Video Article

Time-resolved ElectroSpray Ionization Hydrogen-deuterium Exchange Mass Spectrometry for Studying Protein Structure and Dynamics

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Abstract

Intrinsically disordered proteins (IDPs) have long been a challenge to structural biologists due to their lack of stable secondary structure elements. Hydrogen-Deuterium Exchange (HDX) measured at rapid time scales is uniquely suited to detect structures and hydrogen bonding networks that are briefly populated, allowing for the characterization of transient conformers in native ensembles. Coupling of HDX to mass spectrometry offers several key advantages, including high sensitivity, low sample consumption and no restriction on protein size. This technique has advanced greatly in the last several decades, including the ability to monitor HDX labeling times on the millisecond time scale. In addition, by incorporating the HDX workflow onto a microfluidic platform housing an acidic protease microreactor, we are able to localize dynamic properties at the peptide level. In this study, Time-Resolved ElectroSpray Ionization Mass Spectrometry (TRESI-MS) coupled to HDX was used to provide a detailed picture of residual structure in the tau protein, as well as the conformational shifts induced upon hyperphosphorylation.

Video Link

The video component of this article can be found at https://www.jove.com/video/55464/

Introduction

Over the past several decades, significant advancements have been made in the development of analytical techniques designed to measure protein structure and dynamics[1,2,3,4]. While X-ray crystallography remains the principle tool for determining protein structure, high concentrations of protein are needed and extensive optimization is required to produce diffraction quality crystals. Proteins that are difficult to crystallize, such as membrane-associated and intrinsically disordered proteins have classically been studied by hydrogen-deuterium exchange (HDX) NMR[5]. However, in recent decades, coupling of electrospray ionization mass spectrometry (ESI-MS) to HDX has rapidly gained popularity[6,7].

Mass spectrometry offers a solution to many of the restrictions posed by X-ray crystallography and NMR. In particular, MS is highly sensitive (nM to μM concentrations required), and there is virtually no limit on protein size. In addition, the high duty cycle of MS analysis allows for the possibility of studying proteins as they undergo enzymatic turnover, misfolding, complexation and other biologically-relevant processes. These processes often occur on the millisecond to second time scale and require rapid mixing of reagents prior to analysis.

The development of Time-Resolved ElectroSpray Ionization (TRESI) by Wilson and Konermann in 2003 allowed reactions to be monitored in pseudo-real time by ESI-MS. Their setup incorporated a capillary mixer with a continuously adjustable reaction chamber volume[8]. The device consists of two concentric capillaries, with the inner capillary sealed and a notch cut into its side to allow for mixing within the narrow inter-capillary space from the notch to the end of the inner capillary (typically 2 mm). When applied to HDX experiments, the inner capillary carries the protein of interest, the outer capillary carries the labeling D_2O solution, which then undergoes mixing with the protein before entering the adjustable reaction chamber allowing for HDX labelling prior to direct transfer into the ESI source.

Briefly, HDX relies on backbone amide hydrogens undergoing exchange with deuterium atoms in solution[9,10]. The exchange is base-catalyzed at physiological pH, with acid-catalysis becoming prevalent at pH below approximately 2.6. The rate of exchange is based on four main factors: pH, temperature, solvent accessibility and intramolecular hydrogen bonding. As the former two factors are kept constant throughout the experiment, the rate of exchange, particularly at peptide backbone amide positions, is primarily dependent on protein structure[11]. Tightly folded regions with extensive, stable hydrogen bonding networks in α-helices and β-sheets will take up deuterium at substantially slower rates compared to loops and disordered regions (and sometimes not at all)[12]. This allows for global protein analysis, where perturbations in structure (*e.g.*, upon aggregation or substrate binding) lead to differing deuterium uptake (**Figure 1**).

