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TITLE:

Capillary Electrophoresis-based Hydrogen/Deuterium Exchange for Conformational Characterization of Proteins with Top-down Mass Spectrometry

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SUMMARY:

Presented here is a protocol for a capillary electrophoresis-based hydrogen/deuterium exchange (HDX) approach coupled with top-down mass spectrometry. This approach characterizes the difference in higher-order structures between different protein species, including proteins in different states and different proteoforms, by conducting concurrent differential HDX and electrophoretic separation.

ABSTRACT:

Resolving conformational heterogeneity of multiple protein states that coexist in solution remains one of the main obstacles in the characterization of protein therapeutics and the determination of the conformational transition pathways critical for biological functions, ranging from molecular recognition to enzymatic catalysis. Hydrogen/deuterium exchange (HDX) reaction coupled with top-down mass spectrometric (MS) analysis provides a means to characterize protein higher-order structures and dynamics in a conformer-specific manner. The conformational resolving power of this technique is highly dependent on the efficiencies of separating protein states at the intact protein level and minimizing the residual non-deuterated protic content during the HDX reactions.

Here we describe a capillary electrophoresis (CE)-based variant of the HDX MS approach that aims to improve the conformational resolution. In this approach, proteins undergo HDX reactions while migrating through a deuterated background electrolyte solution (BGE) during the capillary electrophoretic separation. Different protein states or proteoforms that coexist in solution can be efficiently separated based on their differing charge-to-size ratios. The difference in electrophoretic mobility between proteins and protic solvent molecules minimizes the residual non-deuterated

solvent, resulting in a nearly complete deuterating environment during the HDX process. The flow-through microvial CE-MS interface allows efficient electrospray ionization of the eluted protein species following a rapid mixing with the quenching and denaturing modifier solution at the outlet of the sprayer. The online top-down MS analysis measures the global deuteration level of the eluted intact protein species, and subsequently, the deuteration of their gas-phase fragments. This paper demonstrates this approach in differential HDX for systems, including the natural protein variants coexisting in milk.

INTRODUCTION:

Distinguishing protein species in different conformational, binding, or modification states and characterizing their structural differences are important for monitoring the pathways of transitions between these species involved in biological events, ranging from molecular recognition to enzymatic catalysis, and understanding the mechanisms underlying these events. Conventional biophysical techniques do not provide a complete solution due to the limitations such as insufficient resolution and loss of dynamic information in solution. Hydrogen/deuterium exchange coupled with mass spectrometry (HDX MS) is a technique that labels the structural and conformational features of proteins with deuterium (^{2}H) via the exchange between labile hydrogen atoms of proteins and ^{2}H from the deliberately introduced ^2H_2O solution. Protons involved in hydrogen bonding or that are sequestered from the solvent in the protein interior do not exchange readily[1]. Thus, as the exchange rate at an exchangeable site is highly dependent on its involvement in higher-order structures, the protein structures can be revealed at high spatial resolution by MS that probes the extent and rate of ^{2}H-uptake based on the differing atomic masses between ^{1}H and ^{2}H. Over the recent decades, HDX MS has become an outstandingly successful technique for studying protein conformations and dynamics[2].

In the classical bottom-up approach of HDX MS, the ensemble of protein species in different conformational, binding, or modification states is proteolyzed without separation at the intact protein level, making it infeasible to characterize individual species by analyzing the resulting proteolytic fragments with convoluted deuterium contents. In contrast, in the top-down approach, different protein states or proteoforms that have incorporated different deuterium contents give rise to multiple distributions of intact protein masses in an MS scan. This allows individual species to be separated by mass-selection of ions corresponding to each mass distribution using a proper mass filter (such as a quadrupole) and the characterization of their conformational differences in the subsequent tandem MS analysis[3-6]. However, the efficiency of separating protein states or proteoforms in this strategy is limited by the extent of difference in their corresponding mass distributions.

Capillary electrophoresis (CE) provides a means to separate protein species based on their differing charges and hydrodynamic sizes in the solution phase with high efficiency[7]. Combining CE with HDX offers additional separation of protein states or proteoforms in the solution phase. In addition, the small volume of the CE capillary allows the utilization of a fully deuterated solution as the background electrolyte solution (BGE), i.e., the running buffer, rendering the capillary as an HDX reactor for protein samples. Due to the difference in electrophoretic mobility between proteins and protic reagents in the electrophoresis process, conducting HDX during CE results in a nearly complete

deuterating environment for the protein analytes with minimal residual non-deuterated contents, thereby enhancing the sensitivity of the structural analysis using HDX data. As such, we developed a CE-based differential HDX approach coupled with top-down MS to characterize protein higher-order structures in a state- or proteoform-specific manner[8].

This paper describes protocols for this approach by detailing the steps of material preparation, experimental procedure, and data analysis. Factors that may affect the method performance or data quality are listed in short notes. The representative results presented here include differential HDX data of mixtures of different proteins and natural variants of bovine β-lactoglobulin (β-lg), the major whey protein present in milk[9]. We demonstrate separation efficiency, reproducibility, and ^{2}H-labeling performance of the two abundant variants of β-lg, i.e., A and B[10-11] during the CE-based HDX and variant-specific characterization of their conformations.

PROTOCOL:

NOTE: Use high-performance liquid chromatography (HPLC) grade or MS grade reagents whenever possible to minimize the contaminants that may interfere with MS analysis. Do not touch the CE-MS interface with bare hands during the measurement to avoid the possibility of an electrical shock caused by either the electrophoretic voltage or electrospray voltage.

1. Material preparation

1.1. Modification of fused silica capillary for CE

1.1.1. Prepare a 5% (w/w) hydroxypropyl cellulose (HPC) solution by dissolving HPC powder (molecular weight [MW]: 100 kDa) in water with continuous stirring at room temperature on a magnetic stirrer for ~12 h or until complete disappearance of solid particles[12]. Remove any visible air bubbles with an ultrasonicator.

1.1.2. Mount a fused silica glass capillary (internal diameter [ID]: 50 µm, outer diameter [OD]: 360 µm) of approximately 85 cm length into a CE instrument. Rinse the capillary by continuously infusing an organic solvent, such as acetone[13], using the autosampler of CE at an infusion pressure of 40 psi for 10–15 min.

1.1.3. Fill the cleaned capillary with HPC solution using the autosampler at an infusion pressure of 40 psi (which often takes ~40 min). Infuse air into the HPC-filled capillary at 40 psi to ensure free airflow in the capillary, indicated by the air bubbles ejected from the capillary upon immersion in water.

1.1.4. Bake the HPC-coated capillary in a temperature-programmable oven (ideally the temperature-controlled column oven of a temperature-programmed gas chromatograph) with nitrogen gas (25 psi) flowing through the capillary, following the temperature program shown in **Figure 1**.

[Place **Figure 1** here]

1.1.5. Cool the oven to room temperature before taking the capillary out. Use this HPC-modified capillary for CE separation.

