

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 process¹. 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 process 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 H/D-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 pepsin 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.

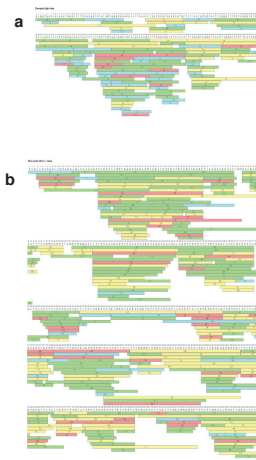
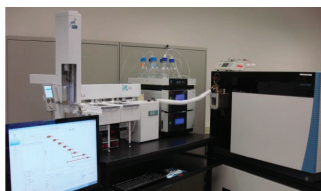
Therapeutic antibody Trastuzumab (Herceptin) aggregation was induced by adjusting the pH to 0.5, 1.5 and 2.5 with HCl and by incubating each sample at room temperature for 30 min. The pH of the samples was subsequently adjusted to pH 9. Both non aggregated and aggregated mAbs were diluted with labeling buffer and incubated for multiple time points. The samples were then quenched and digested online with a pepsin column in a fully automated manner using the H/D-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.

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 analyzed 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

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



RESULTS

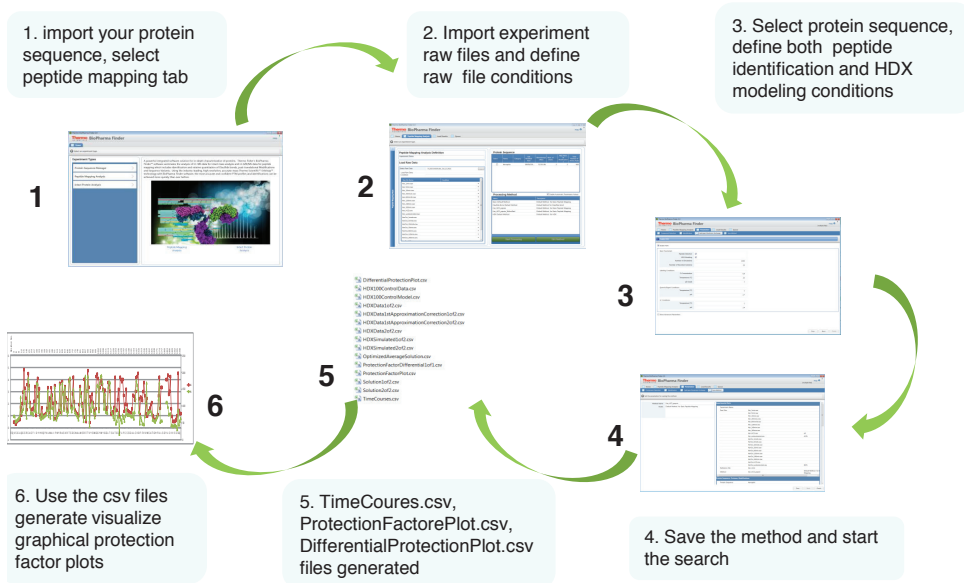
Herceptin and Herceptin Aggregation

Herceptin (Her) and its pH (Her pH0.5) stressed samples were quantified by measuring UV 280 absorption. Aggregations 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 pepsin 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.

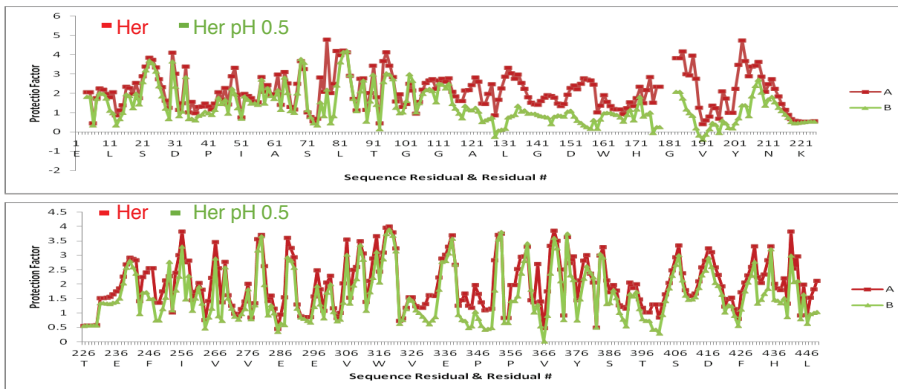
Overall, the control and pH stressed samples showed similar deuterium uptake. However, most of the pH stressed sample peptides showed slightly more deuterium uptake, between 2 and 5%, than for the control sample. The differences could reach up to 15% for different regions of the light chain and could 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 previously².