The kinetic capillary mixer can be incorporated into a microfluidic platform containing a proteolytic chamber for localization of the deuterium uptake. This proteolytic chamber is held at low pH in order to effectively quench the exchange reaction, and requires an immobilized acid

protease in order to digest the protein into localized peptides (**Figure 2**). Monitoring backbone exchange at millisecond to second time scales is especially important for the characterization of conformational changes within difficult to characterize loop regions, molten globules, and intrinsically disordered proteins (IDPs)[13,14]. Alternatively, TRESI-HDX can also be used to characterize proteins that currently do not have a solved atomic structure through the methods of X-ray crystallography and NMR, using deuterium exchange coupled to the COREX algorithm (DX-COREX) approach[15,16]. This detailed protocol will apply TRESI-HDX to study tau, an IDP, in both it's native form as well as it's pathogenic hyperphosphorylated state. While native tau is one of the most well studied IDPs, little is known about its amyloidogenic counterpart[13].

Protocol

NOTE: Please consult all relevant material safety data sheets (MSDS) before use. Fumes produced by laser ablation of poly(methyl methacrylate) (PMMA) can be toxic. Be sure that the laser engraver is connected to a working ventilation system. Use all appropriate safety practices when building the microfluidic device including the use of engineering controls (fume hood, sharps container) and personal protective equipment (safety glasses, face mask, gloves, lab coat, full length pants, closed-toe shoes). It is of utmost importance to use High Performance Liquid Chromatography (HPLC) grade reagents whenever possible, with all being of ACS grade or higher to decrease interfering contaminants during analysis.

1. Preparation of the Microfluidic Device

1. Construction of the PMMA Microfluidic Platform
    1. Obtain a standard PMMA block (8.9 x 3.8 x 0.6 cm) and laser-ablate an input channel for introducing the reagents, a proteolysis chamber, and an output channel using a laser engraver[17,18].
    NOTE: Etch the proteolysis chamber in an elongated oval shape (30 x 5 x 0.05 mm). The input and output channel must extend to the end of the PMMA block and be no larger than 75 µm in both width and depth in order to accommodate a 30 ga capillary.
    2. Cut a 30 ga stainless steel metal capillary into two pieces of approximately 10 cm each using a rotary tool with a 1/64" thick cut-off disc. Use sandpaper to smooth the ends of the capillaries (this can be facilitated by viewing under a light microscope).
    3. Melt the capillaries into the etched poly(methyl methacrylate) block using a soldering iron. The input channel will be connected to automated syringe pumps, and the output channel will be used for coupling into the MS.

2. Construction of the Continuous-Flow Time-Resolved Kinetic Mixer
    1. Obtain a fused silica glass capillary (ID: 75 µm, OD: 150 µm) of approximately 40 cm, and insert it into a 28 ga stainless steel metal capillary of approximately 15 cm. Depending on the reaction time required, use a longer outer metal capillary or one with a larger ID.
    NOTE: Determining the 'true' inner diameter of the metal capillary is critical for obtaining accurate reaction times. This can be done by attaching the metal capillary to an HPLC, flowing solvent though the capillary, and recording the back pressure. A backpressure to inner diameter calculation can be made, and the true inner diameter of the metal capillary determined. This can be calculated using the Molecular Weight Calculator software (for Windows Version 6.49).
    2. Produce a 2 mm notch using low power laser engraver settings on one end of the inner glass capillary and seal this end of the inner glass capillary (**Figure 2**). Alternatively, make the notch using a ceramic glass capillary cutter tool or a rotary grinder with a fine cutting blade.
    3. Line-up the inner glass capillary with the end of the metal capillary (this can be facilitated by viewing under a light microscope).
    4. Attach this kinetic mixer to one end of the mixing tee (**Figure 2**).
    5. Attach a fused silica glass capillary (ID: 75 µm, OD: 150 µm) of approximately 40 cm to the opposite end of the kinetic mixer on the mixing tee. This is used to deliver acid (5% acetic acid, pH 2.4) to quench the reaction.
    NOTE: Reactants are supplied to the device with gas-tight syringes through polytetrafluoroethylene (PTFE) tubing using automated infusion pumps.