1.2. Background electrolyte (BGE) solution and modifier solution[8]

1.2.1. Prepare 1–10 mL of BGE at the desired concentration (e.g., 10 mM) by dissolving the appropriate quantity of ammonium acetate in $^{2}H_2O$. Place 200 µL-aliquots of BGE in separate BGE vials and seal the vials with parafilm to minimize the HDX reaction between BGE and water vapor in the air.

1.2.2. Prepare 10 mL of a modifier solution with 75% (v/v) methanol and 25% (v/v) water, with pH adjusted to 2.5 using formic acid.

NOTE: Use $^{2}H_2O$ and deuterated methanol to prepare the modifier solution if the deuterium atoms in the side chains and unprotected backbone amides should be retained for detection by MS.

1.3. Desalting of protein samples

1.3.1. Prepare an ammonium acetate solution in non-deuterated water at the desired concentration.

NOTE: A concentration less than 100 mM is recommended to avoid a high electric current during electrophoresis and the resulting Joule heating effect.

1.3.2. When necessary, adjust the pH of the ammonium acetate solution to the desired level using formic acid (for pH < 7) or ammonium hydroxide (for pH > 7).

1.3.3. Substitute the original buffers of the protein solution with an ammonium acetate solution (prepared in non-deuterated water at the desired concentration; pH adjusted to 7.5 with ammonium hydroxide) through at least five sequential concentrations and dilution steps at 4 °C using a centrifugal filter with a proper MW cutoff.

NOTE: The protein samples to be desalted may be either from prior production procedures (e.g., purification or formulation) or prepared by dissolving the lyophilized powder of protein. The “salts” to be removed from the sample solutions in this step refer in general to all small ions or molecules that are non-volatile. Although these species can be efficiently separated from proteins during the electrophoresis process, this step is recommended to avoid compromising the electrophoretic resolution and thus minimizing the contamination of the mass spectrometer. When protein analytes should be stabilized by specific salts or additives, include them in the BGE.

1.3.4. Determine the protein concentration using a microvolume UV-Vis spectrophotometer.

2. Operation of CE-based HDX MS analysis

NOTE: The mass spectrometer used in this approach should be equipped with a mass analyzer with ultra-high resolution, such as a Fourier-transform ion cyclotron resonance (FTICR) or orbitrap, a mass-filter, such as a quadrupole that allows mass-selection of precursor ions for fragmentation, and electron-transfer dissociation (ETD) or electron-capture dissociation (ECD) functions to perform top-down analysis with reliable tandem MS data (ideally isotopically resolved signals of fragment ions).

2.1. Optimization of CE and MS settings

2.1.1. Perform a pilot MS measurement using a standard electrospray ionization (ESI) source by spraying either the preloaded sample from a metal-coated borosilicate glass capillary (the "static" nanoESI scheme) or the continuously infused sample from a metal emitter to optimize the MS settings for measurement of intact proteins (MS1) and their gas-phase fragments (MS2). Fragment the protein species of interest by mass-selection of the ensemble of its ions in a single charge state, followed by ETD or ECD of the precursor ions.

NOTE: The essential settings include the parameters that affect desolvation, the mass-selection of precursor ions (to avoid interference from other species), and fragmentation efficiency. Both the center and width of the mass-selection window should be increased to match the resulting mass distribution of the analyte ions after HDX. Because the elution window of a protein species in CE typically ranges from 0.5 min to 2 min, assess the fragmentation efficiency based on MS2 scans accumulated over a comparable time window. The optimal values of these parameters are protein-specific; readers are referred to previously published reports for exemplary settings[8,14].

2.1.2. Perform a pilot CE measurement using a CE instrument equipped with an optical detector, i.e., a photodiode array (PDA) detector or a UV detector to optimize the CE settings for the separation of the protein species and the migration times, which is equivalent to the HDX reaction times.

NOTE: This step is optional depending on the availability of the optical detector of CE. In the absence of an optical detector, the CE settings can be optimized using CE-MS upon completion of section 2.2, following the instructions described in section 2.3. The essential settings include parameters that affect separation efficiency, peak shapes shown in electropherograms, and elution times.

2.2. Pre-conditioning of the CE-HDX setup

2.2.1. Clean the flow-through microvial CE-MS interface with a mixture of 50% methanol, 49% water, and 1% formic acid (v/v) using ultrasonication for at least 30 min at room temperature.

2.2.2. Upon mounting of the HPC-modified capillary on a CE instrument, rinse the capillary with BGE using the autosampler for 10 min and leave the capillary filled with BGE.

2.2.3. Obtain a proper length of unmodified fused silica capillary tubing (ID: 50 µm, OD: 360 µm) as the infusion tubing for the modifier solution. Connect the modifier tubing to a gas-tight glass syringe with a blunt tip using a union and proper sleeve, and rinse the tubing with the modifier solution using an infusion pump for at least 10 min.

2.2.4. Insert the outlets of the HPC-coated CE capillary and modifier tubing, which have been loaded with corresponding solutions, into the cleaned CE-MS interface, as illustrated in **Figure 2**.

[Place **Figure 2** here]

2.2.5. Advance the syringe for the modifier infusion either manually or with the infusion pump to ensure that the modifier solution reaches the tip of the interface. Mount the assembled CE-MS interface on a nanoESI source housing of a mass spectrometer.

2.3. Simultaneous CE separation, HDX reaction, and MS analysis

NOTE: Deuterated BGE is recommended to be used within 1 day after unsealing.

2.3.1. Apply a spray voltage of 3–5 kV to the CE-MS interface.

2.3.2. Start infusing the modifier solution with the infusion pump at a flow rate ranging from 0.1 to 10 µL/min, and ensure a stable electrospray at the tip of the CE-MS interface.

2.3.3. Place the sample vial containing the BGE in the autosampler, and use it in step 2.3.4 to acquire blank electropherograms and blank mass spectra.

2.3.4. Inject the sample solution using the autosampler at 2 psi and for a proper duration to allow the injection of a desired quantity of the sample. Estimate the injection volume using the relationship between injection volume and injection parameters[15] defined by equation (**1**).

$$V_{inj} = \frac{\Delta p \times d_c^2 \times A \times t_{inj}}{32\eta \times L} \qquad (\mathbf{1})$$

Where V_{inj} is the injection volume, Δp is the pressure of injection, d_c is the inner diameter of the capillary, A is the cross-section of the capillary, t_{inj} is the duration of injection, η is the viscosity of the liquid in the capillary, and L is the length of the capillary.

2.3.5. Start the CE separation by applying an electrophoretic voltage of 30 kV and infusion pressure ranging from 0 to 2 psi, and acquire the electropherogram. Meanwhile, start the acquisition of the MS data in the chromatographic mode where the ion current graph is acquired as a function of time, and the corresponding MS scans are not automatically combined into a single spectrum.

NOTE: Proteins undergo spontaneous HDX reaction at the point of contacting ^2H_2O molecules in BGE during their electrophoretic migration in this step. The optical detection for CE can be used in addition to the MS detection. As on-column detection requires the removal of a certain length of polyimide coating at the outlet end of the fused silica capillary, additional care should be taken to avoid capillary damage during the assembly of the CE-MS interface.

