Evaluation of different Hydrogen/Deuterium Exchange data processing software by looking at conformational changes induced by mAb aggregation

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ABSTRACT

Hydrogen/deuterium exchange mass spectrometry (HDX) has emerged as a powerful tool to investigate the conformation of intact proteins, including mAbs. One of the biggest challenges in HDX experiment is data processing. In this study, three HDX processing software, HDExaminer (Sierra Analytics), HDX WorkBench (Scripps) and Thermo Scientific™ BioPharma Finder 2.0 were compared and used to characterize the conformation of Trastuzumab and its aggregation.

INTRODUCTION

Monoclonal antibodies (mAbs) have been increasingly used for detection and treatment of cancer and other diseases. Characterization of critical quality attributes (CQA) of mAb-based drugs is a primary concern for biopharmaceutical development. Structural characterization is used to assess the CQAs of biopharmaceutical products. Protein aggregation is a CQA for monoclonal antibody (mAb) product. The formation of aggregates may impact safety and efficacy of mAbs. Antibody aggregation is a complex, multistep process. Thus, it is important to understand the mechanism of aggregation and the conformational changes of the aggregates. Hydrogen/deuterium exchange mass spectrometry (HDX) has emerged as a powerful tool to investigate the conformation of intact proteins, including mAbs. One of the biggest challenges in HDX is data processing. Accurately measuring the deuterium uptake would yield more precise information of the protein conformation and conformation dynamics. In this study, three HDX processing software, HDExaminer (Sierra Analytics), HDX WorkBench (Scripps) and Thermo Scientific™ BioPharma Finder 2.0 were compared and used to characterize the conformation of Trastuzumab and its aggregation.

MATERIALS AND METHODS

A fully automated HDX platform, based on the HD-X PAL™ system (LEAP Technologies) and the Thermo Scientific™ UltiMate 3000 pump system coupled online with an Thermo Scientific™ Orbitrap Fusion™ Tribrid™ mass spectrometer, was used. Figure 1 shows the HDX work station. It is composed of an autosampler equipped with individually temperature-controlled sample plate and labeling plate. The syringe head and peptin column can also be independently temperature-controlled. Chronos™ control software is fully integrated with the Thermo Scientific™ Xcalibur™ platform and the user. The execution of the whole experiment is completely automated and controlled by Chronos™ software.

Therapeutic antibody Trastuzumab (Herceptin) aggregation was induced by adjusting the pH to 0.5, 1.5 and 2.5 with HCl and by incubating each sample at room temperature for 30 min. The pH of the samples was subsequently adjusted to pH 9. Both non-aggregated and aggregated mAbs were diluted with labeling buffer and incubated for multiple time points. The samples were then quenched and digested online with a peptin column in a fully automated manner using the HD-X PAL system. The digested peptides were injected into a Thermo Scientific™ Acclaim™ Pepmap™ C18 reverse phase column with a 7 min gradient. MS analysis was performed with the Orbitrap Fusion mass spectrometer.

RESULTS

Herceptin and Herceptin Aggregation

Herceptin (Her) and its pH (Her pH 5.5) stressed samples were quantified by measuring UV 280 absorption. Agglomerations around 25% for pH 0.5, 6% for pH 1.5 and 0% for pH 2.5 were observed. The HDX data from the control Her and Her stressed at pH 0.5 samples were used for this study.

MS/MS experiments were first performed using non-deuterated samples for peptide identification. Nearly 100% sequence coverage was achieved for both the Herceptin and Herceptin pH stressed samples. Figure 2 is the peptide map of Herceptin generated by BioPharma Finder software. After online peptin digestion, around 200 and 100 peptides were respectively identified for heavy and light chains. These identified peptides were subsequently used to probe the conformation of the analyzed samples by HDX.

Overall, the control and pH stressed samples showed similar deuterium uptake. However, most of the pH stressed sample peptides showed slightly more deuterium uptake, between 2% and 5%, than for the control sample. The differences could reach up to 15% for different regions of the light chain and could be even higher for the heavy chain. The sample stressed at pH < 0.5 showed more deuterium uptake than the pH = 1.5 sample. Finally, there was no significant deuterium uptake change between Herceptin and the stressed sample pH=1.5 as reported previously.

FIGURE 1. HDX work station coupled online to the Orbitrap Fusion mass spectrometer

Data Analysis

Peptide identification, mapping, PTM analysis and HDX data analysis were performed with Thermo Scientific™ BioPharma Finder™ 2.0 software. HDX experimental data were also analysed with HDExaminer™ software (Sierra Analytics) and HDX WorkBench (Scripps, Florida).

