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Mass Spectrometric Approaches to Study Protein Structure and Interactions in Lyophilized Powders

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Abstract:	Amide hydrogen/deuterium exchange (ssHDX-MS) and side-chain photolytic labeling (ssPL-MS) followed by mass spectrometric analysis can be valuable for characterizing lyophilized formulations of protein therapeutics. Labeling followed by suitable proteolytic digestion allows the protein structure and interactions to be mapped with peptide-level resolution. Since the protein structural elements are stabilized by a network of chemical bonds from the main-chains and side-chains of amino acids, specific labeling of atoms in the amino acid residues provides insight into the structure and conformation of the protein. In contrast to routine methods used to study proteins in lyophilized solids (e.g., FTIR), ssHDX-MS and ssPL-MS provide quantitative and site-specific information. The extent of deuterium incorporation and kinetic parameters can be related to rapidly and slowly exchanging amide pools (Nfast, Nslow) and directly reflects the degree of protein folding and structure in lyophilized formulations. Stable photolytic labeling does not undergo back-exchange, an advantage over ssHDX-MS. Here, we provide detailed protocols for both ssHDX-MS and ssPL-MS, using myoglobin (Mb) as a model protein in lyophilized formulations containing either trehalose or sorbitol.
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Ms Emily Waters
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July 09, 2014

Dear Ms Emily Waters;

I am pleased to submit the manuscript entitled "Mass Spectrometric Approaches to Study Protein Structure and Interactions in Lyophilized Powders" to *Journal of Visualized Experiments*. The manuscript is co-authored by Balakrishnan S. Moorthy, Lavanya K. Iyer and myself. The submission summarizes original research by authors that has not been published previously and is not under consideration for publication in any other journal.

This work demonstrates the detailed protocol for hydrogen deuterium exchange (ssHDX-MS) and photolytic labeling (ssPL-MS) methods for studying protein conformations in lyophilized powders. Though several high resolution methods are available to study protein structure in solution, the methods for characterizing protein in amorphous solid are more limited. Our group has recently developed ssHDX-MS and ssPL-MS methods to assess protein conformations and molecular interactions in lyophilized powders with high resolution. In this study, a model protein (myoglobin, Mb) was co-lyophilized with different excipients and the conformation of Mb in lyophilized samples were assessed using ssHDX-MS and ssPL-MS at both the intact protein and peptide level. Our results show that the methods are capable of distinguishing formulations with different excipients. This study supports the use of ssHDX-MS and ssPL-MS as formulation screening tools for developing protein therapeutics. I believe that this work is suitable for publishing in JoVE's unique multimedia format, as it would provide readers with detailed protocols and video support for performing these measurements.

Thank you for considering our manuscript. Please feel free to contact me if you have any questions about this submission.

Sincerely,



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TITLE:

Mass Spectrometric Approaches to Study Protein Structure and Interactions in Lyophilized Powders

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KEYWORDS:

Amide hydrogen/deuterium exchange, photolytic labeling, mass spectrometry, lyophilized formulations, photo-leucine, solid-state, protein structure, protein conformation, protein dynamics, secondary structure, protein stability, excipients

SHORT ABSTRACT:

Here, we present detailed protocols for solid-state amide hydrogen/deuterium exchange mass spectrometry (ssHDX-MS) and solid-state photolytic labeling mass spectrometry (ssPL-MS) for proteins in solid powders. The methods provide high-resolution information on protein conformation and interactions in the amorphous solid-state, which may be useful in formulation design.

LONG ABSTRACT:

Amide hydrogen/deuterium exchange (ssHDX-MS) and side-chain photolytic labeling (ssPL-MS) followed by mass spectrometric analysis can be valuable for characterizing lyophilized formulations of protein therapeutics. Labeling followed by suitable proteolytic digestion allows the protein structure and interactions to be mapped with peptide-level resolution. Since the

protein structural elements are stabilized by a network of chemical bonds from the main-chains and side-chains of amino acids, specific labeling of atoms in the amino acid residues provides insight into the structure and conformation of the protein. In contrast to routine methods used to study proteins in lyophilized solids (e.g., FTIR), ssHDX-MS and ssPL-MS provide quantitative and site-specific information. The extent of deuterium incorporation and kinetic parameters can be related to rapidly and slowly exchanging amide pools (N_{fast} , N_{slow}) and directly reflects the degree of protein folding and structure in lyophilized formulations. Stable photolytic labeling does not undergo back-exchange, an advantage over ssHDX-MS. Here, we provide detailed protocols for both ssHDX-MS and ssPL-MS, using myoglobin (Mb) as a model protein in lyophilized formulations containing either trehalose or sorbitol.

INTRODUCTION:

Protein drugs are the fastest growing sector of the biopharmaceutical industry and offer promising new treatments for previously intractable diseases, including hormonal disorders, cancers and autoimmune diseases¹. In 2012, the global biotherapeutics market reached \$138 billion and is expected to reach \$179 billion by the year 2018². Proteins are larger and more fragile than conventional small molecule drugs and so are more susceptible to many types of degradation³. To ensure adequate shelf-life and stability, protein drugs are often formulated as lyophilized (i.e., freeze-dried) solid powders. However, a protein may still undergo degradation in the solid state, particularly if its native structure is not preserved during the lyophilization process^{4,5}. Assuring that structure has been retained is feasible only if there are analytical methods that can probe protein conformation in the solid-state with sufficient resolution.

NMR spectroscopy⁶ and X-ray crystallography⁷ are the commonly used high resolution methods to assess protein structure in solution and crystalline solids⁸. Because of the nature of excipients and the processing methods used, lyophilized protein formulations are usually amorphous rather than crystalline⁹. The lack of homogeneity and microscopic order makes the above mentioned techniques impractical for proteins in amorphous solids. Fourier transform infrared spectroscopy (FTIR)¹⁰, Raman spectroscopy¹¹ and near infrared spectroscopy (NIR)¹² have been regularly used by the biopharmaceutical industry to compare protein secondary structure in lyophilized powders to that of the native solution-state structure. However, these methods are low resolution and can only provide information on global changes in secondary structure. Solid-state structural characterization using FTIR has shown either weak^{13,14} or poor¹⁵ correlation with long-term storage stability. These limitations highlight the need for suitable high resolution methods to identify protein structural perturbations in the solid-state.

Chemical labeling coupled with proteolysis and mass spectrometric analysis has emerged as a powerful approach to monitoring protein structure and molecular interactions in aqueous solution. In pharmaceutical development, HDX-MS has been used for epitope mapping in antigen-antibody interactions^{16,17}, to map receptor-drug interactions¹⁸, to monitor the effects of post-translational modifications on the conformation of protein drugs¹⁹, and to compare batch-to-batch variation in developing biosimilars²⁰. Similarly, photoactivatable ligands have been used to identify drug targets and to determine binding affinity and specificity of drug-receptor interactions^{21,22}. To extend the application of these methods to lyophilized formulations, our group has developed solid-state hydrogen deuterium exchange mass spectrometry (ssHDX-MS) and solid-state photolytic labeling mass spectrometry (ssPL-MS) to study protein conformations

and excipient interactions in lyophilized samples with high resolution.

In both ssHDX-MS and ssPL-MS, the protein is labeled under ideal reaction conditions in lyophilized solids, and the samples are then reconstituted and analyzed by mass spectrometry with or without proteolytic digestion. ssHDX-MS provides information on main chain exposure to deuterium vapor, while ssPL-MS provides information on the environment of side chains (**Figure 1**). The two methods thus can provide complementary information about protein conformation in the solid-state. Here, we provide a general protocol for studying proteins in lyophilized solids using ssHDX-MS and ssPL-MS (**Figure 2**), using Mb as a model protein. We show the ability of the two methods to distinguish differences in formulations with two different excipients.

[Place Figure 1A and 1B here]

[Place Figure 2A and 2B here]

PROTOCOL:

1. Sample preparation and lyophilization

1.1) Dialyze the required volume of Mb stock solution against a suitable buffer and filter through a 0.22 μm sterile filter.

1.2) Prepare the required volume of excipients and photo-leucine (L-2-amino-4,4-azipentanoic acid; pLeu) stock solutions in suitable buffer. Filter the stock solutions through a 0.22 μm sterile filter.

1.3) Prepare formulations as shown in Table 1 using the stock solutions of protein, excipients, pLeu, and buffer.

1.4) Filter samples through a 0.22 μm sterile filter to remove any particles formed at step 1.3. Fill the samples separately as 0.2 ml into 2 ml glass vials. Use glass vials that are transparent to UV (365 nm) light in order to activate pLeu in the ssPL-MS studies.

1.5) Load vials in a lyophilizer and initiate lyophilization by designing an appropriate lyophilization cycle.

1.5.1) Here, freeze samples at $-40\text{ }^{\circ}\text{C}$, followed by primary drying under vacuum (70 mTorr) at $-35\text{ }^{\circ}\text{C}$ for 12 h and secondary drying at $25\text{ }^{\circ}\text{C}$ for 12 h. Other lyophilization cycles and drying methods (e.g., spray drying) may also be used.