2.3.6. Save the blank electropherogram and mass spectra as references.

NOTE: Blank data are to be used for troubleshooting rather than baseline subtraction.

2.3.7. Place the sample vials containing the desired concentrations of the protein sample solutions in the autosampler. Acquire the electropherograms and mass spectra for the protein samples following steps 2.3.4–2.3.5. Collect an adequate number of MS scans to obtain MS1 spectra of the electrophoretically separated and ^{2}H-labeled protein species.

2.3.8. Perform tandem MS measurements for the species of interest either after acquiring the MS1 spectra within the same run or in a subsequent, separate run.

2.3.9. When necessary, adjust the migration times/HDX reaction times by changing the infusion pressure or the length of the CE capillary. If the HDX reaction time must be shorter than the migration time, use the approach described previously[8], which employs both deuterated and non-deuterated BGE in the capillary during the CE process.

2.3.10. Flush the CE capillary with BGE at a pressure of 20 psi for at least 10 min after each measurement.

2.3.11. Upon completion of the experiments, clean the CE-MS interface and all tubing for storage.

2.3.12. Acquire a data set of the HDX "endpoint" sample (which can be prepared using approaches described previously[6,16]) with MS in direct infusion mode.

NOTE: This step is only required when a deuterated modifier solution is used for CE-based HDX.

3. Data analysis

3.1. Analysis of CE data

3.1.1. Use one of the following plots as the electropherogram to determine the electrophoretic characteristics, including the number of peaks, migration times, and separation efficiency: (a) UV absorbance vs. migration time, acquired by the optical detector of CE instrument (when available); (b) the total ion current (TIC) graph acquired by MS; (c) the extracted ion current (EIC/XIC) graph acquired by MS.

NOTE: EIC/XIC provides the optimal signal/noise ratio (S/N), in general, among the aforementioned formats of electropherograms. It is noteworthy that even in the absence of any instrumental biases, while UV absorbance is proportional to the mass concentration of protein, the MS signal is proportional to the molar concentration. Hence, it is reasonable to observe differences in peak patterns between CE- and MS-derived electropherograms.

3.1.2. Use the area under the curve (AUC) of the peaks shown in the electropherograms for semi-quantitation. For samples involving protein complexes, use the approach described previously[17] to deduce the mass concentration data from the TIC/EIC electropherograms.

3.2. Analysis of MS data

3.2.1. Obtain the MS1 and MS2 spectra by combining the MS1 and MS2 scans acquired within the corresponding elution windows, respectively.

3.2.2. Determine the masses of the intact protein ($M(\text{intact protein})$) and fragments by either of the following two methods.

3.2.2.1. Calculate the average masses of the ions giving rise to the isotopically resolved signal clusters.

3.2.2.2. Use the center of the Gaussian-like curves resulting from the fitting of the corresponding isotopic envelopes[6].

3.2.3. Use software such as Biopharma Finder, ProSight[18], or MASH Suite[19] to generate the mass list of the fragment ions and identify them.

3.3. Analysis of HDX data

3.3.1. Determine the overall deuteration level of an intact protein species using equation (**2**).

$$D(\text{intact protein}) = \frac{M^*(\text{intact protein}) - M(\text{intact protein})}{M(\,^2\text{H}) - M(\,^1\text{H})} \qquad (\mathbf{2})$$

where M (^{2}H) or M (^{1}H) are atomic weights of ^{2}H or ^{1}H. The asterisk indicates data of the ^{2}H-labeled sample.

3.3.2. Determine the cumulative protection or cumulative deuteration of backbone-amides of a specific segment.

3.3.2.1. For data acquired with a deuterated modifier solution, use equations (**3**) and (**4**) to determine the cumulative protection level.

$$P(\text{S}_k(\text{N})) = \frac{M^{**}(\text{c}_{k-1}) - M^*(\text{c}_{k-1})}{M(\,^2\text{H}) - M(\,^1\text{H})} \qquad (\mathbf{3})$$

$$P\big(\text{S}_m(\text{C})\big) = \frac{M^{**}(\text{z}_{m+1}) - M^*(\text{z}_{m+1})}{M(\,^2\text{H}) - M(\,^1\text{H})} \qquad (\mathbf{4})$$

Where $P(S_k(N))$ is the total protection of the N-terminal segment spanning residues 1 through k, $P(S_m(C))$ is the total protection of the C-terminal segment comprising m residues, M (^2H) or M (^1H) are atomic weights of ^2H or ^1H, and M (c_i) or M (z_i) are the molecular weights of c_i or z_i ions.

NOTE: The double-asterisk indicates data of the HDX "endpoint" sample.

3.3.2.2. For data acquired with a non-deuterated modifier solution, use equations (**5**) and (**6**) to determine the cumulative deuteration level.

$$D(S_k(N)) = \frac{M^*(c_{k-1}) - M(c_{k-1})}{M(^2H) - M(^1H)} \quad (\mathbf{5})$$

$$D(S_m(C)) = \frac{M^*(z_{m+1}) - M(z_{m+1})}{M(^2H) - M(^1H)} \quad (\mathbf{6})$$

Where $D(S_k(N))$ is the cumulative deuterium uptake of the N-terminal segment spanning residues 1 through k; $D(S_m(C))$ is the cumulative deuterium uptake of the C-terminal segment comprising m residues.

3.3.3. Determine the deuteration level at a local backbone amide group

3.3.3.1. For data acquired with a deuterated modifier solution, use equations (**7**), (**8**), (**9**), (**10**), and (**11**) to determine the local protection level.

for data deduced from *c*-ions

$$P(R_i) = P(S_i(N)) - P(S_{i-1}(N)) \quad (\mathbf{7})$$

for data deduced from *z*-ions

$$P(R_i) = P(S_{total+1-i}(C)) - P(S_{total-i}(C)) \quad (\mathbf{8})$$

Where $P(R_i)$ is the protection of a backbone amide at residue i, and the subscript "total" denotes the total residue number of the protein.

For residue sites where subsequent fragment ions were missing, assign $P(R_i)$ using equations (**9**) and (**10**).

for data deduced from *c*-ions

$$P(R_i) = P(R_{i+1}) = \cdots = P(R_{i+j-1}) = \frac{P(S_{i+j}(N)) - P(S_i(N))}{j} \quad (\mathbf{9})$$

for data deduced from *z*-ions

$$P(R_i) = P(R_{i+1}) = \cdots = P(R_{i+j-1}) = \frac{P(S_{total+1+j-i}(C)) - P(S_{total-i}(C))}{j} \quad (\mathbf{10})$$

Then, determine the deuteration level $D(R_i)$ at a local backbone amide group using equation (**11**).

$$D(\mathrm{R}_i) = 1 - P(\mathrm{R}_i) \qquad (\mathbf{11})$$

3.3.3.2. For data acquired with a non-deuterated modifier solution, use equations (**12**), (**13**), (**14**), and (**15**) to determine the local protection level.

for data deduced from *c*-ions

$$D(\mathrm{R}_i) = D(\mathrm{S}_i(\mathrm{N})) - D(\mathrm{S}_{i-1}(\mathrm{N})) \qquad (\mathbf{12})$$

for data deduced from *z*-ions

$$D(\mathrm{R}_i) = D(\mathrm{S}_{\mathrm{total}+1-i}(\mathrm{C})) - D(\mathrm{S}_{\mathrm{total}-i}(\mathrm{C})) \qquad (\mathbf{13})$$

Where $D(R_i)$ is the protection of a backbone amide at residue *i*, and the subscript "total" denotes the total residue number of the protein.

