurements from several laboratories, which have measured the error associated with HX MS experiments [42–44]. In the majority of the published cases, the inclusion of robotics (e.g., reference [45]) was essential to obtaining a large enough dataset to quantify the reproducibility and to making the measurements as reproducible as possible. Chalmers et al. [45] found that following triplicate experiments, the average standard deviation of the mass measurement across 52 different deuterated peptides was 0.08 Da, with a mean standard deviation of the percent deuterium incorporated of 1.4%. Chalmers et al. more recently [44] validated the use of HX MS for ligand screening and obtained data for 127 replicate measurements for a large number of deuterated peptides over an eight

month period. Based on their measurements, 96% of all measured percent deuterium values (4039 measurements in all) were within 10% of the mean value obtained during that period, and 3452 values (or 82% of all measurements) were within 5% of the mean. Burkitt et al. [42] tested the reproducibility of an automated system over a 2-mo period, and they found the intraday repeatability in measured exchange values of deuterated peptides to be between 0.2% and 0.9% deuterium [coefficients of variation (CVs) for intraday repeatability ranged from 0.3% to 1.5%]. For interday reproducibility, the variation was between 1.0% and 2.2% deuterium [CVs of 1.9%–3.8%]. Houde et al. [43] found the global error of each mass measurement of deuterated peptides to be about  $\pm 0.14$  Da, and this deviation was shown to be independent of peptide size, deuteration time, or the magnitude of the mass difference between undeuterated and deuterated. They calculated with 98% confidence that differences of  $\pm 0.5$  Da between relative measurements were significant differences and well outside the error range of the experiment. Consistent with the Burkitt et al. and Chalmers et al. reports [42, 44, 45], Houde et al. described comparability studies and also concluded that interday analyses were significantly more variable than intraday analyses. This implies that as discussed above, the experimental conditions all must be precisely controlled before interday comparisons can be conducted with high reliability. The studies that were just illustrated included robotics, which were extremely valuable for reducing variability to the lowest levels. Robots can be helpful in all sample handling steps, including labeling and subsequent LC/MS analysis.

Another way to reduce variability is to make relative measurements. Relative measurements are found throughout analytical chemistry, for example in double-beam UV-vis spectrometers where the solvent is automatically compensated for with a cuvette in a reference beam, or in the thermal conductivity detector in gas chromatography where the reference flow gas is compared with the column flow via a Wheatstone bridge [46]. We have long been advocates of relative measurements in HX MS [9], wherein a reference protein condition is compared with a test condition. That is, HX is performed on two states of the protein in question (bound versus free, wild-type versus mutant, buffer A versus buffer B, etc.) using the most similar conditions possible—same protein stock solution, same buffers, same instrumental setup, analyses done very close in time, etc. The difference between HX into the two states is monitored with the goal of first locating where HX differences exist, and second what the magnitude of the differences is. In relative measurements, many variables then cancel out, for example minor changes in temperature, pH, time of digestion, amounts of back-exchange, etc. apply equally to both the reference state and to the test state. It is still very important to know the error of each data point even in a relative experiment (see reference [43]).

We think reproducibility is presently not a limitation to the technique of HX MS.

## Is HX MS Just Too Difficult to Do and Does It Take Too Long?

For historical reasons, HX MS is often perceived as a method that “...just takes way too long and is just way too hard” (anonymous, personal communication). If these perceptions are true, one might see how it would be difficult to justify adopting HX MS, especially in industrial settings where there is often not the luxury of doing an experiment in which it may take weeks or even months to develop the methods and interpret the results. These perceptions about HX MS might have been true in the early days, but they are certainly not true in 2012. The following section explores some of the issues and where we stand currently.

### *Are There Too Many Variables to Control?*

As described above in the section on reproducibility, there are a lot of variables one must keep under control to be successful. This can be a daunting task to the uninitiated, but it just requires careful planning to surmount. In the words of Norman Vincent Peale, “*Plan your work—work your plan*” [47]. One way to ease the control over all the variables is to automate. A number of recent articles have described robotics that can significantly reduce variability [42, 43, 45, 48–50]. One caveat is that the initial setup of the sample handling system takes more time than simply pipetting deuterium into an Eppendorf tube. However, once set up, fully operational, and well understood, robotic systems can help control the many variables and make HX MS experiments significantly easier to perform reproducibly.

### *Are the Quench Conditions Too Restrictive?*

Another complaint is that because of the restrictive conditions one must adopt in order to preserve the deuterium label, namely short analysis times and chromatography at zero degrees, it is too difficult to do the experiment and maintain the deuterium label (i.e., too much back-exchange). For those used to long separation times and for not being fastidious with the details of the separation, adapting to the fast separation times required can be a challenge. It is not just doing a separation fast; it is also doing a *high-quality, efficient* separation fast and at 0 °C (see next section). One solution that may seem to simplify the problem is to use MALDI instead of LC electrospray MS, thereby eliminating the entire LC step. While MALDI can be used successfully in HX MS (e.g., [51–55]), the disadvantage is that there is more back-exchange than well controlled LC electrospray MS experiments and that all peptides one wishes to study are in a single spectrum. Lack of chromatography limits the protein size that can be analyzed owing to peak capacity problems and ionization efficiencies. Another reported solution to get around back-exchange was to use super-critical fluid chromatography (SFC) [56], but this solution is not practical as it requires a new separation column for each injection. We think the restrictive quench conditions actually sound much harder than they are.