1.6) Backfill the vials containing lyophilized samples with nitrogen before capping.

[Place Table 1 here]

2. ssHDX-MS for intact protein

2.1) Add a saturating amount of K_2CO_3 to 200 ml of D_2O previously placed in the lower compartment of a desiccator. Seal the desiccator air-tight and allow it to equilibrate until a relative humidity (RH) of 43% is reached. Other RH values of interest can be obtained by selecting different saturated salt solutions^{23,24}.

2.2) Initiate ssHDX reactions by placing uncapped vials containing the lyophilized protein in the upper compartment of the desiccator. Seal the desiccator air-tight and incubate at 5 °C to allow HDX to occur (**Figure 2A**).

2.3) Collect ssHDX samples at various times in triplicate. For Mb formulations, collect samples at nine time points 1, 2, 4, 8, 16, 32, 56, 92 and 144 h.

2.4) Cap the vials immediately after withdrawing from the desiccator and quench the reactions by flash freezing the vials in liquid nitrogen. Store vials at -80 °C until mass spectrometric analysis.

2.5) Analyze samples using a suitable high resolution liquid chromatography-mass spectrometry (LC-MS) method. Design or purchase a suitable refrigerated LC system to minimize back-exchange during sample analysis. Use the setup of the column refrigeration unit and LC-MS method previously reported²⁵.

Note: Since the rate of amide proton exchange depends on pH and temperature, deuterons incorporated in the protein can exchange with hydrogen present in the mobile phase (“back exchange”), causing a loss of information. Although an acidic pH (pH 2.5) of quench buffer and HPLC solvents can minimize back-exchange to a large extent, reducing the temperature (≤ 0 °C) by means of a suitable column refrigeration system can further protect the protein from back-exchange.

2.6) Connect the sample loop and protein trap to the valve that automatically controls the desalting and elution process. Calibrate the mass spectrometer by injecting a TOF tuning mix in acetonitrile solution into the mass spectrometer in the m/z range of 200-3200. The immobilized pepsin column and analytical column are not required for intact protein analysis.

2.7) Set the temperature in the refrigerated system to ≤ 0 °C and wait for the system to reach a stable operating temperature of ~ 0 °C.

2.8) Quickly transfer the samples from -80 °C into liquid nitrogen for mass spectrometric analysis. Using forceps, carefully withdraw each vial from liquid nitrogen and reconstitute the sample by adding a specific volume of ice cold quench buffer containing 0.2% formic acid (FA) (pH 2.5) and 5% methanol to the vial.

2.9) Program a suitable HPLC and mass spectrometry method using the control software. For Mb formulations, desalt sample containing 20 pmol Mb in the protein trap for 1.7 min with 5% acetonitrile, 95% water and 0.1% formic acid (FA), and elute using a gradient increased to 80% acetonitrile, 20% water and 0.1% FA in 3.3 min. Collect mass spectra over the m/z range 200-

3200.

2.10) To determine the mass of intact protein, acquire data for an undeuterated protein sample (i.e., protein not subjected to ssHDX) in aqueous solution using the method of step 2.9.

2.11) Obtain the masses of undeuterated and deuterated samples by deconvoluting the raw spectra using data analysis software. Here, set the mass range to 15,000-18,000 Da, the mass resolution to 1.0 Da, and the peak height to 90% for calculating the mass of Mb.

2.12) Calculate the number of deuterons incorporated in the intact protein (here, Mb) by subtracting the mass of undeuterated protein from the mass of deuterated protein at each exchange time point.

2.13) Calculate the percent deuterium uptake relative to the theoretical maximum using the following equation (Equation 1)

$$\% \text{ Deuterium Uptake} = \frac{\text{Number of deuterons exchanged}}{\text{Total number of exchangeable amides}} \times 100 \quad (1)$$

where total number of exchangeable amides = total number of amino acids – number of proline residues – 2 (“2” accounts for the N-terminal amino group and amide hydrogen that undergo rapid back-exchange).

2.14) Fit the ssHDX kinetic data using a suitable exponential equation. A biexponential equation (Equation 2) is usually the simplest that provides a reasonable fit to the ssHDX data. In this study, for MbT and MbS, fit the data to a biexponential model which assigns deuterons to “fast” and “slow” exchanging pools.

$$\text{Deuterium Uptake} = N_{\text{fast}}(1 - e^{-k_{\text{fast}}t}) + N_{\text{slow}}(1 - e^{-k_{\text{slow}}t}) \quad (2)$$

where N_{fast} and N_{slow} are the number of exchangeable amides in the “fast” and “slow” exchanging pools, respectively, and k_{fast} and k_{slow} are the first-order rate constants associated with the two pools.

3. ssHDX-MS for protein at peptide level

3.1) Perform ssHDX by following steps 2.1 to 2.8, with the following modifications in step 2.6. Connect the immobilized pepsin column and analytical column to the valve as reported previously²⁵ and replace the protein trap connected to the valve with a peptide trap.

Note: During the reconstitution step (step 2.8), a reducing agent and denaturing agent can be included in the quench buffer to facilitate pepsin digestion of proteins with disulfide bonds (e.g., monoclonal antibodies).

3.2) Program an appropriate HPLC and mass spectrometry method using the control software. For Mb formulations, digest samples containing 20 pmol Mb online with 100% water and 0.1% FA, trap and desalt peptides for 1.7 min with 25% acetonitrile, 90% water and 0.1% FA in a peptide trap. Elute the fragments onto the analytical column with a gradient increase to 60% acetonitrile, 40% water and 0.1% FA in 4.0 min. Acquire mass spectra over the m/z range 100–1700.

3.3) Identify the peptic fragments by MS/MS analysis of an undeuterated protein sample. Use mass spectrometry software to compare experimental masses of peptide fragment ions to the predicted masses of peptide fragments in a custom database. Set a mass cut-off point (e.g. 10 ppm) to identify masses with low error. For peptides matched, prepare a list comprised of (i) peptide sequence, (ii) charge state, and (iii) retention time.

3.4) Use the list generated in step 3.3 to map and determine the average number of deuterons incorporated for each pepsin digest fragment. This can be achieved by employing suitable HDX-MS data analysis software²⁴.

3.5) To calculate the percent deuterium uptake and to fit the ssHDX kinetic data for each of the peptic fragments, follow steps 2.13 and 2.14. In this study, HDX kinetic data for each of six non-redundant pepsin digest fragments from MbT and MbS formulations were fitted to a biexponential association model (Equation 2).

4. ssPL-MS for intact protein

4.1) To begin the photolytic labeling reaction, first switch on the UV crosslinker and allow the lamps to warm up for 5 min. Make sure the UV source is equipped with lamps of wavelength 365 nm to activate the diazirine group of pLeu.

CAUTION: Never open the door of the UV crosslinker when the lamps are on. Protect eyes and skin from exposure to UV light if the source is not enclosed by a UV-protective glass door.

4.2) Switch off the UV crosslinker before opening the door. Once the lamps are turned off, uncap the vials containing the lyophilized formulation and place them inside the UV crosslinker chamber as shown in **Figure 2B**. Irradiate the samples with UV light for 40 min.

4.3) Perform control experiments by following step 4.1 to 4.3 for (i) samples lyophilized without pLeu and (ii) samples lyophilized with pLeu reconstituted in water.

4.4) Cap and store the vials at -20 °C until mass spectrometric analysis.

4.5) Reconstitute the solid samples by adding a suitable volume of mass spectrometry grade distilled water to bring the concentration to 2 µM.

4.6) To begin sample analysis, follow steps 2.6 and 2.9.

Note: Since back-exchange is not an issue with covalent labeling, ssPL-MS does not require a special refrigerated LC system.

4.7) To determine the mass of the intact protein, acquire data for a protein sample that has not been subjected to ssPL by following step 2.9. Obtain the masses of unlabeled and labeled samples by deconvoluting the raw spectra as explained in step 2.11.

4.8) Calculate the number of pLeu incorporated using the following formula:

$$\text{Number of pLeu incorporated} = \frac{M_L - M_N}{115} \quad (3)$$

where M_L is the mass of labeled protein, M_N is the mass of native protein and 115 is the average mass (Da) added to native protein following single pLeu incorporation. Note that the labeling reaction occurs with the loss of N_2 (28 Da). The monoisotopic mass of pLeu is 143.07.

4.9) Calculate the percentages of protein populations with different numbers of labels using peak heights from the extracted ion chromatograms.

$$\% L_i = \frac{PH_i}{(PH_i + PH_u)} \times 100 \quad (4)$$

where “i” denotes the number of labels, PH_i denotes the peak height for labeled protein L_i and PH_u denotes the peak height of the unlabeled protein as observed by mass spectrometry.

5. ssPL-MS for protein at the peptide level

5.1) Perform ssPL by following steps 4.1 to 4.4.

5.2) For peptide level analysis, reconstitute solid samples in ammonium bicarbonate buffer (100 mM, pH 8.0).

5.3) After reconstitution, mix the labeled protein solution with trypsin (10:1 or 20:1 molar ratio of protein to trypsin) and incubate at 60 °C for 16 h.

5.4) Quench the reaction by adding 0.1% FA in water to the sample.