For residue sites where subsequent fragment ions were missing, assign $D(R_i)$ using equations (**14**) and (**15**).

for data deduced from *c*-ions

$$D(\mathrm{R}_i) = D(\mathrm{R}_{i+1}) = \cdots = D(\mathrm{R}_{i+j-1}) = \frac{D(\mathrm{S}_{i+j}(\mathrm{N})) - D(\mathrm{S}_i(\mathrm{N}))}{j} \qquad (\mathbf{14})$$

for data deduced from *z*-ions

$$D(\mathrm{R}_i) = D(\mathrm{R}_{i+1}) = \cdots = D(\mathrm{R}_{i+j-1}) = \frac{D(\mathrm{S}_{\mathrm{total}+1+\mathrm{j}-i}(\mathrm{C})) - D(\mathrm{S}_{\mathrm{total}-i}(\mathrm{C}))}{j} \qquad (\mathbf{15})$$

REPRESENTATIVE RESULTS:

Changing the infusion pressure of BGE allows the adjustment of both separation efficiency and migration time, which is equivalent to the HDX reaction time of the proteins to be separated (**Figure 3**). A lower infusion pressure results in better separation of CE peaks at the cost of the duration of the experiment (**Figure 3A**). A longer migration/HDX reaction time results in a higher level of deuteration of the protein analytes (**Figure 3B–D**). At the HDX timescale of minutes, the deuteration difference should primarily reflect the differing exchange extents at the structurally protected sites instead of the fast-exchangeable sites. According to the trend of the deuteration-time functions shown by either protein species, the migration time difference is unlikely to be the major contributor to the deuteration difference. Indeed, in differential CE-HDX of holo- and apo-myoglobin (Mb)[8], the earlier eluted apo-Mb shows a higher deuteration level than holo-Mb, clearly suggesting that the conformational difference is the primary factor determining the measured deuteration difference.

Correction for the deuteration difference introduced by the migration time difference can be made via curve-fitting for the data of the deuteration level vs. HDX time (**Figure 3D**). The variants A and B of β-lg differ by only two amino acid residues in their sequence (D64G and V118A)[20]. These variants gave rise to two adequately separated peaks in the EIC-derived electropherogram (**Figure 4A**).

Reproducible separation profiles were obtained from experiments conducted by different operators using different instruments at different facilities (**Figure 4A**). The resulting distinct mass distributions of ions corresponding to the differentially ^{2}H-labeled variant (**Figure 4C**) allow mass-selection of each variant using a quadrupole mass filter for the subsequent top-down MS analysis, without interference from the cation-adduct ions of the other variant.

Tandem MS spectra of representative fragment ions are shown in **Figure 5**. The unique disulfide linkage and conformation of β-lg limit the fragmentation efficiency between Cys82 and Cys176 because additional fragmentation energy is required to cleave the disulfide bonds that enclose this region[14], resulting in a lower number and relative abundance of *z*-ions (C-terminal) than *c*-ions (N-terminal) (**Figure 5A,B**). This problem can be solved by combining with disulfide reduction approaches[21-24]. While most of the fragment ions produced from β-lg A and β-lg B exhibit a similar extent of deuterium uptake (**Figure 5A,B**), larger segments that cover the sequence variation sites (represented by ions such as c137) from β-lg A are deuterated to significantly greater extents than β-lg B (132 vs. 119 ^{2}H atoms; **Figure 5C**). These results are in agreement with the CE profile and the crystallography characterization results of these variants. The CE profile indicates higher electrophoretic mobility of β-lg B due to lower structural flexibility. The crystallography characterization results of these variants indicate that small changes in backbone conformation take place in the vicinity of D64G on loop CD (residues 61–67)[11].

FIGURE AND TABLE LEGENDS:

Figure 1: A recommended temperature program for capillary baking.

Figure 2: Schematic illustration of the CE-based HDX MS setup. This figure has been modified from[8]. Abbreviations: BGE = background electrolyte solution; CE = capillary electrophoresis; MS = mass spectrometry; HDX = hydrogen/deuterium exchange; ESI = electrospray ionization; FTICR = Fourier-transform ion cyclotron resonance; ETD = electron-transfer dissociation; ECD = electron-capture dissociation.

Figure 3: CE-based HDX MS analysis of a mixture of myoglobin and ubiquitin with different HDX reaction times. (**A**) Electropherograms (EIC-based) of ^{2}H-labeled Mb (red) and Ub (blue) from a mixture, acquired with different BGE infusion pressures. (**B**) Mass spectra of ^{2}H-labeled $[Mb]^{16+}$ (red) and $[Ub]^{8+}$ (blue) acquired with different HDX times. Overlaid on top are reference spectra of unlabeled $[Mb]^{16+}$ and $[Ub]^{8+}$ ions (gray). (**C**) Migration time of Mb (red) and Ub (blue) as a function of BGE infusion pressure. (**D**) Deuteration level of Mb (red) and Ub (blue) as a function of HDX reaction time (equivalent to the migration time). Data acquired with a CESI 8000 plus capillary electrophoresis system and a Q Exactive UHMR mass Spectrometer. Abbreviations: BGE = background electrolyte solution; CE = capillary electrophoresis; MS = mass spectrometry; HDX = hydrogen/deuterium exchange; Mb = myoglobin; Ub = ubiquitin; EIC = extracted ion current.

Figure 4: CE-based HDX MS analysis of a natural mixture of β-lg A and β-lg B from bovine milk. (**A**) Electropherograms (EIC-based) of ^{2}H-labeled β-lg A (blue) and β-lg B (red) from bovine milk, acquired with different BGE infusion pressures. Data acquired with a CESI 8000 plus CE system and a Q Exactive UHMR Mass Spectrometer. Overlaid on top is an electropherogram from a measurement performed

at a different facility (gray), with a PA 800 Plus Pharmaceutical Analysis CE System and an Orbitrap Fusion Lumos mass spectrometer. (**B**) Mass spectra of ^{2}H-labeled [β-lg A]$^{14+}$ (blue) and [β-lg B]$^{14+}$ (red) acquired with BGE infusion pressure of 1 psi. Overlaid on top are reference spectra of unlabeled [β-lg A]$^{14+}$ and [β-lg B]$^{14+}$ ions (gray). Abbreviations: BGE = background electrolyte solution; CE = capillary electrophoresis; MS = mass spectrometry; HDX = hydrogen/deuterium exchange; Ig = immunoglobulin.