Figure 2. Peptide map of Herceptin by BioPharma Finder a) Light chain b) Heavy Chain
Ultimate 3000 pump system coupled online with an Thermo ScientificTM Orbitrap FusionTM TribridTM mass spectrometer, was used. Figure 1 shows the HDX work station. It is composed of an autosampler equipped with different Hydrogen/Deuterium Exchange data processing software by looking at conformational changes induced by individually temperature-controlled sample plate and labeling plate. The syringe head and pepsin column can also be generated by BioPharma Finder software. After online pepsin digestion, the digested peptides were injected into a Thermo Scientific™ Acclaim™ T3 C18 2.1x50 chromatography column and eluted with linear acetonitrile gradient. Peptide identification, mapping, PTM, and protein quantification were performed with Thermo Scientific™ PeptideWorks WorkBench software.  Protein Monoclonal antibodies (mAbs) have been increasingly used for detection and treatment of cancer and other diseases. In this study, three HDX processing software, HDExaminer (Sierra Analytics), HDX WorkBench (Scripps) and Thermo Scientific™ BioPharma Finder 2.0 were used.

One of the biggest challenges in HDX is data processing. Accurately measuring the deuterium uptake would yield more meaningful conclusions. To overcome this, we focused on the development of three HDX data processing software and used the results to compare their efficiency. The BioPharma Finder is a comprehensive software package that can perform both peptide identification and HDX data analysis. The HDX tool in mass analyzer has been implemented into BioPharma Finder 2.0. The HDX MS data can be processed in an automated fashion by BioPharma Finder 2.0. The unlabeled protein samples must be analyzed with data-dependent MS/MS for peptide identification. Deuterium labeling is performed at different time intervals to obtain time courses for each condition. The software uses a 5-parameter equation to fit the curves to calculate the variance in the data. Ideally, a 0% and 100% deuteration control should be performed for appropriate back exchange modeling. Figure 3 shows the HDX data process workflow of BioPharma Finder. First, import the protein sequence in fasta format, including all chains of the protein into the protein sequence manager, then select peptide mapping analysis tab. Browse in all the raw files and define the experiment name, the file conditions and search parameters. There are two major steps involved once the data analysis process started, peptide detection and HDX modeling. The peptide detection including feature extraction, retention time alignment, gap-filling and peptide identification. The HDX modeling including deuteration level calculation, back exchange modeling and full HDX modeling. HDXData, TimeCourses, HDSiluation, ProtectionFactorPlot, Differential Protection Plot .csv result files were created for analyzed protein including all the chains, ie, light and heavy chains for mAb. To evaluate the overall protein conformation properties at different conditions, visualized deuteration uptake and protection factor plots can be generated from the csv files.

The HDX data from Her and stressed Her samples were processed by Biopharma Finder. The heavy chain protection factor plots of Her and stressed Her samples were generated from the BioPharma Finder searched csv file as shown in figure 4. In most of the regions Herceptin showed slightly higher protection factor than the stressed sample. The differences were not uniform along the sequence. From amino acid 110 to 210 and 240 to 255, Herceptin had a much higher protection factor than Herceptin pH stressed sample which would reflect in figure 5 the deuteration uptake profile. One peptide with less deuteration uptake difference, peptide 49-60, and two peptides with more significant deuteration uptake changes, 149-159 and, 244-255, were selected to evaluate the deuteration uptake profiles as shown in figure 5. The deuteration uptake differences range from 0.1 to around 2 Daltons. These results indicated that aggregation would induce more conformational changes in the Fab region and less changes for the Fc region.

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Figure 3. Biopharma Finder HDX data analysis workflow

1. import your protein sequence, select peptide mapping tab
2. Import experiment raw files and define raw file conditions
3. Select protein sequence, define both peptide identification and HDX modeling conditions
4. Save the method and start the search
5. TimeCourses.csv, ProtectionFactorPlot.csv, DifferentialProtectionPlot.csv files generated
6. Use the csv files generate visualize graphical protection factor plots

The HDX tool in mass analyzer has been implemented into BioPharma Finder 2.0. The HDX MS data can be processed in an automated fashion by BioPharma Finder 2.0. The unlabeled protein samples must be analyzed with data-dependent MS/MS for peptide identification. Deuterium labeling is performed at different time intervals to obtain time courses for each condition. The software uses a 5-parameter equation to fit the curves to calculate the variance in the data. Ideally, a 0% and 100% deuteration control should be performed for appropriate back exchange modeling. Figure 3 shows the HDX data process workflow of BioPharma Finder. First, import the protein sequence in fasta format, including all chains of the protein into the protein sequence manager, then select peptide mapping analysis tab. Browse in all the raw files and define the experiment name, the file conditions and search parameters. There are two major steps involved once the data analysis process started, peptide detection and HDX modeling. The peptide detection including feature extraction, retention time alignment, gap-filling and peptide identification. The HDX modeling including deuteration level calculation, back exchange modeling and full HDX modeling. HDXData, TimeCourses, HDSiluation, ProtectionFactorPlot, Differential Protection Plot .csv result files were created for analyzed protein including all the chains, ie, light and heavy chains for mAb. To evaluate the overall protein conformation properties at different conditions, visualized deuteration uptake and protection factor plots can be generated from the csv files.