5.5) Connect the sample loop, peptide trap and analytical column to the valve linked to the HPLC system.

5.6) Program a suitable HPLC and mass spectrometry method using the control software. For Mb formulations, inject 20 pmol of the digested protein into the sample loop, and desalt the peptides in a peptide trap for 1.5 min with 5% acetonitrile, 95% water and 0.1% FA, followed by an elution in the analytical column with gradient increase to 55% acetonitrile, 45% water and 0.1% FA in 22 min. Collect mass spectra over the m/z range 200-3200.

5.7) Prepare a theoretical mass list for peptide-pLeu adducts using an online tool such as ExPASy²⁶ with numbers of pLeu incorporated previously calculated from the intact protein analysis. Include at least 4 missed cleavages. Note that the labeling reaction occurs with the loss of N_2 . Therefore, the mass of peptide-pLeu adduct = mass of unlabelled tryptic peptide + n (mass of pLeu) – n (mass of N_2), where “n” is the number of pLeu incorporated.

Note: If the mass analysis of intact protein showed up to three labeled populations of protein, consider up to three possible pLeu labels per peptide. For a peptide with mass of 1000 Da, the theoretical mass of peptide-pLeu adduct with 1 pLeu incorporation would be 1000 + 1(143) - 1(28) = 1115 Da. Similarly, the theoretical masses of peptide-pLeu adducts with 2 pLeu, 3 pLeu, etc. would be 1230 Da, 1345 Da, etc., respectively.

5.8) Use the mass analysis software to match the theoretical mass list generated in step 5.7 with

the masses observed experimentally. Set a mass cut-off point (e.g., 50 ppm) to identify masses with low error.

5.9) For peptides matched, determine the actual number of pLeu incorporated using the formula under step 4.8 (Equation 3), where M_L and M_N are the masses of labeled and native peptide, respectively.

REPRESENTATIVE RESULTS:

Here, ssHDX-MS and ssPL-MS have been used to study the effect of excipients on the conformation and solid-state interactions of lyophilized Mb formulations. The concentrations of protein and excipients used in this study are given in Table 1. Representative results from the ssHDX-MS and ssPL-MS analysis of lyophilized Mb obtained by following the above protocols are presented.

Deuterium uptake at intact protein level

ssHDX-MS is able to distinguish between Mb formulations at intact level. The deconvoluted mass spectra of intact Mb following 144 h of ssHDX from formulation MbS showed greater deuterium uptake than formulation MbT (**Figure 3A**). On an average, MbS showed 46% greater deuterium uptake than MbT (**Table 2**).

[Place Figure 3A and 3B here]

The deuteration kinetics for intact MbS and MbT are similar at early time points (1-4 h), but MbS showed increased deuterium exchange with increase in time (8-144 h) (**Figure 3B**). This suggests the importance of selecting longer time points for ssHDX at lower RH and temperature conditions. Also, the D₂O sorption and diffusion process may affect the rate of ssHDX at the early time points. Our previous studies have shown that moisture sorption in ssHDX is complete in a period of hours, and has minimal contribution to exchange kinetics beyond this time. The observed rate and extent of exchange therefore are not simply measures of D₂O adsorption^{27,28}. The small error bars in Figure 3B, indicating standard deviations from three independent ssHDX-MS samples, show that the experiment is highly reproducible.

[Place Table 2 here]

The regression parameters (N_{fast} , N_{slow} , k_{fast} and k_{slow}) for deuterium uptake kinetics for MbT and MbS are given in **Table 2**. Though the N_{fast} and N_{slow} values are larger for MbS than MbT, differences in the N_{slow} values were greater than differences in the N_{fast} values. Specifically, the N_{fast} value is only 18% greater in MbS than in MbT, whereas the N_{slow} value is 75% greater in MbS than in MbT. This suggests that the smaller N_{slow} values in MbT may be due to the higher retention of Mb structure or protection of amide groups by excipients that are exposed to D₂O in MbS. However, the detailed mechanisms are not clearly understood. The rate constants (k_{fast} and k_{slow}) for both formulations are very similar.

Deuterium uptake at the peptide level

Following pepsin digestion, a total of 52 peptides were identified. Six non-redundant fragments corresponding to 100% of the Mb sequence were used for the analysis reported here. Additional information can be obtained by using overlapping fragments, as reported by our group previously²⁴. The percent deuterium uptake for each peptide was calculated and the results from 144 h samples plotted (**Figure 4A**). HDX kinetics for the six peptic fragments showed biexponential behavior (**Figure 4B**), consistent with subpopulations of amide hydrogens undergoing “fast” and “slow” exchange.

[Place Figure 4A and 4B here]

Regression parameters for the nonredundant peptides are presented in **Figure 5**. As the fitted rate constants for peptide fragments are not the average rate constant for individual amides, the observed rate constants for the peptic fragments cannot be linearly related to those for the intact protein. The N_{fast} values for most of the peptic fragments (except fragment 56-69) in formulations MbS were slightly greater than those in MbT (**Figure 5A**). Similarly, the k_{fast} values generally showed little difference between formulations and in different regions of the Mb molecule (**Figure 5B**). However, the N_{slow} and k_{slow} values for MbS are significantly greater in all fragments than for MbT (**Figure 5C and 5D**). The considerable increase in N_{slow} and k_{slow} for MbS may reflect greater mobility of amide groups in the “slow” exchanging pools.

[Place Figure 5A, 5B, 5C and 5D here]

Photolytic labeling at intact protein level

Mb irradiated in the presence of 20x excess pLeu formed multiple Mb-pLeu adducts, as detected by LC-MS (**Figure 6A**). The deconvoluted spectra for MbT irradiated for 40 min with 20x pLeu showed up to 3 labels with the addition of +115, +230 and +345 Da to the mass of unlabeled Mb. MbS irradiated similarly with 20x pLeu showed less pLeu uptake at the intact level, with up to 2 labeled populations detected by LC-MS.

[Place Figure 6A, 6B and 6C here]

In kinetic studies, the percent of labeled protein increased exponentially for both MbT and MbS with increasing irradiation time (**Figure 6B**). MbS showed less pLeu uptake than MbT at every irradiation time. Both formulations appeared to reach a plateau at 40 min. Thus, a kinetic study can be useful to determine the duration of irradiation needed to obtain complete pLeu activation. Labeling kinetics were also studied as a function of pLeu concentration (**Figure 6C**). The percent of labeled protein increased with pLeu concentration for both MbT and MbS. However, at 20.7 % w/w pLeu, MbT showed a decrease in pLeu uptake. This may be due to exclusion of pLeu from the surface of the protein at high pLeu concentration. Hence, a study with varying concentration of pLeu should be performed to select the appropriate pLeu concentration that allows for sufficient labeling across the protein surface without surface exclusion. In this study, 20x excess pLeu was selected for further peptide-level studies.

The overall decreased labeling observed for MbS suggests poor side-chain accessibility to the matrix containing pLeu. This is consistent with a conformational change in the presence of

sorbitol that results in reduced labeling.

Photolytic labeling at the peptide level

Based on the intact protein labeling studies, 20x excess pLeu was selected to compare MbT and MbS at the peptide level. Labeled samples were digested with trypsin and analyzed by LC-MS. A total of 40 peptides corresponding to 100% of the Mb sequence were detected for MbT and MbS samples. In some cases, tryptic digestion may provide limited protein sequence coverage if Lys and/or Arg residues are heavily labeled. To improve sequence coverage, a mixture of trypsin and chymotrypsin can be used to digest the labeled protein.

[Place Figure 7A and 7B here]

ssPL-MS with trypsin digestion provides qualitative information about the peptides being labeled. Given the different labeled populations at the intact level, the promiscuous mechanism of pLeu labeling and differences in ionization efficiencies of labeled and unlabeled peptides, it is difficult to obtain quantitative metrics for ssPL-MS after digestion. However, the qualitative information can still provide insight into protein conformational changes at the peptide level. In this study, both MbT and MbS formulations showed pLeu uptake across most of the protein surface. When compared to MbS, peptide fragments 32-42, 134-139 and 146-153 from MbT showed pLeu labeling (**Figure 7**). This suggests that the side-chains of these amino acids are exposed to pLeu, as the helices in these regions are intact in the MbT matrix. In contrast, protection from pLeu labeling in the MbS matrix is consistent with structural perturbations in these regions.

Overall, the results from ssHDX-MS and ssPL-MS suggest that the methods can provide complementary high-resolution peptide-level information about backbone (ssHDX-MS) and side-chain (ssPL-MS) exposure and excipient effects in lyophilized protein formulations.

Table 1. Composition of lyophilized Mb formulations.

^a Formulations used for the ssHDX-MS study.

^b Formulations used for the ssPL-MS study.

^c L-2-amino-4,4-azipentanoic acid or photo-leucine (pLeu). pLeu at five different concentrations (14.3×10^{-3} to 1.43 mg/ml) corresponding to 1x, 10x, 20x, 50x and 100x molar excess relative to Mb were co-lyophilized with MbT and MbS formulations.

Table 2. Quantitative measures of deuterium uptake in ssHDX studies of Mb formulations.