Figure 5: Tandem MS spectra of representative fragment ions produced from β-lg A (blue) and β-lg B (red). (**A**) c10 ions are abundant and deuterated to similar extents; (**B**) z29 ions are less abundant and deuterated to similar extents; (**C**) c137 ions cover the sequence variation sites and are deuterated to significantly different extents in β-lg A and B. The locations of the corresponding segments are illustrated as orange-colored portions of the crystal structure of β-lg B (PDB ID: 5IO5). Abbreviations: MS = mass spectrometry; Ig = immunoglobulin.

DISCUSSION:

The objectives of coating the inner wall of the CE capillary include the minimization of the electroosmotic flow and protein absorption during the CE process[13]. Although electroosmotic flow is beneficial for conventional CE analysis of small molecules owing to its capability of driving neutral or oppositely charged species to the detector, it compromises the separation efficiency of protein species with similar sizes and net charges in solution. Coating the capillary with HPC minimizes the electroosmotic flow caused by the silanol groups on the inner wall of the capillary. In addition, masking these silanol groups reduces their interaction with proteins, avoiding slowed migration or even complete retention of proteins in the capillary.

During the electrophoresis, both the analytes and the cations and anions from the BGE undergo electrophoretic migration. Upon coupling with MS, a reservoir of the BGE at the negative voltage side of the CE capillary is replaced with a CE-MS interface with a different composition. Applying a pressure that continuously infuses fresh BGE into the capillary from the reservoir at the positive voltage side at a specific flow rate minimizes the concentration gradient of the BGE content throughout the capillary, which is beneficial for the separation performance.

HDX reaction time is an essential parameter in determining the exchange rate at a given site/segment and characterization of the dynamics of higher-order structures of proteins. In a CE-based HDX scheme, when the capillary is filled with deuterated BGE, the HDX reaction time is dependent on the inner volume of the capillary and the migration velocity of the analytes. Although the inner volume of the capillary may be adjusted by changing the length or ID of the capillary, the extent of the adjustment using this method is limited by factors such as the minimal length required for connecting the CE and MS instruments and the additional backpressure and risk of clogging caused by the decreased ID.

In contrast, changing the BGE infusion pressure is a practically effective way to adjust the HDX reaction time over a wide range. However, it is still challenging to lower the HDX time to values at the sub-min because a high flow rate compromises the desolvation at the ESI interface. To achieve a lower HDX time, the desired quantity of non-deuterated BGE can be injected into the capillary that

has been filled with deuterated BGE before sample injection. This will help reduce the length of the deuterated BGE section that the analyte proteins should pass through and interact with during their migration. This approach allows the reduction of the effective HDX time to the second scale[8].

Simulation of the velocity field and concentration distribution when the analyte is constantly infused into the microvial reveals that the CE-flow is efficiently diluted by the modifier solution at the flow-through microvial, and that the traveling duration of the analyte in this mixing region is on the second scale in the absence of electroosmotic flow[25]. Although HDX of the structurally protected backbone amide sites is "quenched" upon mixing with the acidified modifier, such a mixing scheme results in the loss of deuterium labels at the fast-exchangeable hydrogen atoms (including those at the side chains) when a non-deuterated modifier is used. Accordingly, $^{2}H_2O$ and deuterated methanol should be used to prepare the modifier in measurements that require the deuterium labels at the fast-exchangeable sites to be retained for MS analysis.

The limitations of the current scheme of this CE-based HDX MS approach are associated with (1) the HDX time regulation and (2) further hydrogen exchange between the protein analytes and the modifier solution at the flow-through interface. The determination of the effective flow rate is based on the estimation using its empirical correlation with parameters such as infusion pressure, capillary parameters, and solution parameters (see step 2.3.4), Because of this and because it is not feasible to accurately measure the inner volume of the capillary (either modified or unmodified, homemade or commercial), the HDX time cannot be deliberately set with high accuracy by adjusting the operation parameters. However, the experimentally resulting HDX time can be accurately measured.

The modifier solution used in this approach includes an organic solvent, which facilitates tandem MS by unfolding the proteins, and acid to minimize further exchange reactions by lowering the pH to 2.5. Because it is not feasible to avoid using protic solvents, exchange between proteins and the modifier solution occurs upon their mixing at the CE-MS interface. When a non-deuterated modifier solution is used, fast-exchangeable sites lose their deuterium labels at this stage, and only well-protected sites remain labeled, limiting the sensitivity in comparing proteins with minor conformational differences. Such effects can be partially calibrated by measuring the fully deuterated protein in a reference sample.

Performing HDX in CE provides a means to separate protein species in solution during HDX to avoid interference from ions of neighboring species in top-down MS characterization of individual species and an approach of initializing the HDX reaction. In this HDX reaction, the proteins to be deuterated completely leave the original non-deuterated environment due to their differing mobilities. This contrasts with the conventional dilution operation, where a fraction of non-deuterated contents (typically ranging from 1% to 10%) is retained. Considering the advantages of recent developments of the top-down MS technique, we expect to improve this approach further so that it can be included in the reliable toolbox for differential characterization of protein higher-order structures.

ACKNOWLEDGMENTS:
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Laboratory, China; Jiangsu Collaborative Innovation Center of Biomedical Functional Materials; and Jiangsu Key Laboratory of Biomedical Materials at Nanjing Normal University, China.

DISCLOSURES:

D. D. Y. Chen is one of the founders of the Knowledge for Health Institute for Biomolecules, which is commercializing the flow-through microvial CE-MS interface. Other authors have nothing to disclose.