3. Pepsin Activation
    1. Weigh out 20 mg of pepsin from porcine gastric mucosa and suspend it in 1 mL of coupling buffer (0.1 M sodium phosphate, 0.15 M NaCl, pH 6.0).
    2. Weigh out 50 mg of *N*-hydroxysuccinimide (NHS)-activated agarose beads, add to the re-suspended protease and rotate gently overnight at 4 °C.
    3. Spin down at 1,000 x g for 2 min at room temperature to collect the resin.
    4. Aspirate the unbound protease.
    5. Incubate the agarose in 1 mL of blocking buffer (1 M Tris-HCl, pH 6.0) and rotate gently at room temperature for 1 h.
    6. Spin down at 1,000 x g for 2 min at room temperature to collect the resin.
    7. Aspirate the blocking buffer.
    8. Incubate the pepsin-agarose with 1 mL of 5% acetic acid, pH 2.4 for 5 min.
    9. Spin down at 1,000 x g for 2 min to collect the resin.
    10. Aspirate the supernatant.
    11. Repeat steps 1.3.8 - 1.3.10 for a total of three washes.
    12. Store the beads in 1 mL acetic acid at 4 °C for long-term use.

4. Device Assembly
    1. Fill the proteolysis chamber with the slurry of activated pepsin-agarose beads in 5% acetic acid using a sterile spatula.
    2. Place the PMMA microfluidic platform in between two blank PMMA blocks as a cover to seal the device, lined with silicone rubber to create a liquid tight seal.
    3. Use metallic clamping plates to pressure-seal the device.
    NOTE: The clamp was custom made in order to fit the microfluidic platform and is composed of two plates measuring 10.1 cm x 7.4 cm x 1.2 cm.

4. Flow 5% acetic acid, pH 2.4 at a rate of 10 µL/min. Flow 50 mM ammonium acetate buffer (pH 6.9) though the protein line at a rate of 1 µL/min.
   NOTE: It is extremely important that the acetic acid is continuously flowing through the device throughout the entirety of the experiment. Ensure that there are no leakages and that fluid is exiting only from the output channel, which will serve as the ESI source.
5. Couple the device to the front end of a modified quadrupole time-of-flight (Q-TOF) mass spectrometer using an adjustable insulated stage to achieve optimal electrospray conditions.
   NOTE: A bypass switch is introduced in order to simulate the presence of a commercial ESI source.[17]

2. Time-resolved ElectroSpray Ionization Hydrogen-deuterium Exchange

1. Acquisition of Pepsin and Protein Only Spectra
   NOTE: ESI-MS acquisition is carried out in positive ion mode with a voltage of +4,500 to +5,000, 60-V declustering potential, and 250-V focusing potential. Spectra are acquired over a range of 350-1,500 m/z with a scanning rate of 1 s^{-1}.
   1. Acquire a pepsin only spectrum. Any peaks appearing in this spectrum are subtracted once the protein is added.
   2. Introduce 50-100 µM tau/phospho-tau protein (purify and prepare as previously described[13,19]) at a rate of 1 µL/min where the 50 mM ammonium acetate buffer was previously flowing.
   3. Acquire a protein only spectrum.

2. Acquisition of Time Points
   1. While 100 µM tau/phospho-tau protein is flowing at 1 µL/min, introduce D_2O at a rate of 3 µL/min via a tee connector and allow to react in the kinetic mixer. Allow for the system to equilibrate for at least 10 min before acquisition of the spectrum.
      NOTE: After the exchange, the labelling reaction is quenched by the flow of acetic acid pH 2.4 at 10 µL/min and digestion of the labelled protein occurs in the proteolytic chamber.
   2. In order to increase the labelling time, manually pull back the position of the inner glass capillary to achieve mixing times of 42 ms to 8 s. Allow for the system to equilibrate for at least 10 min in between each pull-back.

3. Data and Statistical Analysis

1. Identifying Peptides and Calculating the Percentage of Deuterium Exchange
   1. Perform MS spectra analyses using mMass software, version 5.5.0[20].
   2. Identify peptides using the ExPASy FindPept proteomic server and confirm by collision-induced dissociation (CID) when required[21].
      NOTE: Here, deuterium uptake incorporation was measured using an in-house developed FORTRAN software for isotopic distribution analysis[22,23].
   3. Calculate the theoretical intrinsic rates based on the primary sequence using the SPHERE web tool[22,24].
   4. Fit the data using single exponential non-linear regression and normalize using a graphing and statistical software (*e.g.*, SigmaPlot).
      NOTE: The ratio of k_{int} / k_{obs} yields the Protection Factor (PF), a semi-quantitative measure of the degree that a particular region is structured within the conformational ensemble.