^a See Table 1 for composition.

^b Percent deuterium uptake relative to theoretical maximum by intact Mb after 144 h of HDX at 5 °C, 43% RH (n = 3, mean ± SD).

^c Parameters determined by nonlinear regression of ssHDX-MS kinetic data. Time course of deuterium exchange for intact Mb was fitted to a biexponential association model (Eqn. 2). Values in parentheses are standard errors of the regression parameters.

^d The percent change in measurements were calculated as $100 \times [(value\ from\ MbS - value\ from\ MbT) / (value\ from\ MbT)]$.

Figure 1: ssHDX and ssPL measure protein structure in lyophilized solids through different labeling mechanisms. (A) In HDX, the backbone amide hydrogens exchange with deuterium as a function of protein structure and D₂O accessibility. In the solid-state, the rate and extent of deuterium exchange depend on the level of D₂O sorption, protein mobility (unfolding and refolding events) and the nature of the excipients present in the solid matrix. (B) In PL, UV irradiation at 365 nm initiates the formation of a reactive carbene intermediate from the diazirine functional group of pLeu and is inserted non-specifically into any X-H bond (X= any atom), or added across a C=C bond in its immediate vicinity. In the solid-state, the rate and extent of labeling depend on the local concentration of the labeling agent, irradiation time, protein structure and the nature of excipients present in the solid matrix. Figure A and B show the maximum theoretically possible labeling that can occur on backbone and side-chains respectively in protein.

Figure 2: Schematic showing solid-state HDX-MS (A) and PL-MS (B) for protein in lyophilized formulation.

Figure 3: ssHDX-MS for intact Mb: (A) Deconvoluted mass spectra of deuterated intact Mb from formulations MbT (solid line) and MbS (dashed line) following 144 h of ssHDX. The deconvoluted mass spectrum of undeuterated intact Mb is also shown (dotted line). (B) ssHDX kinetics for intact Mb in formulations MbT (solid line) and MbS (dashed line). The time course of ssHDX was fitted to an equation for two phase exponential association using Graph Pad Prism software version 5 ($n = 3, \pm \text{SD}$).

Figure 4: ssHDX-MS for Mb at the peptide level: (A) Percent deuterium uptake for 6 non-redundant peptic fragments from Mb in formulations MbT (gray) and MbS (white) following 144 h of HDX. (B) ssHDX kinetics for six non-redundant peptic fragments from Mb in formulations MbT (solid line) and MbS (dashed line). The time course of ssHDX was fitted to an equation for two phase exponential association using Graph Pad Prism software version 5 ($n = 3, \pm \text{SD}$).

Figure 5: ssHDX kinetic parameters for Mb peptic peptides: N_{fast} (A), k_{fast} (B), N_{slow} (C) and k_{slow} (D) values obtained from nonlinear regression of ssHDX-MS kinetic data for six non-redundant peptic peptides from Mb in formulations MbT (gray) and MbS (white) ($n = 3, \pm \text{SE}$).

Figure 6: ssPL-MS for intact Mb: (A) Deconvoluted mass spectra for MbT (solid line) and MbS (dashed line) labeled with 20x excess (5 % w/w) pLeu. Deconvoluted mass spectrum of native Mb (Mb lyophilized and irradiated in the absence of pLeu) is shown as the dotted line. U denotes a population of protein that remains unlabeled after irradiation. Populations of protein carrying 1, 2 and 3 pLeu labels are represented as 1L, 2L and 3L respectively. (B) ssPL-MS kinetics for intact Mb in formulations MbT (closed circles) and MbS (open circles) as a function of pLeu concentration. All samples were irradiated for 40 min. Error bars are within the symbols. (C) ssPL-MS kinetics for intact Mb in formulations MbT (closed circles) and MbS (open circles) lyophilized and irradiated in the presence of 100x excess pLeu (20.7 % w/w) as a function of irradiation time. Error bars are within the symbols.

Figure 7: ssPL-MS for Mb at peptide level: Cartoon representation of Mb labeled with 20x

excess pLeu (5 % w/w) in the presence of trehalose (**A**) and sorbitol (**B**). The labeled protein was digested with trypsin and labeled peptides were mapped on to the crystal structure of Mb (PDB ID 1WLA). The labeled and unlabeled regions are colored magenta and green, respectively.

DISCUSSION:

Several studies have suggested that the local environment in lyophilized samples affects protein degradation^{5,29,30}. However, establishing a direct relationship between protein structure and stability in the solid state has not been feasible due to the lack of high-resolution analytical methods. The application of existing high resolution methods such as HDX and PL to lyophilized powders requires modification of solution protocols and careful data interpretation. HDX-MS and PL-MS have been successively adopted to monitor protein conformations in the solid-state. The results presented here and elsewhere^{27,28,31-33} have demonstrated the ability of these methods to monitor protein conformation with high resolution in the solid environment. Though the critical steps in data analysis do not vary from labeling in solution³⁴⁻³⁶, important considerations during experimental setup and data interpretation are required for solid-state chemical labeling.

Selection of the labeling reagent must be based on size and mechanism of labeling. The small size of deuterium allows the peptide backbone to be probed easily, whereas the relatively larger size of pLeu limits labeling to the side-chains. Both ssHDX and ssPL show no preference for any amino acid, so that labeling depends only on backbone and side-chain exposure to the matrix. To effectively probe protein conformations in solid-state, the external factors affecting the labeling process must be carefully controlled. The total amount and the spatial distribution of labeling agent in lyophilized solids is different from aqueous solutions.

In ssHDX, the amount of D₂O in the solid matrix may affect the rate of protein unfolding (or partial unfolding), refolding, and deuterium exchange. This is not the case with solution HDX, in which the protein sample is normally diluted with an ample volume of D₂O. Careful screening of the effects of hydration on the ssHDX rate can inform the selection of ideal RH conditions. To control the rate of moisture sorption and avoid collapse of the powder in formulations containing hygroscopic excipients (e.g. sucrose and trehalose), ssHDX may be carried out under refrigerated conditions (2-8 °C). Our previous study on hydration effects showed increased rate and extent of exchange with increase in moisture content, as expected. In much of our work, an intermediate RH of 43% at 5 °C has proven to be ideal to distinguish formulations in a reasonable time²⁴. The reaction is usually carried out until a plateau is reached. This ensures that moisture sorption and diffusion into the solid do not control the HDX rate. The use of small solid sample sizes with pre-lyophilization volume of ≤ 2 ml also helps to ensure that D₂O vapor sorption is essentially complete early in the exchange period. Though ssHDX-MS provides quantitative information on the conformation of protein in solid-state, there are certain conditions where interpretation of data cannot be entirely based on the ssHDX study alone. It is possible that the decreased deuterium uptake observed in a sample (when compared to control) may be due to the higher retention of protein structure or the significant amount of protein aggregates present in the sample. In such case, interpretation of ssHDX data requires results from other complementary methods. Peak broadening in deuterated mass spectra was observed for several Mb formulations^{27,28}. This could be due to various factors like the presence of partially unfolded protein population, spatial heterogeneity in the sample, or the spatial gradients in the D₂O

concentration. However, these factors were not distinguished in ssHDX-MS and needs further investigation.

As ssPL-MS is relatively new when compared to other methods, continuous learning about its applications and limitations is required. In ssPL, the photo-cross-linker is lyophilized with the protein. The lack of moisture limits the mobility of components within the solid matrix, and the partial structural relaxation that may occur with moisture sorption in ssHDX is not a phenomenon in ssPL. This limits labeling in ssPL to the immediate vicinity of the photo-cross-linker. However, unlike HDX-MS, MS/MS analysis of the covalently labeled protein can provide residue-level structural information. Since ssPL labeling is covalent and irreversible, back exchange does not occur and samples can be prepared and analyzed without concern for loss of label. To facilitate diffusion of labeling agent and improve labeling efficiency in solid-matrix, ssPL may be performed with increasing % RH. pLeu uptake can also be improved by increasing the concentration of photoreactive agent. The molar ratio of protein to pLeu can be varied as desired. In general, a 100x molar excess of pLeu to protein will ensure adequate labeling. However, high pLeu concentration may result in loss of protein tertiary structure in the solid matrix. Hence, In addition to labeling kinetics and formulation composition, selection of pLeu concentration must also be based on maintaining protein structural integrity. As pLeu nonselectively labels X-H (where, X = C, N, O) group, it is possible that excipients with similar labeling sites can greatly affect the level of protein labeling. The interference of excipients in the availability of pLeu for protein labeling is yet to be characterized. It is known that the carbene generated from diazirine activation is not residue-specific, however one study reports bias towards Asp and Glu³⁶. While it is good to learn about residue-specific interactions, peptide-level information is also useful and can be used to design excipients to block regions with high matrix exposure in the solid state. ssPL-MS provides detailed qualitative information, however quantitative data needs to be obtained and robust metrics need to be developed to analyze formulation differences across a variety of lyophilized systems.