### *Is Separation Too Poor at Quench Conditions?*

Related to the previous section, chromatographic performance at low temperature is not stellar. One way to fix this is to use higher performance separations systems such as UPLC/UHPLC [57, 58]. The most important factor is to do the chromatography with sub-2-micron particles, where the separation efficiency at 0 °C is vastly superior to that of more conventional HPLC particles of 3.0–3.5 microns in diameter. With sub-2-micron particles, the deuterium recovery is generally higher since the gradient time can be made shorter while still maintaining separation quality. The Waters Corporation commercialized (both pumps and columns) the use of 1.7 micron particles in 2004 [59], and there are now many other instrument vendors that also provide commercial systems compatible with small diameter separation particles. We adopted UPLC separations for HX MS [60, 61], and now use UPLC exclusively; sub-2-micron particles are now also used by other groups practicing HX MS (e.g., [62–64]). Peptides from the digestion of protein systems in excess of 200 kDa of unique sequence can be efficiently separated using UPLC at HX MS quench conditions [64]. Other alternatives to the traditional 3.5 micron reversed-phase HPLC particles could also be used to significantly improve the separation efficiency. We have recently explored whether additional resolving power could be incorporated into the overall HX MS experiment after the chromatography step by using ion mobility spectrometry (IMS) [65]. Including IMS to increase the resolving power is not a new concept (e.g., [66–69]). The deconvolution of overlapping peptides unresolved in the chromatographic separation or isotope distributions that become unresolved with addition of deuterium can be separated in many cases with IMS. More work in this area will hopefully see IMS routinely incorporated to increase resolving power in the overall HX MS experiment, especially now that commercially available MS IMS instruments have appeared.

### *Are Both LC and MS Skills Required?*

In the early days of HX MS, now some 15 years or so ago, combining LC with ESI was not as routine as it is now, in 2012. Because both an LC system and mass spectrometer must be working well at the same time, and interfaced together, there were some difficulties for those who were not skilled in making this entire two-instrument setup function all at the same time. Those problems are mostly overcome at this point and many operators are very skilled in the combination of LC with MS.

### *Is It Too Hard to Identify Pepsin Fragments?*

Because the digestion step must be done with an acid protease active in quench conditions [4, 70, 71], pepsin is often used (see also below). Pepsin digestion is not predictable like digestion with trypsin or other enzymes (e.g., Asp-N, Glu-C, etc.) and likely has much to do with the acid-induced molten globule conformation at low pH. Therefore, all the peptide

fragments must be identified. Modern MS/MS techniques, many of them automated, have made this task straightforward and much less of an impediment than it once was. Even as recently as 10 years ago, the rapid identification of pepsin fragments was often challenging.

### *Is Digestion Efficiency a Problem?*

To retain as much deuterium as possible in labeled samples, the time for analysis must be short—this includes digestion time. There is no luxury of digesting overnight at 37 °C. To improve digestion efficiency, many have adopted online pepsin digestion (first described in 1999 [72] and expanded upon later [73]) because the enzyme:protein ratio can be made much higher than in-solution digestions. Alternatives to pepsin have also impacted efficiency [74–78]. Another modification that improves the digestion efficiency is including denaturants and reducing agents in the digestion step, first described by Woods [79] and used by many since (e.g., [80, 81]). With these modern digestion techniques, it is possible to digest very resistant proteins completely within 20–30 s at 0°, and produce a number of overlapping peptides (see also below).

### *Are the Mass Spectrometers Good Enough?*

Historically, instrument sensitivity was a problem for proteins that were only available in small quantities. Huge improvements in instrument sensitivity and duty cycle in recent years have made this almost a non-issue. Analysis can be done currently with as little as 1–5 pmol of protein per injection, which is perhaps an improvement of 50- to 100-fold over that of a decade or more ago [4, 82–86]. We find that response is still variable depending on the protein, so the values listed here are not a hard rule. Given the high-quality and sensitive mass spectrometers available today, instrument sensitivity is a very minor issue for HX MS.

### *Does It Take Too Long?*

An HX MS experiment can be broken into three main components/stages. They are deuterium labeling, mass analysis, and data processing. The maximum amount of time required for the deuterium labeling portion of the experiment is a function of the longest time point of deuterium exposure, typically 4–8 h or more. The amount of time required to acquire the MS data is a function of the digestion, chromatography, and system recovery time. The time required for both of these first two components of the total HX MS experiment has stayed relatively the same since the first experiments were reported, although these two parts can be done in parallel (i.e., LC/MS analysis of shorter labeling times while the longer labeling times are incubating in D<sub>2</sub>O, etc.) to save some time. Better technology, including faster computers, better operating systems and instrument control software, and user-friendly graphic interfaces have all contributed to making the analysis easier and faster. There have been huge reductions in the time

required for the final component, the data analysis step. Data processing and analysis software have significantly reduced the amount of time required for interpreting the results. Before covering the software advancements in detail, we will take a slight detour to discuss protein size limits as that is related to the overall issue of analysis time.