Representative Results

Digestion profiles of native and phospho-tau were similar, yielding a sequence coverage of 77.1 and 71.7% respectively. Deuterium uptake values of each peptide was determined by fitting the observed isotopic distributions with the theoretical distributions generated using an in-house developed FORTRAN software. The best fitting distributions are shown (**Figure 3a**) along with the associated deuterium uptake values. Uptake kinetic profiles are then generated, and were well described by single exponential expressions. Kinetic profiles for all peptides observed at 3 or more labelling times (n = 3) were analyzed. These single exponential fits to the observed uptake values yield the exchange rate constant k_{obs} for each peptide. This is compared to the primary-sequence dependent "random coil" intrinsic rate constant k_{int}. The calculated PF (k_{int} / k_{obs}) at 1.52 s is mapped onto representative structures of native and hyperphosphorylated tau (**Figure 4**).

As expected, no PFs were observed in the range normally associated with secondary structure elements confirming that native tau exhibits weak internal hydrogen bonding. Significant protection is observed at the N and C-termini, the central domain and the aggregation-prone (hexapeptides I and II) regions (**Figure 4**). After 1.52 s, 90% of the protein is deuterated, with full exchange occurring in under 2 min.

The native protein can be compared to an altered state, in this case the amyloidogenic hyperphosphorylated state, where we observe a general increase in deuterium uptake across the protein. There are significant increases at the N- and C-termini and in the H2 region. The general increase in uptake supports the current hypothesis that hyperphosphorylation causes the protein to adopt an extended structure. Of the two hexapeptides, H2 shows the greatest increase in deuterium uptake, from being one of the most highly protected regions in the native state to almost no protection in the hyperphosphorylated state. This suggests that exposure of the H2 domain is crucial for amyloidogenesis. On the other hand, some of the areas exhibiting a decrease in uptake include regions spanning L114 to Q124, and G326 to S352. The latter region corresponds to the microtubule-associated R3 and R4 regions in the repeat domain. This stretch of newly formed residual structure agrees with earlier studies demonstrating that hyperphosphorylated tau has a decreased affinity for microtubule binding[25]. The region spanning L114 to Q124 is identified in the native state as having a significant propensity for helical structure, and the decrease in uptake may be attributed to a higher prevalence of secondary structure in the hyperphosphorylated state.

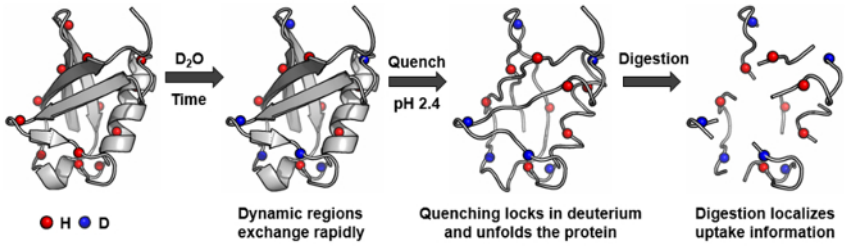

Figure 1. Schematic depiction of a protein undergoing HDX prior to mass spectrometry analysis. The protein of interest is diluted into a solution of deuterium oxide (D_2O), allowing for labile backbone hydrogens to exchange with deuterium over time. Highly dynamic regions will exchange rapidly, and must be monitored on the millisecond to second time scale. Other regions containing more rigid secondary structures will exchange more slowly. The exchange reaction is terminated by quenching with acid (pH 2.4), which also unfolds the protein allowing for digestion with an acid protease (*e.g.*, pepsin). Digestion localizes the level of deuterium uptake across different areas of the protein. Please click here to view a larger version of this figure.