The use of a residue-specific label combined with MS/MS analysis can further enhance resolution to the amino-acid level. Labeling reagents such as 2, 3-butanedione to label Arg, N-hydroxysuccinimide derivatives for Lys and N-alkylmaleimide derivatives for Cys can be used to precisely map molecular interactions in lyophilized powder. However these reagents are pH-dependent and the reactions may not be as well-controlled as photolytic labeling in solid-state. An alternate approach is to incorporate the photo-cross-linker into the protein sequence with the use of auxotrophic cell lines, site-directed mutagenesis or side chain derivatization.

Our previous ssHDX-MS and ssPL-MS studies have shown that labeling of protein depends on the nature and amount of excipients used^{24,27,28,31-33,37,38}. ssHDX-MS of Mb co-lyophilized with guanidine hydrochloride (Gdn.HCl) showed greater deuterium uptake than Mb co-lyophilized with low-molecular-weight sugars³². In a separate ssPL-MS study, Mb co-lyophilized with Gdn.HCl showed greater protection from photolytic labeling than Mb with sucrose³³. Further, quantitative measurements from ssHDX-MS have been highly correlated with the stability of protein during long-term storage²⁸. These studies suggest that ssHDX or ssPL of protein reflects the extent of structural retention of the protein in lyophilized powder. We believe that the retention of secondary structure in lyophilized powders provides favorable environment for side chain labeling with pLeu and protection of amide hydrogen from deuterium exchange. However,

detailed comparison of the informative content from these methods needs to be performed in the future. Though establishing the utility of ssHDX-MS and ssPL-MS as a formulation screening tool will ultimately require that it be applied to many proteins, results from our recent studies supports its wider adoption. With further development, these methods are expected to be widely useful for characterizing solid-state protein formulations in the biopharmaceutical industry.

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DISCLOSURES:

The authors declare that they have no competing financial interests.

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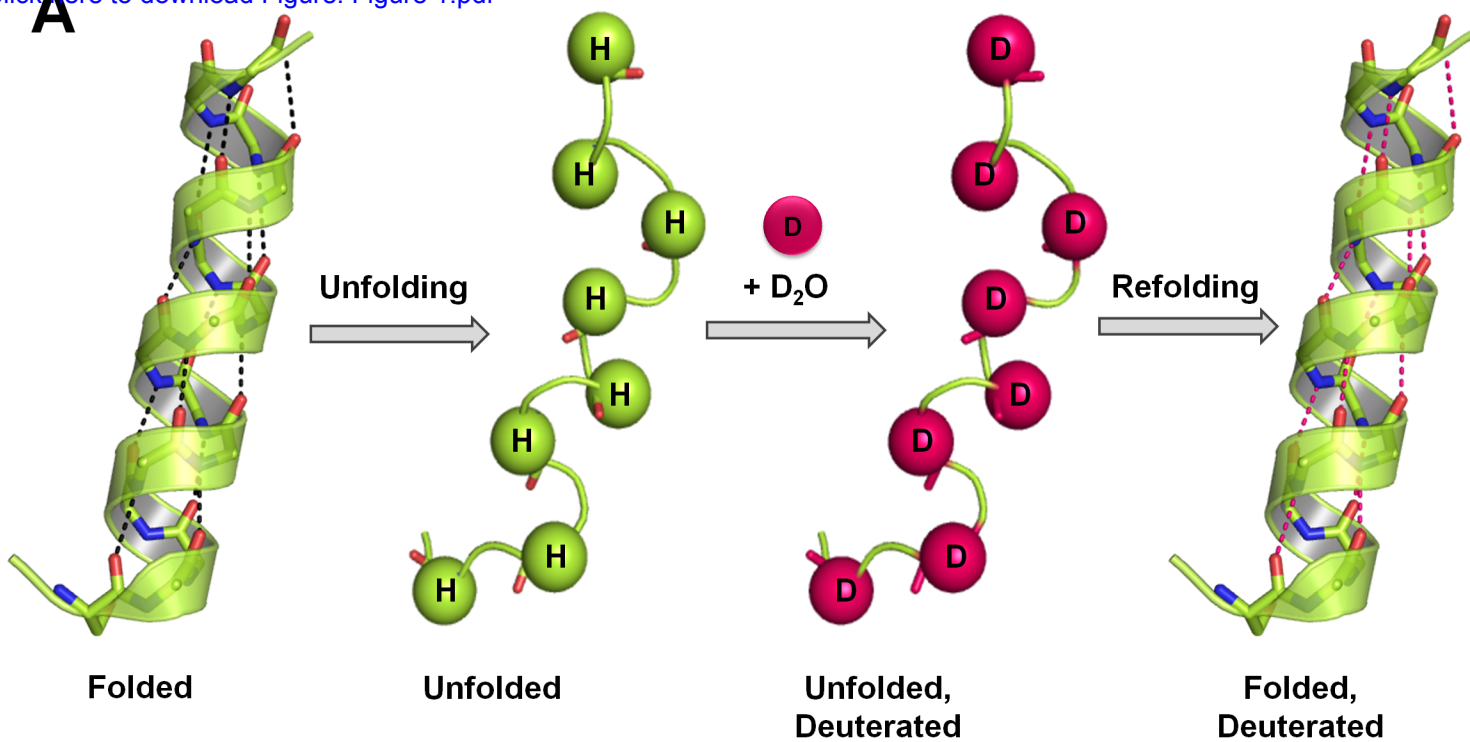
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Figure 1
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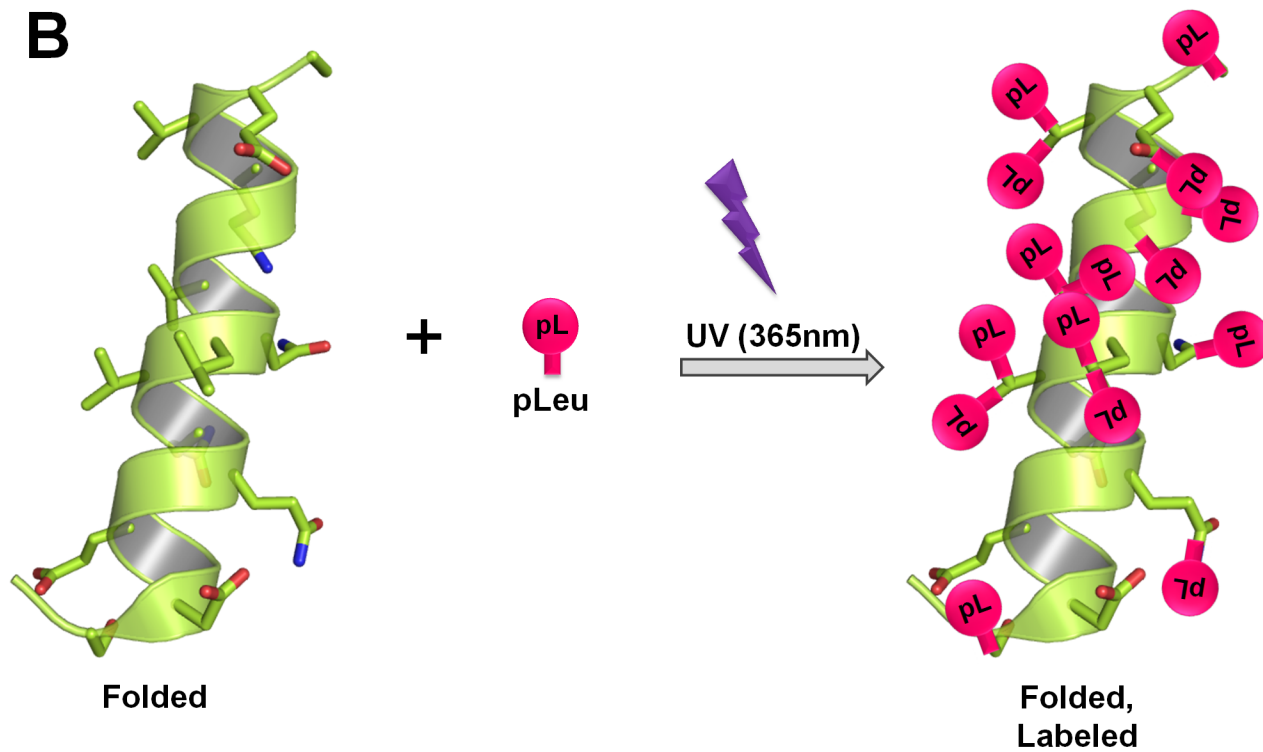
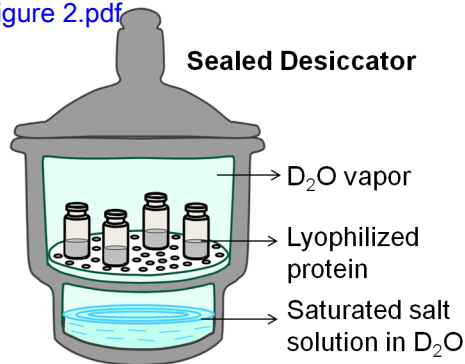