Figure 4. Herceptin and pH 0.5 stressed sample heavy chain protection factor comparison Top: residual 1-225 Bottom: residual 226-449

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Figure 5. Example deuteration uptake plots generated by Biopharma Finder
**MATERIALS AND METHODS**

Chronos™ software is fully integrated with the Thermo Scientific™ H/D Exchanger Workbench. The deuterium uptake heat map of the protein, the peptide deuterium uptake plots, different protein states deuterium uptake mirror and residual plots would be created to probe the protein conformation and conformational changes.

The peptide identification list csv file of Her and Her pH 0.5 stressed samples from the BioPharma Finder was exported and used directly to process the HDX data by HDExaminer. The heavy chain peptides deuterium uptake residual plots in both %D (top left) and absolute #D (top right) were shown in figure 7. In general, Herceptin had less deuterium uptake compared to the stressed sample and the differences were not uniform. Peptides with more significant change, were identified between amino acids 70 and 100 and this observation was consistent with the BioPharma Finder protection factor prediction shown in figure 4. Figure 7a), b), c) were the deuterium uptake plots from the same three peptides shown in figure 5. The deuterium uptake of the three selected peptides was very similar to the plots shown in figure 6.

**REFERENCES**

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A fully automated HDX platform, based on the H/D-X PAL™ system (LEAP Technologies) has emerged as a powerful tool to investigate the conformation of intact proteins, including mAbs.

Evaluation of different Hydrogen/Deuterium Exchange data processing software by looking at conformational changes induced by pH 0.5 stress. The same peptide deuterium uptake plots were shown in figure 10. Figure 11 was the single residue consolidated perturbation sequence coverage of the heavy chain.

Figure 8. HDX WorkBench data analysis workflow

1. Peptides identification by PD or BF, use the identified peptides create CSV peptides list with defined format.
2. Protein Definition
3. Define experiment conditions. Import time series of MS data, initial detection of peptides within undeuterated control data files.
4. Search, review peptides
5. Data Review & Visualization
6. Experiment Comparison
5. Analyze overall protein and peptide deuterium uptake behavior via perturbation view and various visualization and interrogation tools.
6. Comparison of result data from multiple experiments.

HDX WorkBench from Scripps is an integrated software platform for analysis of HDX mass spectrometry data. Figure 8 was the HDX WorkBench HDX data analysis workflow. The first step was to build a specific format csv peptide list based on the peptide identification results (the list from BioPharma Finder) as indicated in step 1. The next step was to define the protein and to import the csv peptides list. The protein could be edited to add secondary structure features. The experimental conditions and labeling time series of the identified peptide in the undeuterated sample would be defined in the experimental set up. The different chains of the protein, heavy and light chain of mAb would be treated as two proteins under the same experiment. Only the identified peptides from the undeuterated sample would be used to start the HDX data analysis. The software would create a very comprehensive results dashboard including: peptide list, chromatogram, peptide deuterium update plot, peptide spectra, and time points statistics as shown in figure 9.

A csv file with the specific format was generated by using the BioPharma Finder search results and used in HDX WorkBench to process the data from the Her and Her pH 0.5 stressed samples. Similar results were obtained with HDX Workbench and the two previous software packages. The same three peptide deuterium uptake plots were shown in figure 10. Figure 11 was the single residue consolidated perturbation sequence coverage of the heavy chain.

CONCLUSIONS

• The HDX data from Her and Her pH stressed samples were successfully processed by all three software packages and the results were consistent.
• The BioPharma Finder is a comprehensive software package that can perform both peptide identification and HDX data analysis. HDX model simulates the HD exchange and back exchange process at the single residue level. The protection factor plot can be used to evaluate the whole protein conformational property.
• HDXExaminer can directly import the .mz or .csv files from a peptides identification results. The HDX data process is straightforward and results are easy to implement. The peptides deuterium uptake residual plots can be used to probe the protein deuterium uptake behavior.
• HDX WorkBench generates a very comprehensive results dashboard. It can yield a consolidated amino acid level perturbation sequence coverage map.

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PN64890-EN 11/16S