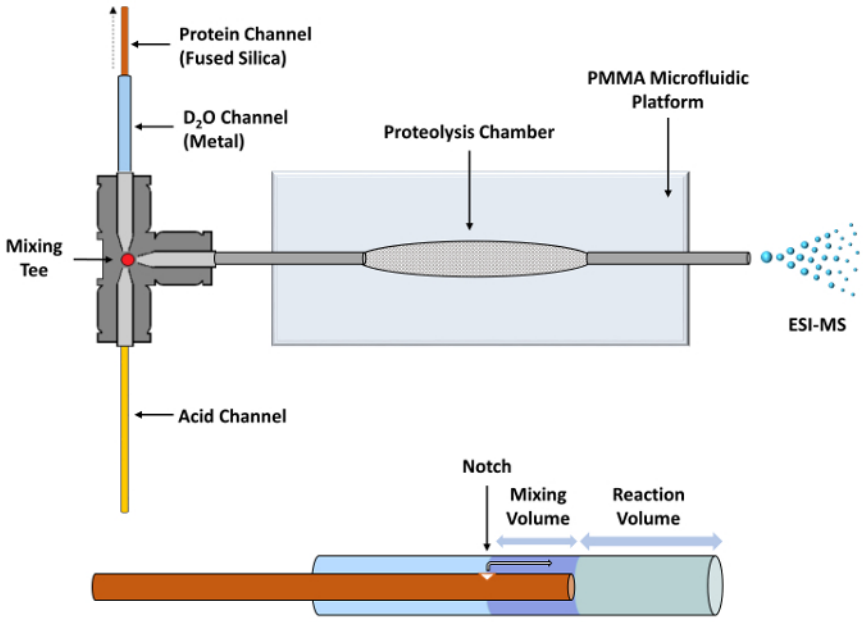

Figure 2. Experimental set-up for TRESI-HDX-MS. A detailed view of the kinetic mixer is shown at the bottom. The mixer is composed of two concentric capillaries, an inner fused silica glass capillary within an outer metal capillary. A notch is made 2 mm from the end of the inner capillary with a plugged end, allowing for protein to exit the notch and mix with the incoming deuterium. This kinetic mixer is attached to a mixing tee. On the opposite side of the mixer, a channel delivering 5% acetic acid (pH 2.4) is attached. Following quenching of the reaction, the deuterated protein passes through the proteolysis chamber packed with pepsin-agarose beads in order to produce peptic peptides. The distal capillary is used as an ESI source. Please click here to view a larger version of this figure.

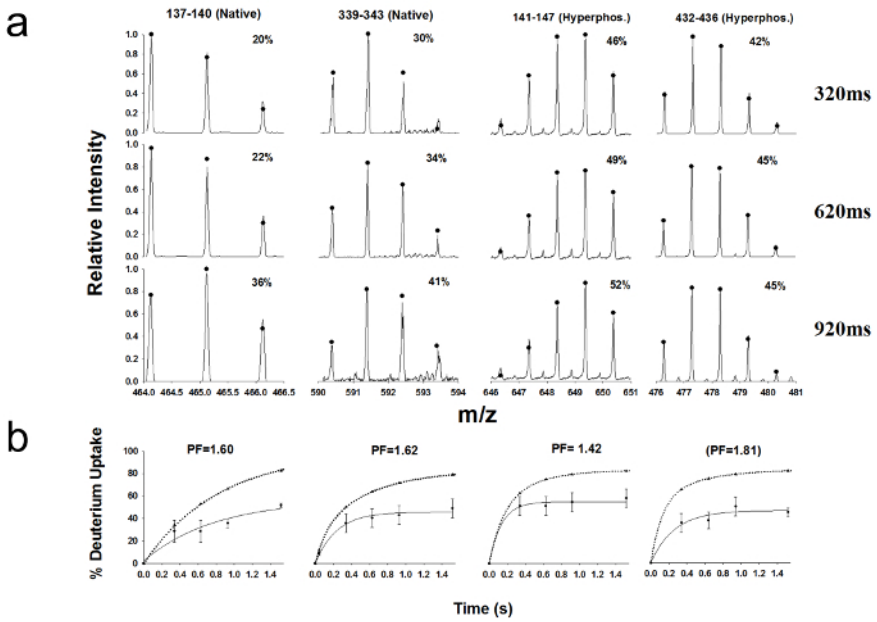

Figure 3. Typical workflow from raw data to kinetic plots for selected peptides from the native and hyperphosphorylated protein. (**a**) Raw spectrum for four peptides (columns) are fitted to predicted isotopic distributions to determine % of deuterium uptake at three different time points (rows). (**b**) Observed kinetic plots of % deuterium uptake vs. time for each peptide (solid line) used to extract k_{obs}. Kinetic plots for all peptides were observed at 3 or more labelling times (n = 3), error bars represent S.E. The calculated "random coil" profile (dotted line) is shown for comparison. Reproduced from Zhu *et al.*[8] with permission. Please click here to view a larger version of this figure.