Figure 2

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A

Protein lyophilized
with excipients



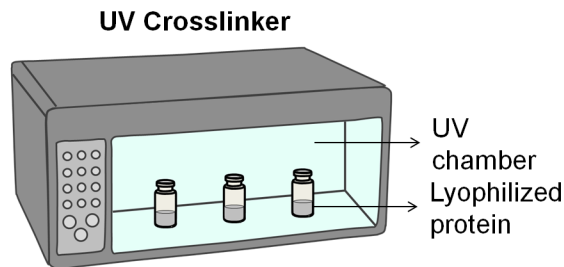
Solid-State HDX

Quenching &
Reconstitution

Mass Spectrometric
Analysis at Intact
and Peptide level
Resolution

B

Protein lyophilized
with excipients and
photoreactive agent



Solid-State Photolytic
Labeling

Quenching &
Reconstitution

Figure 3

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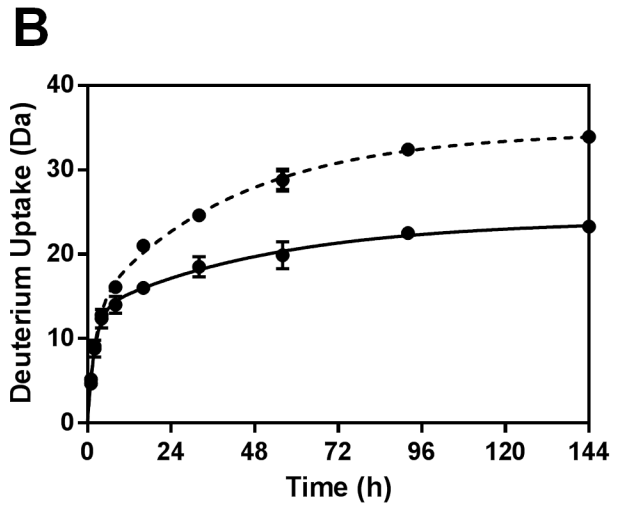
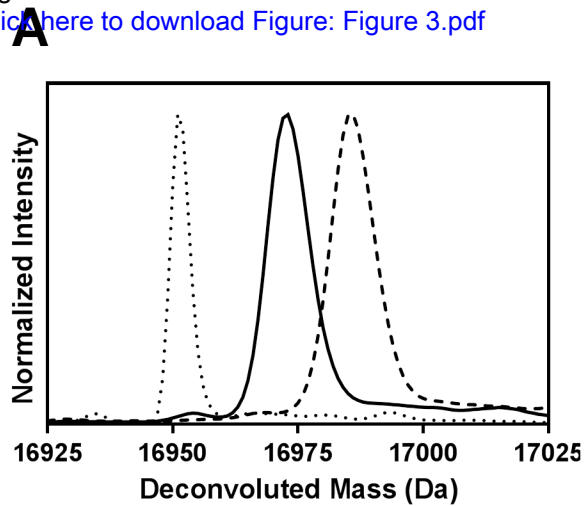
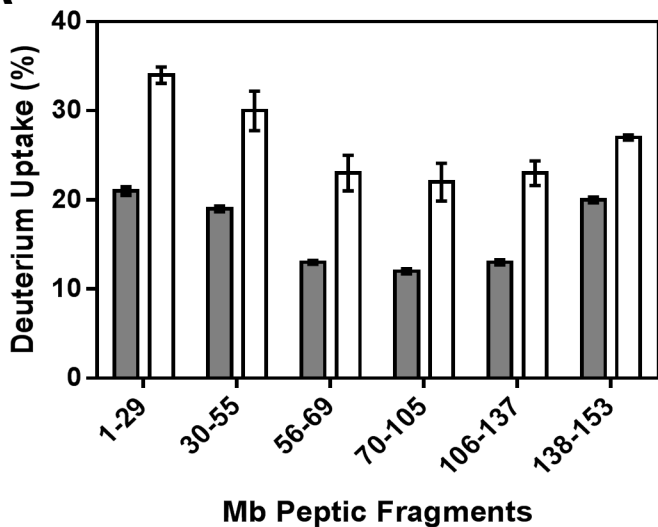


Figure 4

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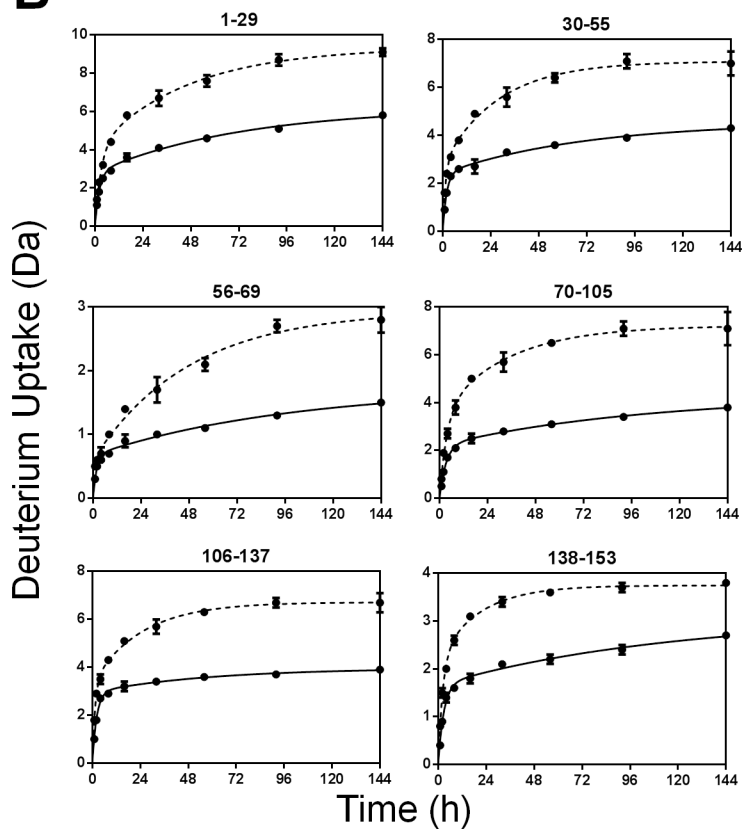