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Figure 1

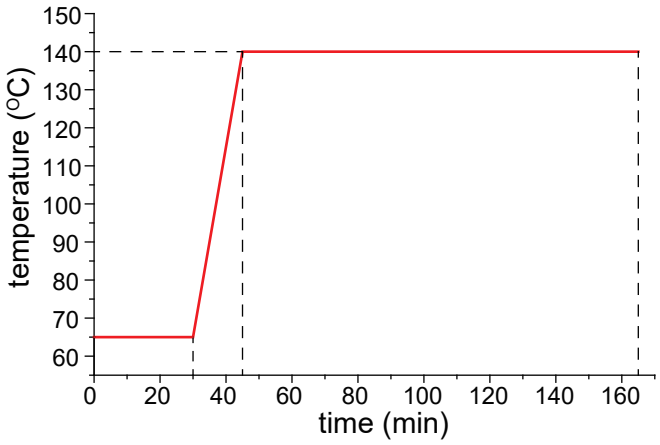


Figure 2

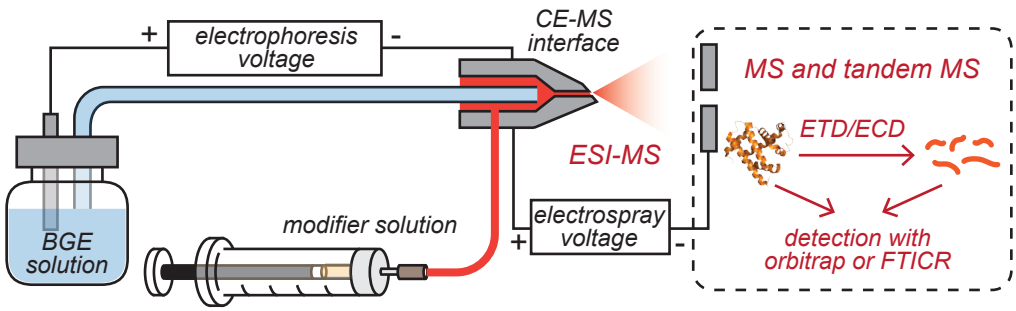
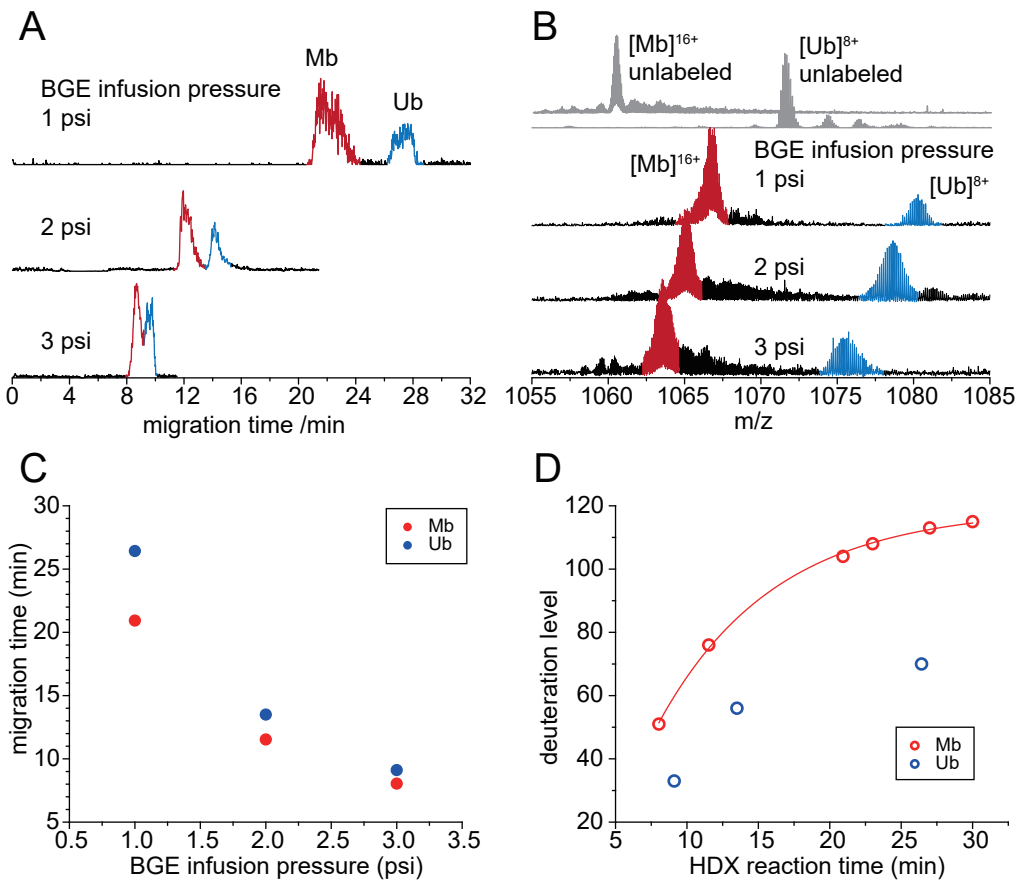

+
electrophoresis voltage
-
CE-MS interface
MS and tandem MS
ETD/ECD
ESI-MS
BGE solution
modifier solution
electrospray voltage
+
-
detection with orbitrap or FTICR

Figure 3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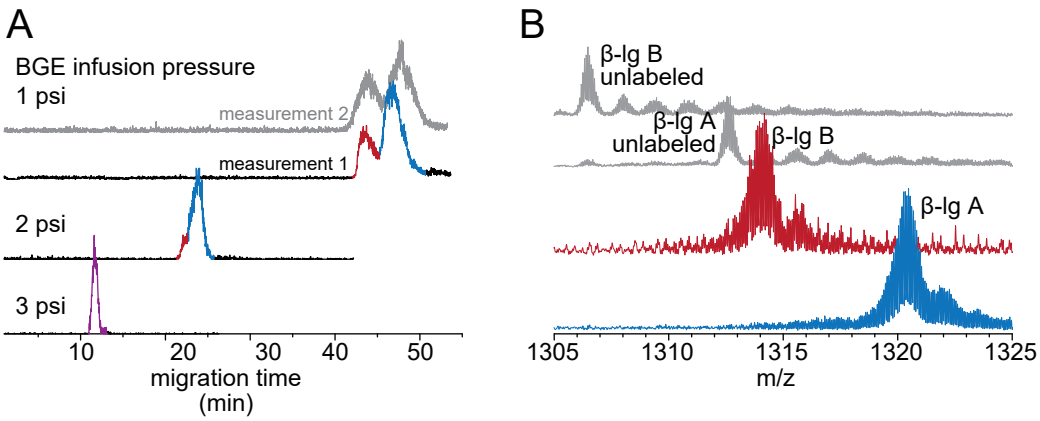
BGE infusion pressure
1 psi
Mb
Ub
2 psi
3 psi
0 4 8 12 16 20 24 28 32
migration time /min
B
[Mb]16+
unlabeled
[Ub]8+
unlabeled
[Mb]16+
BGE infusion pressure
1 psi
[Ub]8+
2 psi
3 psi
1055 1060 1065 1070 1075 1080 1085
m/z
C
migration time (min)
30 25 20 15 10 5
Mb
Ub
0.5 1.0 1.5 2.0 2.5 3.0 3.5
BGE infusion pressure (psi)
D
deuteration level
120 100 80 60 40 20
Mb
Ub
5 10 15 20 25 30
HDX reaction time (min)