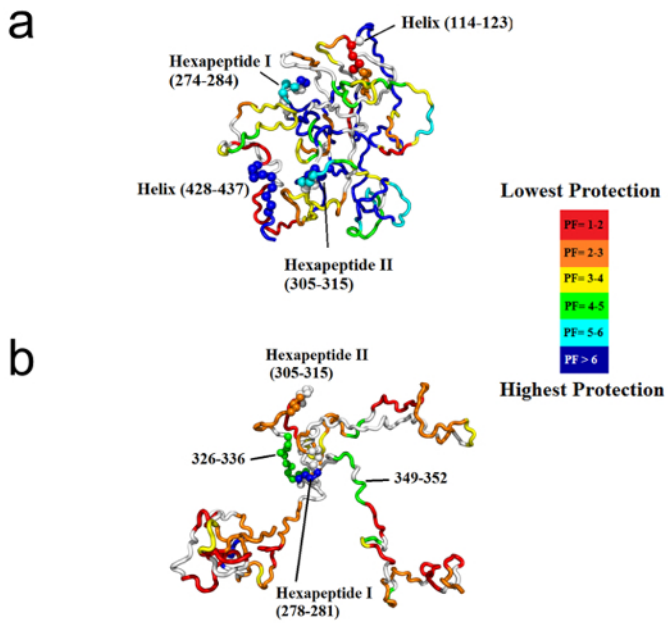

Figure 4. HDX protection factor data mapped onto representative structures of (a) native full length tau and (b) hyperphosphorylated tau. Structure of hyperphosphorylated tau was generated using FRODAN simulation with subsequent refinement using VADAR. Degree of protection factors are colored as a rainbow scheme as shown in the legend (left). Reproduced from Zhu *et al.*[8] with permission. Please click here to view a larger version of this figure.

Discussion

While structural biology methods such as X-ray crystallography and NMR are advantageous because they provide extremely detailed structures of proteins, these pictures are often static. The characterization of transient species and weakly structured domains continues to be elusive when studied by these conventional methods. Therefore, in order to gain dynamic insights on these types of systems it is important to work at rapid time scales. We have successfully applied TRESI-HDX-MS to obtain detailed insights on the conformational changes occurring within a well-studied IDP on a localized peptidic level. One of the key limitations to this technique is that pepsin is an unspecific protease and, on its own,

rarely produces full sequence coverage. One way to help improve sequence coverage and specificity is to combine protease type XIII from *Aspergillus saitoi* to the digestion chamber[26].

It is critical that the digestion chamber is kept at a pH of 2.4 during the entirety of the experiment in order to effectively quench the exchange reaction and reduce the amount of back-exchange[11]. It is imperative that any proteolysis steps and subsequent analysis be done as quickly as possible. Previous studies have optimized the size of the digestion chamber and suggested flow rates in order to keep the level of back-exchange to negligible levels (≤5%) at room temperature[22]. It is strongly recommended that the size of the chamber is no larger than the dimensions listed within this protocol. A highly structured protein will not take up significant levels of deuterium on the millisecond to second time scale and will be difficult to digest in the time allowed by the proteolysis chamber. Such proteins are not recommended for study using this technique.

While we recommend the use of 50 mM ammonium acetate buffer (pH 6.9) for the protein sample, not all proteins will be compatible with this concentration or pH value. It is important to note the pI of the protein, and adjust the buffer accordingly to be at least 1 pH unit above or below. This will ensure that the protein has the required surface charge needed for proper folding and prevent aggregation. Increasing the concentration of ammonium acetate is recommended for aggregation prone proteins in order to increase solubility, however concentrations above 500 mM is to be avoided.