Figure 5
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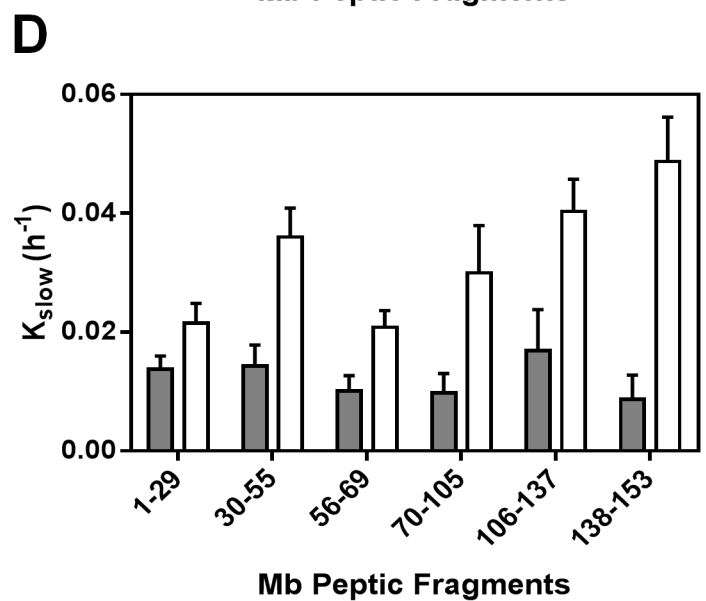
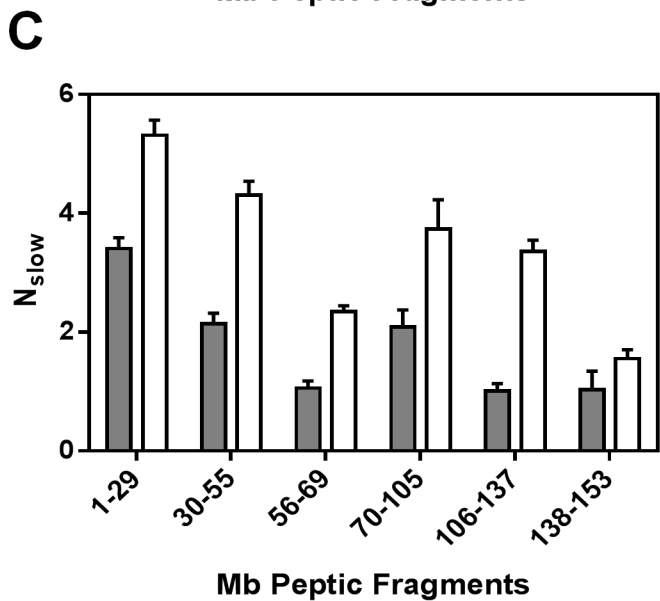
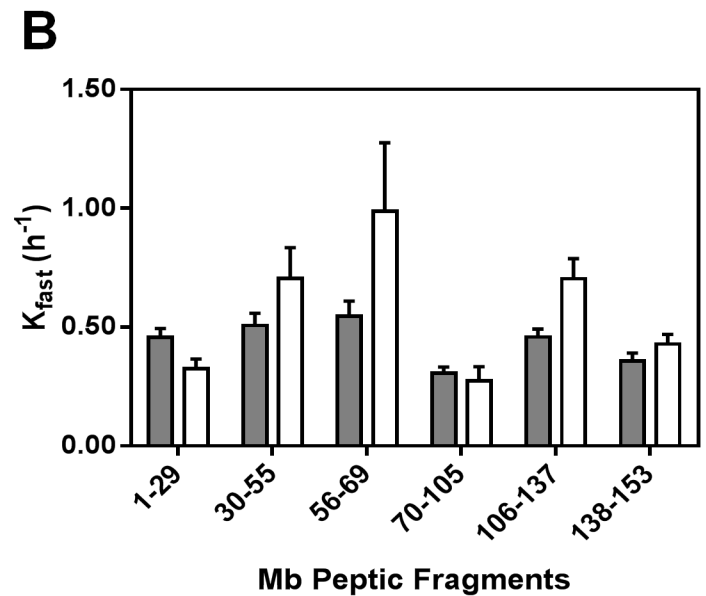
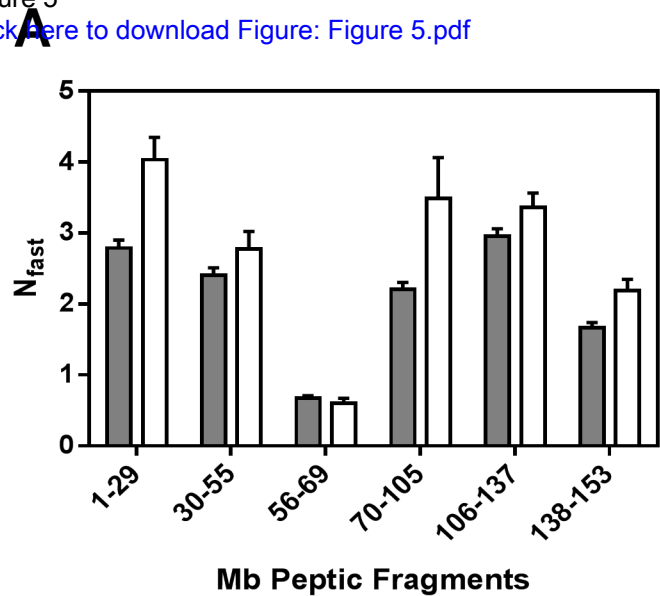
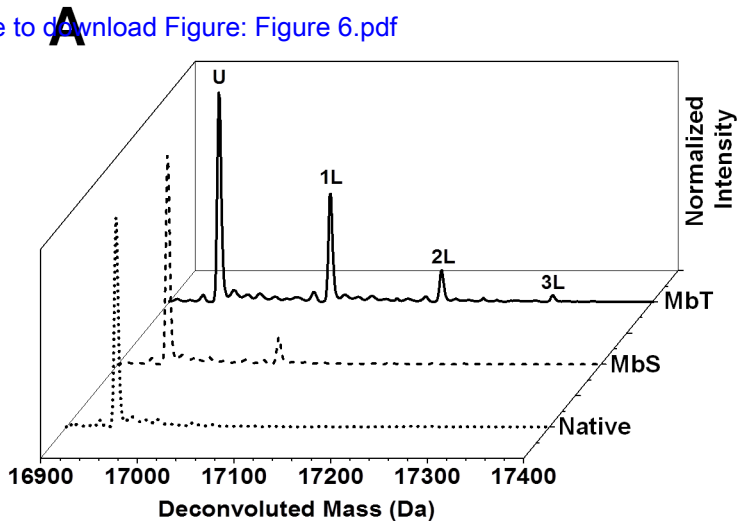
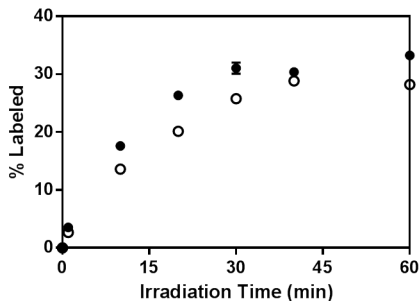


Figure 6

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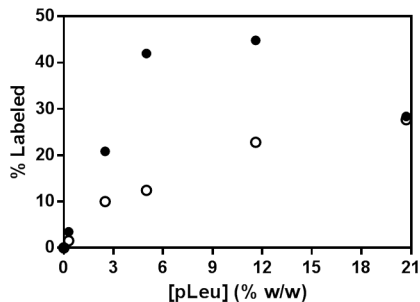
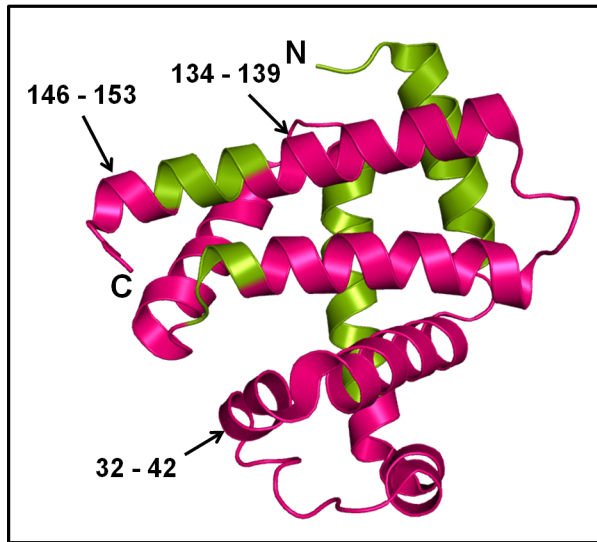


Figure 7

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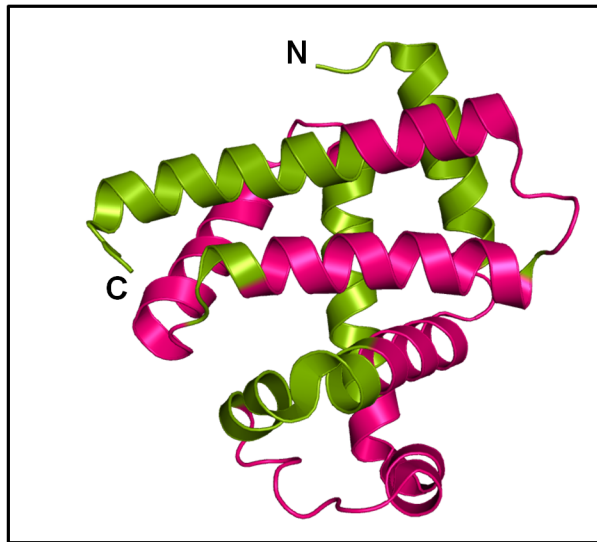


Table 1

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Formulations	Composition (mg/mL) prior to lyophilization				
	Mb	Trehalose	Sorbitol	pLeu ^c	Potassium Phosphate, pH 7.4
MbT ^a	1.7	3.4	-	-	0.4
MbS ^a	1.7	-	3.4	-	0.4
MbT + pLeu ^b	1.7	3.4	-	14.3 x 10 ⁻³ to 1.43	0.4
MbS + pLeu ^b	1.7	-	3.4	14.3 x 10 ⁻³ to 1.43	0.4

Table 2
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	Deuterium Uptake (%) ^b	N _{fast} ^c	k _{fast} ^c	N _{slow} ^c	k _{slow} ^c
MbT ^a	15.9 ± 0.5	13.1 (0.8)	0.43 (0.03)	11.0 (0.9)	0.019 (0.001)
MbS ^a	23.2 ± 0.5	15.4 (0.7)	0.49 (0.04)	19.2 (0.6)	0.024 (0.002)
% change ^d	46%	18%	14%	75%	26%

Name of Material/ Equipment	Company	Catalog Number
Equine myoglobin	Sigma-Aldrich	M0630-5G
D-(+)-Trehalose dihydrate	Sigma Aldrich	#T9531
D-Sorbitol	Sigma Aldrich	#240850
L-Photo-leucine	Thermo Scientific	#22610
Potassium phosphate monobasic	Sigma-Aldrich	#P0662
Potassium phosphate dibasic	Sigma-Aldrich	#P3786
Deuterium Oxide	Cambridge Isotope Laboratories	#DLM-4-PK
Immobilized pepsin	Applied Biosystems	#2-3132-00
Trypsin	Promega	#V511A
Water, Optima LC/MS grade	Fisher Chemical	#7732-18-5
Acetonitrile	Sigma-Aldrich	#34998
Formic acid	Thermo Scientific	#28905
ESI-TOF Calibrant	Agilent Technologies	#G1969-85000
Protein microtrap	Michrom Bioresources	TR1/25108/03
Peptide microtrap	Michrom Bioresources	TR1/25109/02
Analytical column	Agilent Technologies	Zorbax 300SB-C18
Freeze dryer	VirTis AdVantage Plus	
Stratalinker equipped with five 365 nm lamps	Stratagene Corp.	Stratalinker 2400
HPLC	Agilent Technologies	1200 series LC
ESI-qTOF MS	Agilent Technologies	6520 qTOF
HDExaminer (HDX-MS data analysis software)	Sierra Analytics	

Comments/Description

Alternate (Cat. No.: 151882, Sigma-Aldrich)

Chymotrypsin (Cat. No.: #V1062, Promega) can be additionally used

Highly flammable liquid

Refrigerated LC system for HDX-MS

<http://www.massspec.com/HDExaminer.html>



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*The sections below detail our responses to the editorial and peer review comments in a point-by-point fashion. In the manuscript, revisions are indicated in **blue** font.*

Editorial Comments:

Editorial Comment 1: Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

***Response:** The manuscript has been thoroughly proofread to ensure that there are no spelling and grammar errors.*

Editorial Comment 2: Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. The highlighted steps should form a cohesive narrative with a logical flow from one highlighted step to the next. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

***Response:** No changes were made, as the steps highlighted (Yellow) in the current version is already 2.75 pages. The highlighted steps will tell the most cohesive story.*

Editorial Comment 3: Headers were highlighted. Currently there is 3.25 pages of highlighted text that must be reduced to 2.75 pages.