Figure 4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BGE infusion pressure
1 psi
measurement 2
measurement 1
2 psi
3 psi
10
20
30
40
50
migration time
(min)
B
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β-lg B
β-lg A
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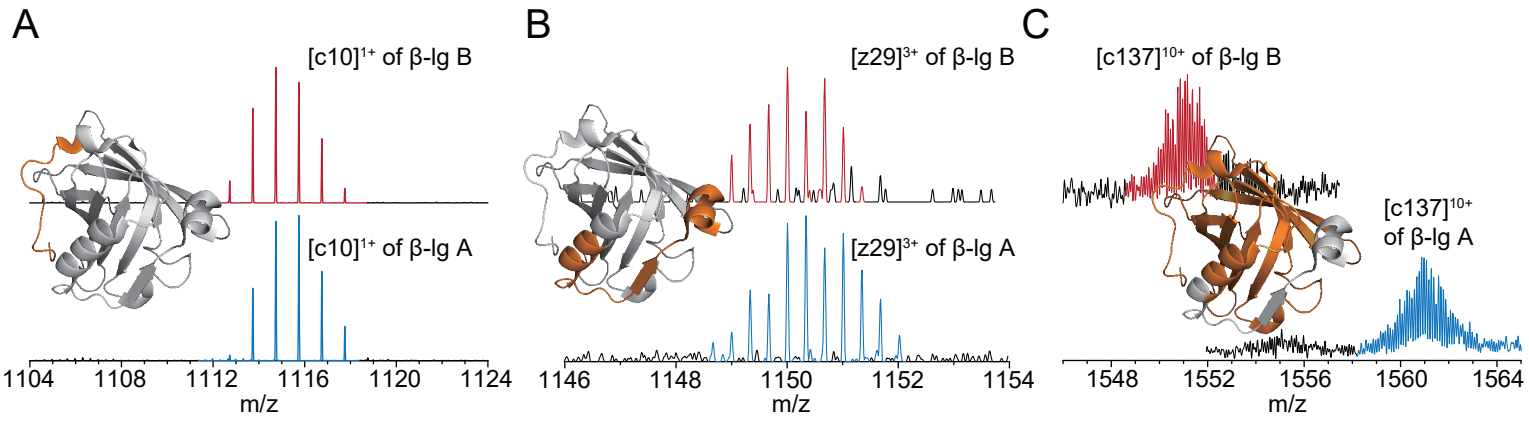
1320
1325
m/z

Figure 5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m/z
C
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[c137]10+
of β-lg A
1548
1552
1556
1560
1564
m/z

Table of Materials

Click here to access/download;Table of Materials;JoVE_Materials_Chaihu et al_CE HDX_20210312.xls

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
ammonium acetate	Fisher Chemical	A/3446/50	≥99%
CESI 8000 plus capillary electrophoresis system	Sciex, USA		
centrifuge	Eppendorf	5406000097	
centrifugal filter	Merck	UFC201024	10 kDa cutoff
deuterium oxide	Energy Chemical	E090001	99.9 % D
formic acid	Acros Organics	270480250	
fused silica glass capillary	Polymicro Technologies	1068150017	ID 50μm, OD 360μm
gas chromatography	Agilent	GC6890N	
hydrochloric acid	Sigma Aldrich	258148	
hydroxypropyl cellulose	Aladdin	H113415	MW 100000
magnetic stirrers	DLAB	8030101212	
methanol	Fisher Chemical	A456-4	MS grade
microvolume UV-Vis spectrophotometer	DeNovix	84677JK7731	
myoglobin	Sigma Aldrich	M1882	
Orbitrap Fusion Lumos mass spectrometer	Thermo Fisher Scientific, USA		
PA 800 Plus Pharmaceutical Analysis CE System	Beckman Coulter, USA		
Q Exactive UHMR mass Spectrometer	Thermo Fisher Scientific, Germany		
sodium hydroxide	Sigma Aldrich	S5881	
ubiquitin	Sigma Aldrich	U6253	
ultrasonicator	SCIENTZ	SB-5200	
β-lactoglobulin	Sigma Aldrich	L0130	

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May. 10th, 2021

Dr. Vineeta Bajaj, Review Editor
Journal of Visualized Experiments

RE: *revision of Manuscript* ***JoVE62672***

Dear Dr. Bajaj
or to whom it may concern,

Thank you very much for giving us the opportunity to revise our manuscript JoVE62672 (titled "*Capillary Electrophoresis-based hydrogen/deuterium exchange for conformational characterization of proteins with top-down mass spectrometry*"). We also thank the reviewers for their very constructive and helpful comments. We have carefully revised the manuscript in accordance with reviewers' comments and suggestions. A tracked changes version of the manuscript was uploaded. Provided below is a detailed point-by-point response to the reviewers' comments and concerns.

We hope that this revised version of our manuscript measures up to the high standards of JoVE. Thank you very much.

Sincerely,

Guanbo Wang

DETAILED POINT BY POINT RESPONSE

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

- We have thoroughly proofread the revised manuscript.

2. Please provide an email address for each author.

- Email addresses of all authors have been listed on the cover page of the manuscript file.

3. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points

- The manuscript file has been hereby reformatted.

4. Please include a Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Presented here is a protocol …"

- The summary has been included in the manuscript file.

5. Please ensure that the long Abstract is within 150-300-word limit and clearly states the goal of the protocol.

- The current length is within the limit.

6. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."

- We prepared the protocol section following the instruction.

7. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, alphabets, or dashes.

- We followed the instruction when numbering the steps.

8. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? This can be done by including mechanical actions, button clicks in the software, knob turns in the instrument, command lines, etc.

- We added more details in several protocol steps.

9. 1.1.3: How do you infuse the organic solvent. Flow rate if any?

- 1.1.3 has been updated with these details (infused with the autosampler of the CE instrument, at the specified infusion pressure).

10. 1.2: Please include volume/concentrations/compositions of the solutions used in your experiment.

- 1.2 has been updated with these details (infused with the autosampler of the CE instrument, at the specified infusion pressure).

11. 1.3: Please include details on the protein samples.

- Details of protein samples have been included in the NOTE following 1.3.

12. 2.1.1: Please include how this is done.

- More details have been included in 2.1.1.

13. 2.3: Please include button clicks wherever applicable.

- Sufficient details have been included in 2.3. Since the instrumental design varies among different models of CE instruments, we provided detailed descriptions of operations rather than specific button clicks.

14. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step.

- We simplified the protocol, and shortened the occasionally long paragraphs.

15. There is a 10-page limit for the Protocol, but there is a 3-page limit for filmable content. Please highlight 3 pages or less of the Protocol (including headings and spacing) that identify 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.

- We have highlighted the essential steps of the protocol that should be filmed (a bit shorter than 3 pages).

16. Calculation steps cannot be filmed.

- We are aware of that.

17. Please ensure the results are described in the context of the presented technique. e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. Data from both successful and sub-optimal experiments can be included.

- We double-checked the results included the manuscript and believe they can convey the expected information.

18. Please include a title and a description of each figure and/or table. All figures and/or tables showing data must include measurement definitions, scale bars, and error bars (if applicable). Please include all the Figure Legends together at the end of the Representative Results in the manuscript text.

- We moved the Figure Legends (which were previously prepared following the instruction) to the end of the Representative Results.

19. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol

b) Any modifications and troubleshooting of the technique

c) Any limitations of the technique

d) The significance with respect to existing methods

e) Any future applications of the technique

- We reduced the number of paragraphs in Discussion to 5. The listed aspects care covered in the Discussion.

20. All figures should be uploaded separately to your Editorial Manager account with panels combined into a single image file.

- The figure files to be uploaded were prepared following the instruction.

21. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

- Figure 2 of this manuscript is adapted from a previously published version. We have obtained the permission and have uploaded it.