TRESI-HDX-MS is best applied to systems composed of large loop regions, molten globules, or transient secondary structure elements[22]. In addition, there are various economic advantages to this technique as it is simple and relatively inexpensive to implement. Recently, there has been a surge in the use of HDX-MS in the pharmaceutical industry for drug discovery and development[27]. Over the past several decades there has been a rapid growth of biological macromolecules, most notably monoclonal antibodies (mAbs), being approved by FDA. Compared to small molecule drugs, higher order structures of proteins and their conformational dynamics play a large role in the determination of drug efficacy. HDX-MS has successfully been used as a confirmation tool for the production of biosimilar antibodies[28], as well as the characterization of antibody-antigen[29,30,31], and protein-ligand interactions[32]. Advances in software development and automation of experiments will speed up analysis times allowing for further expansion of this technique in the near future.

Disclosures

We have nothing to disclose.

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References

1. Sapienza, P. J., & Lee, A. L. Using NMR to study fast dynamics in proteins: methods and applications. *Curr. Opin. Pharmacol.* **10,** 723-730 (2010).
2. Neira, J. L. NMR as a tool to identify and characterize protein folding intermediates. *Arch. Biochem. Biophys.* **531,** 90-99 (2013).
3. Xu, A., Li, F., Robinson, H., & Yeung, E. S. Can protein conformers be fractionated by crystallization? *Anal. Chem.* **85,** 6372-6377 (2013).
4. Keedy, D. A. *et al.* Mapping the conformational landscape of a dynamic enzyme by multitemperature and XFEL crystallography. *eLife.* **4,** e07574 (2015).
5. Englander, S. W., Sosnick, T. R., Englander, J. J., & Mayne, L. Mechanisms and uses of hydrogen exchange. *Curr. Opin. Struct. Biol.* **6,** 18-23 (1996).
6. Engen, J. R., & Smith, D. L. Investigating protein structure and dynamics by hydrogen exchange MS. *Anal. Chem.* **73,** 256A-265A (2001).
7. Hoofnagle, A. N., Resing, K. A., & Ahn, N. G. Protein analysis by hydrogen exchange mass spectrometry. *Annu. Rev. Biophys. Biomol. Struct.* **32,** 1-25 (2003).
8. Wilson, D. J., & Konermann, L. A Capillary Mixer with Adjustable Reaction Chamber Volume for Millisecond Time-Resolved Studies by Electrospray Mass Spectrometry. *Anal. Chem.* **75,** 6408-6414 (2003).
9. Wales, T. E., & Engen, J. R. Hydrogen exchange mass spectrometry for the analysis of protein dynamics. *Mass Spectrom. Rev.* **25,** 158-170 (2006).
10. Konermann, L., Pan, J., & Liu, Y.-H. Hydrogen exchange mass spectrometry for studying protein structure and dynamics. *Chem. Soc. Rev.* **40,** 1224-1234 (2011).
11. Morgan, C. R., & Engen, J. R. Investigating solution-phase protein structure and dynamics by hydrogen exchange mass spectrometry. *Curr. Protoc. Protein Sci.* **Chapter 17,** Unit 17.6.1-17 (2009).
12. Katta, V., & Chait, B. T. Conformational changes in proteins probed by hydrogen-exchange electrospray-ionization mass spectrometry. *Rapid Commun. Mass Spectrom. RCM.* **5,** 214-217 (1991).
13. Zhu, S. *et al.* Hyperphosphorylation of intrinsically disordered tau protein induces an amyloidogenic shift in its conformational ensemble. *PloS One.* **10,** e0120416 (2015).
14. Lento, C., Ferraro, M., Wilson, D., & Audette, G. F. HDX-MS and deletion analysis of the type 4 secretion system protein TraF from the Escherichia coli F plasmid. *FEBS Lett.* **590,** 376-386 (2016).
15. Hilser, V. J., & Freire, E. Structure-based calculation of the equilibrium folding pathway of proteins. Correlation with hydrogen exchange protection factors. *J. Mol. Biol.* **262,** 756-772 (1996).
16. Liu, T. *et al.* Quantitative Assessment of Protein Structural Models by Comparison of H/D Exchange MS Data with Exchange Behavior Accurately Predicted by DXCOREX. *J. Am. Soc. Mass Spectrom.* **23,** 43-56 (2012).