***Response:** We don't agree with this comment. The steps highlighted (Yellow) in the current version is already 2.75 pages with single line spacing and space between each paragraph/bullet point as given in the Manuscript Instructions for Authors.*

Reviewers' comments:

Reviewer #1:

Reviewer's Comment 1: Some minor concerns I have include the discussion of the results. Although the results section demonstrates differences between the MbS and MbT proteins with both methods, it would be nice to see a comparison of these results using both methods discussed further in the discussion section.

***Response:** Comparison of results from these two methods has not been performed before. This has been included in discussion (lines 595-599).*

Reviewer #2:

Reviewer's Comment 1: p.2 line 54...the complementarity of covalent labeling (CL) to HX comes from the fact that CL labels side chains mostly and HX monitors the polypeptide backbone. The lack of back exchange in CL is just an advantage.

Response: *We agree with the reviewer's comment, we have changed this statement in the manuscript (line 54).*

Reviewer's Comment 2: p.9 line 400/403...figures 6B/C seem to be reversed.

Response: *We thank the reviewer for notifying this error. This has been fixed.*

Reviewer's Comment 3: p. 10 line 435 and p.13 line 568-572...while decreased labeling in the MbS sample is consistent with conformational change, it could also be that sorbitol may be more susceptible to labeling from the pLeu than trehalose? This is a well-known phenomenon in hydroxyl radical labeling, where solution additives can greatly affect the amount of labeling [Tong et al. (2008) Anal. Chem. 80, 2222]. Previous work from the authors suggests this caveat may exist in pLeu labeling as well [Iyer et al. (2013) Mol. Pharmaceutics 10, 4629]. One could investigate this by monitoring the ratio of labeled:unlabeled excipient, or alternatively using a protein that could not undergo large conformational changes, such as lysozyme with its intact S-S bonds. The authors should address this question in their discussion, as it could easily lead to artifactual data interpretations.

Response: *We agree with the reviewer's comment. As the samples were desalted before injecting into mass spectrometry, we were unable to detect pLeu-excipient adducts in both MbT and MbS formulations. The interference of excipients in the availability of pLeu for protein labeling is yet to be characterized. This has been added to the manuscript (lines 569-572).*

Reviewer's Comment 4: p.12 line 523...previous pLeu studies [Jumper et al. (2012) Anal. Chem. 84, 4411] have shown that there is a labeling bias towards negatively charged amino acid side chains. The authors should address this bias and potential ways to modulate the bias. The fact that in this study the pLeu is lyophilized with the protein, one could imagine a concentration effect of the label in the vicinity of Asp and Glu side chains.

Response: *Jumper et al. (2012) showed that there is some bias for diazirine distribution near carboxylates (Asp and Glu), but solvent accessibility of these residues is also a limiting factor. They suggest that once the diazirine is activated, the singlet carbene is able to sample its local environment and label carboxylates to a greater extent than other residues. This bias was somewhat modulated by controlling ionic strength and temperature. In this paper, we are not looking for residue-specific information. We are looking for peptide/region specific labeling and labeling differences as a function of excipient type. Formulation comparison will not be affected by residue bias, only by excipient effect on protein conformation.*

Reviewer's Comment 5: Figure 1B...the amount of pLeu labeling on the α -helix is deceiving. Figure 6A shows a maximum of 3 labels per protein. The authors should reduce the number of labels in Fig 1B.

Response: *Figure 1A and 1B depicts the maximum theoretically possible labeling in protein. This has been added to the figure legend (lines 469-471).*

Reviewer's Comment 6: Figure 6...panels B and C seem to be inverted with respect to their descriptions in the text.

Response: *We thank the reviewer for notifying this error. This has been fixed.*

Reviewer's Comment 7: The techniques described herein are very useful. While the data from the two labeling methods are complementary, both have distinct caveats. Given that this is a methods paper, the drawbacks and pitfalls of labeling and data interpretation should be more explicitly addressed (especially in the case of the pLeu covalent labeling, which is much newer and not as well characterized as HX)

Response: *We agree with the reviewer's comment. Additional information has been provided in the discussion (lines 543-553 and 572-578).*

Reviewer #3:

Reviewer's Comment 1: I don't understand "This ensures that moisture sorption and diffusion into the solid do not control the reaction rate (line 168)", because the humidity is considerably different between at the beginning and ending of the HDX.

Response: *We do not agree with the reviewer's comment, because the humidity within the desiccator was controlled and maintained constant (lines 141-144).*

Reviewer's Comment 2: Table 2 is confusing. For example, neither N (number, percentage, or something else?) nor k (min⁻¹ or h⁻¹?) is defined appropriately.

Response: *We do not agree with the reviewer's comment. The parameters are already explained under step 2.13 in the protocol and under the description of Table 2.*

Reviewer's Comment 3: Do the different values for MbS and MbT (not being defined, either) mean that there are amide sites completely protected from HDX (extremely slow=no deuterium incorporation) and those completely exposed (extremely fast=no deuterium incorporation due to fast back-exchange), and that some of N(fast)s in MbT become N(slow)s or vanishing (too fast or too slow) in MbS (or vice versa)?

Response: *The composition of formulations MbS and MbT are already given in Table 1. The smaller N_{slow} values for MbT at both intact and peptide level may suggest either higher structure retention or protection of these amide hydrogens by excipients that are exposed to D₂O in MbS.*

However, the detailed mechanisms are not clearly understood. This has been included in the text (lines 361-363). The back-exchange event happens only after the HDX reactions were quenched and has no effect on the N_{fast} or N_{slow} values.

Reviewer's Comment 4: what is "% change" meant?

***Response:** The details on how percent change was calculated are already provided under description of Table 2.*

Reviewer's Comment 5: in equation (1), total number of exchangeable amide cannot be equal to total number of amino acids (-Pro-2) but there are amide protons of Asn and Gln, the other NHs of Trp and His, and the OHs of Ser, Thr, and Tyr, all of which are potential HDX sites contributable to N_{fast} and k_{fast} values unless they are involved in hydrogen bonds.

***Response:** The N_{fast} s and N_{slow} s include amide hydrogens present in the protein back-bone and are different from hydrogens present in the side-chains. The side-chain hydrogens that exchange with deuterons will be back-exchanged during sample analysis and are usually not included in the calculation.*

Reviewer's Comment 6: carbene resulting from pLeu on photolysis is claimed to be non-specific (line 496), but it is definitely specific; it reacts with the OH and the NH groups to form the corresponding ester, ether, and alkylamine much faster than it does with C-H bonds (please see the standard textbooks in organic chemistry). This point can be clarified by examining the MS/MS spectra of the peptide with an increased mass to pinpoint the residue modified; this is really site-specific (Abstract).

***Response:** We do not agree with the reviewer's comment. MS/MS analysis was carried out on a selected tryptic peptide from myoglobin (Iyer et al Mol. Pharm. (2013) 10, 4629) and no specific residue was found to be labeled. This suggests multiple possible targets for pLeu labeling. Since the abundance of labeled peptides detected was low and MS/MS did not yield complete b- and y-ion data, it was not possible to perform MS/MS successfully for all peptides.*

Reviewer's Comment 7: The most disappointing point is that it does not suggest any advantageous feature over the conventional solution HDX-MS. Even if the readership is limited to pharmacologists who deal with lyophilized protein preparations, what is the benefit of using this technique? A few notable results include the difference between sorbitol and trehalose as additives to Mb. The result of the experiment is described, but the cause of such a difference is not discussed. So which (or anything else) is the better additive to store the lyophilized protein?

***Response:** HDX-MS and PL-MS experiments has been modified and adopted to study structure and conformation of protein in lyophilized powders. As these techniques are specifically developed to study proteins in solid-state, we have no purpose to compare ssHDX-MS or ssPL-MS with solution based methods. However, the drawbacks of other methods and the advantages*

of ssHDX-MS and ssPL-MS in characterizing lyophilized proteins has already been explained in introduction (lines 70-81) and discussion (lines 588-602) respectively. As the scope of JoVE limits the discussion section to method and protocol, the differences observed between MbT and MbS were not discussed in this work. However, for detailed discussion of results, readers are encouraged to read similar studies that have been cited in this work.