### *What About Protein Size?*

Given that manual data processing and interpretation can be an extremely long and laborious process in HX MS (taking weeks or months perhaps), there was an artificial limit placed on the maximum size of protein that people wanted to work with—big: it will take “forever” to process the data so it is not a practical/manageable experiment; small: data processing was manageable. Until several years ago, before the advent of software to aid in processing, the “too big” limit was something around 50 kDa, whereas today, with progress in software, the limit is closer to 300 kDa. Therefore, a benefit of developing software for data processing is that it raises the size limit imposed by the processing burden. A goal of ours is for the maximum size/complexity that can be analyzed with HX MS to be limited by separation power rather than by processing capability. We are approaching that point now, hence, the need to further improve separation capacity as described above.

### *Is There Software for HX MS Data Analysis?*

Without data processing software, routine HX MS is generally out of reach for many, especially in an industrial setting. Starting around 8–10 years ago, a number of software platforms began to become available to address these issues (Supplementary Table S1). These software advancements have changed data processing from weeks to hours, and many software platforms include visualization tools for easier interpretation. All of the available software is not created equal, however, so caution is still required. Just as one would test-drive and compare mass spectrometers to see which is a good fit, one should test-drive and compare HX MS software platforms, many of which are freely available.

We think that HX MS is not too difficult and that it does not take too long. Many of the issues that led to this reputation have been resolved, and we think it is now a practical and robust method.

## **Can a Complete HX MS Experiment Be Done in One Day?**

One goal would be complete labeling, data acquisition and analysis within a single day, or even within the span of the longest time point of labeling. Is this feasible? The answer is, it depends on multiple factors such as the size of the protein, digestion efficiency, operators’ experience, prior knowledge about the protein(s) that is/are interrogated, software availability, etc. In well-understood protein systems with which the analyst has some experience, one could do an

HX MS experiment and data analysis in a single day. But realistically speaking, it generally does not happen that a completely new protein that has never been worked with before can be completely analyzed in a single day, including replicate analyses. Given the technological progress in HX MS, we think it is currently realistic for the average laboratory to do an entire set of HX MS experiments (including 3+ replicates) within a week for proteins of modest (<30–50 kDa) size. More sophisticated setups and researchers will be able to realize much more in a shorter amount of time.

## Is Single Amino Acid Resolution Possible?

In HX detected by NMR, exchange can be measured for individual backbone amide hydrogens [87, 88]. The quality of the NMR spectra dictates if every amide hydrogen can be measured but, in general, the spatial resolution of HX NMR is significantly higher than HX MS that relies solely on pepsin digestion. A long-standing goal has been to improve the spatial resolution of HX MS experiments in order to locate the deuterium with, at best, single backbone amide hydrogen resolution. Two ways to improve the resolution are to make shorter and shorter pepsin fragments (including perhaps overlapping fragments), or to use MS/MS to fragment deuterium-labeled peptides in the gas phase inside the mass spectrometer and locate the deuterium in the resulting fragment ions.

The aspiration of the HX MS community to achieve single-residue resolution with MS/MS started early on, with the first papers appearing in 1999 [89, 90]. It was found that collision-induced dissociation (CID) led to deuterium scrambling, whereas ECD and ETD fragmentation did not, under the right experimental conditions (see reference [91–94] and references therein for a comprehensive summary). The issue at hand now for ETD fragmentation as it applies to HX MS experiments is widespread implementation, software development to ease data processing, and better defining the expectations that non-experts should have about this methodology. Although seemingly straightforward, especially as presented for model peptides and small proteins [95], routine ETD implementation is far from trivial [96]. Fragmentation efficiency, precursor ion selection in complex samples, electrospray ionization parameters, and lack of software for ETD data processing are issues that need to be addressed [96]. It seems to us, and to others, that a targeted approach wherein only selected peptides are interrogated by ETD after HX and LC is the most viable option.

## Conclusions

We hope that with this critical insight, readers will have a better idea where HX MS stands now as a method and what can be expected today when implementing HX MS as a routine technique for interrogating protein structure and dynamics. Compared with even a few years ago, things have greatly progressed in the field, and many groups now employ the methodology on a routine basis. Some practical concerns

remain, as do other ways to further improve the technique. Obtaining the protein of interest in its highest purity possible is an ongoing challenge and is protein-dependent, dealing with interpreting data for heavily post-translational modified proteins (such as heterogeneity due to glycosylation) remains challenging, ETD fragmentation to obtain amino acid resolution needs to become routine, and complete automation (including easy-to-use but reliable software) for higher throughput is an ongoing endeavor. Overall, we can, without a doubt, acknowledge that HX MS has overcome many of its most stringent challenges and can currently be applied as a routine technique in a multitude of environments. From our perspective, we are out of the quicksand.

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