17. Rob, T., & Wilson, D. J. A versatile microfluidic chip for millisecond time-scale kinetic studies by electrospray mass spectrometry. *J. Am. Soc. Mass Spectrom.* **20,** 124-130 (2009).
18. Liuni, P., Rob, T., & Wilson, D. J. A microfluidic reactor for rapid, low-pressure proteolysis with on-chip electrospray ionization. *Rapid Commun. Mass Spectrom.* **24,** 315-320 (2010).
19. Barghorn, S., Biernat, J., & Mandelkow, E. Purification of recombinant tau protein and preparation of Alzheimer-paired helical filaments in vitro. *Methods Mol. Biol. Clifton NJ.* **299,** 35-51 (2005).
20. Strohalm, M., Kavan, D., Novák, P., Volný, M., & Havlícek, V. mMass 3: a cross-platform software environment for precise analysis of mass spectrometric data. *Anal. Chem.* **82,** 4648-4651 (2010).
21. Gattiker, A., Bienvenut, W. V., Bairoch, A., & Gasteiger, E. FindPept, a tool to identify unmatched masses in peptide mass fingerprinting protein identification. *PROTEOMICS.* **2,** 1435-1444 (2002).
22. Rob, T. *et al.* Measuring Dynamics in Weakly Structured Regions of Proteins Using Microfluidics-Enabled Subsecond H/D Exchange Mass Spectrometry. *Anal. Chem.* **84,** 3771-3779 (2012).
23. Ferguson, P. L. *et al.* Hydrogen/Deuterium Scrambling during Quadrupole Time-of-Flight MS/MS Analysis of a Zinc-Binding Protein Domain. *Anal. Chem.* **79,** 153-160 (2007).
24. Bai, Y., Milne, J. S., Mayne, L., & Englander, S. W. Primary structure effects on peptide group hydrogen exchange. *Proteins.* **17,** 75-86 (1993).
25. Buée, L., Bussière, T., Buée-Scherrer, V., Delacourte, A., & Hof, P. R. Tau protein isoforms, phosphorylation and role in neurodegenerative disorders1. *Brain Res. Rev.* **33,** 95-130 (2000).
26. Zhang, H.-M. *et al.* Enhanced Digestion Efficiency, Peptide Ionization Efficiency, and Sequence Resolution for Protein Hydrogen/Deuterium Exchange Monitored by FT-ICR Mass Spectrometry. *Anal. Chem.* **80,** 9034-9041 (2008).
27. Deng, B., Lento, C., & Wilson, D. J. Hydrogen deuterium exchange mass spectrometry in biopharmaceutical discovery and development - A review. *Anal. Chim. Acta.* (2016).
28. Pan, L. Y., Salas-Solano, O., & Valliere-Douglass, J. F. Conformation and dynamics of interchain cysteine-linked antibody-drug conjugates as revealed by hydrogen/deuterium exchange mass spectrometry. *Anal. Chem.* **86,** 2657-2664 (2014).
29. Zhang, Q. *et al.* Epitope mapping of a 95 kDa antigen in complex with antibody by solution-phase amide backbone hydrogen/deuterium exchange monitored by Fourier transform ion cyclotron resonance mass spectrometry. *Anal. Chem.* **83,** 7129-7136 (2011).
30. Engen, J. R. Analysis of protein complexes with hydrogen exchange and mass spectrometry. *The Analyst.* **128,** 623-628 (2003).
31. Lu, J. *et al.* IL-1beta epitope mapping using site-directed mutagenesis and hydrogen-deuterium exchange mass spectrometry analysis. *Biochemistry (Mosc.).* **44,** 11106-11114 (2005).
32. Konermann, L., Rodriguez, A. D., & Sowole, M. A. Type 1 and Type 2 scenarios in hydrogen exchange mass spectrometry studies on protein-ligand complexes. *The Analyst.* **139,** 6078-6087 (2014).