Reviewer 1

The manuscript is overall well-written and describes a protocol that is relevant to many future users as methods in MS are moving more and more towards top-down approaches. A few minor points are listed below that should be addressed prior to publication acceptance.

- We are very grateful for the reviewer's comments. They are very helpful for improving this manuscript and the follow-up studies.

Minor Concerns:

l.32 modification

l.46-54 - slight English editing required

l.75 - that may interfere with MS analysis.

l.82 an ultrasonicator

l.86 for ~10-15 min.

l.89 specify which portion

l.167 ~3-5 kV

l.303 required

l.310 of these variants

l.323 undergo

- We corrected these typos and rewrote the paragraph in Line 46-54.

Reviewer 2

Comments:

The manuscript by Guanbo Wang and coworkers described an innovative CE-based HDX-MS approach. In this method, labeling of proteins was carried out during migration through deuterated BGE solution, at the same time, different proteins or different conformers of one protein were being separated, when proteins reached the interface, quenching and ionization were performed in an on-line fashion and MS analysis was executed immediately with intact analysis giving global D uptake and to-down experiment giving local level information. This JoVE manuscript has explained in detail how the apparatus was constructed, how the experiment was performed, and how the results were analyzed.

Major Concerns:

The reviewer agrees that this method provides a means to probe the higher order structure of proteins by on-line CE-based HDX and mass spectrometry. Perhaps it would be helpful to dedicate more discussion toward the quenching and back exchange at the end of the CE separation. The reviewer is curious about the mixing efficiency and subsequently, the time protein samples spent with modifier solution before MS analysis. Such discussion could be critical in understanding how much of side-chain deuterium and fast-exchanging backbone deuterium were retained and detected by MS analysis.

- We are very grateful for the reviewer's comments. They are very helpful for improving this manuscript and the follow-up studies.
- The characteristics of modifier mixing at the outlet of the CE-MS interface has been investigated in a previous study[1]. We added a paragraph to discuss on this topic in the Discussion section.

Also, the reviewer is also wondering how the migration time difference should be taken into consideration when comparing D uptake or protection. For example, it took Ub more migration time than Mb, Ub had

more D uptake which could be explained by a more unprotected conformation. However, the higher extent of deuterium exchange could also be resulted from longer exchange time with BGE. How should the data be analyzed to correct for the longer exchange time?

- We updated Figure 3 by making new Panel C and Panel D, which we believe can present the data in a more reader-friendly way.
- As illustrated in Figure 3C, the difference in migration time between two species can be adjusted by changing the BGE infusion pressure. As illustrated in Figure 3D, significant differences in deuteration level (vertical scale) can be observed at slight migration time differences (horizontal scale). At the HDX time scale of minutes, the deuteration difference should primarily reflect the differing exchange extents at the structurally protected sites, instead of the fast-exchangeable sites. According to the trend of the deuteration-time functions shown by either protein species, the migration time difference is unlikely to be the major contributor to the deuteration difference. Correction for the deuteration difference introduced by the migration time difference can be made via a curve fitted for the data of deuteration level vs. HDX time (Figure 3D).
- In differential CE-HDX of holo- and apo-myoglobin reported in our previous article (Anal Chem 2019)[2], the earlier eluted apo-Mb shows a higher deuteration level than holo-Mb, clearly suggesting that the conformational difference is the major factor determining the measured deuteration difference.
- We added the discussion on this topic in the Representative Results section.

Finally, Z29 ion demonstrated bi-modal distribution in its isotopic pattern, could it be an experimental artifact or real conformers that were still mixed together despite CE separation? It would help to discuss briefly.

- Since the cleavage site responsible for z-29 ion is within the enclosing region of the disulfide bond Cys82-Cys176, the appearance of the isotopic pattern of the z-29 ions is compromised by the low signal intensity of z-29 ions. Here we intend to show that (1) disulfide formation can compromise the data quality in direct top-down analysis; (2) even without prior reduction, fragments that were formerly enclosed by disulfide bonds can still be produced in direct top-down MS analysis, as we previously reported[3]. Several disulfide reduction approaches[4-7] can be used to solve this problem; but they may be beyond the scope of this protocol.
- Following the reviewer's suggestion, we added the discussion on this topic in the Representative Results section.

Minor Concerns:

The reviewer would recommend authors to conduct a proof-reading and harmonize the terms used throughout the manuscript. For example, both "HDX MS" and "HDX-MS" were used in various context. For another example, there were multiple terms used for to describe the same parameter: "back pressure", "backpressure" and "pressure".

- We removed all hyphens between HDX and MS.

- We realized “back pressure” or “backpressure” are improper terms in the Protocol section; we changed them to “infusion pressure”. However, the “backpressure” mentioned in the third paragraph of the Discussion section is retained because it refers to the resistance opposing the desired flow of fluid through the capillary, rather than the infusion pressure of BGE.

Reviewer 3

Comments:

The manuscript presents protocol for a CE based HDX approach coupled with top-down mass spectrometry analysis. The topic is novel. The protocol is straightforward to follow. Therefore, my recommendation is to accept the manuscript. But I would suggest some further edits for potential improvement:

1. The steps for performing a pilot MS measurement using a nanoESI source are not crystal clear. For example, it's not clear what MS parameters needs tune or optimization. Is it possible to share the detailed MS method parameters including desolvation, mass selection, fragmentation, etc. in the manuscript as reference?

- We are very grateful for the reviewer’s comments. They are very helpful for improving this manuscript and the follow-up studies.
- We included more details in Step 2.1.1. The optimal values of MS parameters are protein-specific; we cited several previously published reports as the reference for exemplary settings[2-3].

2. For the data analysis step 3.2.3, the description is a little bit vague. Does manual data interpretation is needed or the data analysis/fragment ion identification fully automated by softwares such as Biopharma Finder, Prosight, or MASH Suite? Thanks!

- We used the commercial top-down MS software to identify the fragment ions; however, at present, none of them can be used to automatically deduce reliable HDX data. We used a semi-automated approach (with a lab-made program in combination with Origin Pro) to analyze the masses of deuterated fragment ions, and are making effort to develop an automated program. We plan to report our program in a separate research article following further development of it.

Minor Concerns:

It's not a concern. But I am wondering if other top down fragmentation methodologies such as UVPD, EtHCD are considered. Are those top-down methods out of the scope for discussion? ETD and ECD are only two out of many top-down methods.

- It has been reported that hydrogen scrambling (rearrangement of deuterium labels) is associated with collision-activation involving events; and ETD and ECD are the only two fragmentation methods that have been demonstrated to minimize hydrogen scrambling. It remains to be further investigated whether EThcD or UVPD can be used to generate reliable top-down HDX data. We decided to leave the discussion on this topic outside the scope of this manuscript

because a lengthy paragraph may be needed to explain the hydrogen scrambling phenomenon and the effect of other fragmentation techniques on it. And we will make effort to assess the applicability of other fragmentation techniques in our follow-up studies.

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