Figure 3. Biopharma Finder HDX data analysis workflow



The HDX tool in mass analyzer⁹ 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 data⁹. 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 deuterium level calculation, back exchange modeling and full HDX modeling. HDXData, TimeCourses, HDXSilulation, 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 deuterium 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



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 deuterium uptake profile. One peptide with less deuterium uptake difference, peptide 49-60, and two peptides with more significant deuterium uptake changes, 149-159 and 244-255, were selected to evaluate the deuterium uptake profiles as shown in figure 5. The deuterium 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.

Figure 5. Example deuterium uptake plots generated by Biopharma Finder

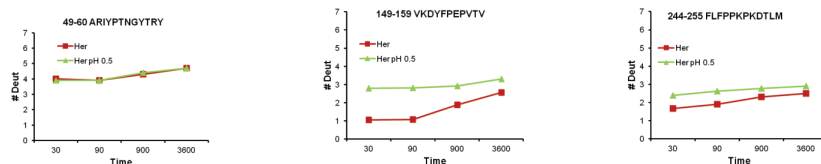


Figure 6. HDExaminer HDX data analysis workflow

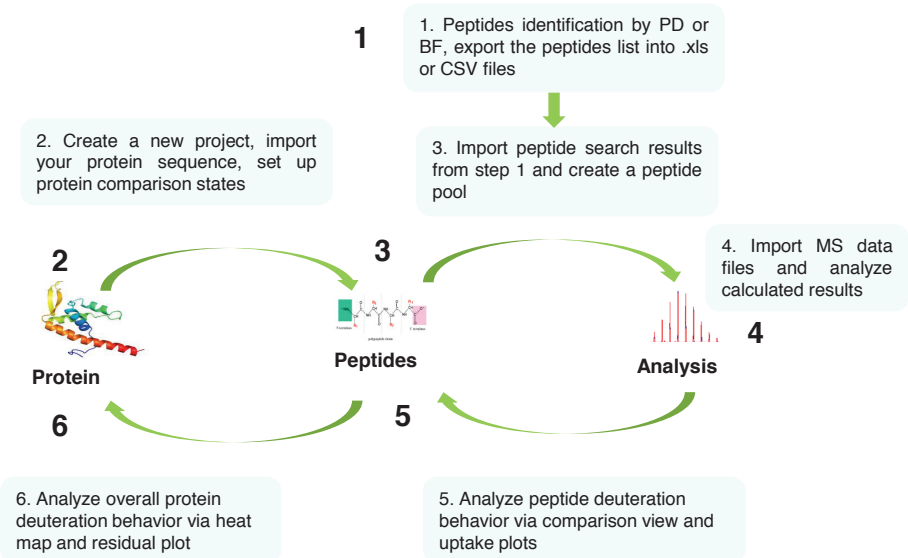
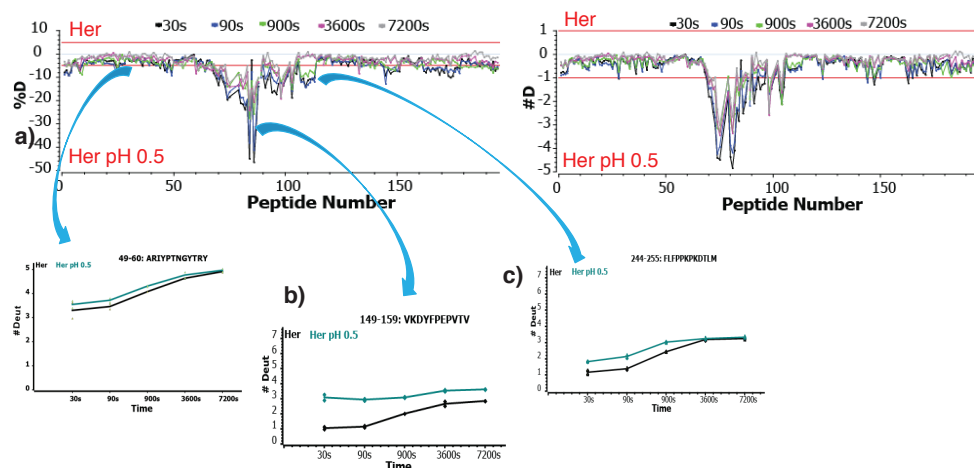


Figure 6 is the HDX data process workflow of HDExaminer. To process the HDX data with HDExaminer, a .xls or csv file had to be generated by exporting the peptide identification list from the peptide identification search results. Similar to BioPharma Finder, the protein sequence had to be defined first in the protein tab (the software would only process one protein at a time, the different chains of the same protein would be treated as different protein and would be processed separately), the .xls or csv peptide list would be directly imported into a peptide pool for peptide deuterium uptake measurement in the peptides tab. The undeuterated and deuterated raw files at different time points would be loaded in the analysis tab to start the data analysis. 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 stressed samples from the BioPharma Finder was exported and directly used 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, the Herceptin had less deuterium uptake compare to 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 acids in figure 4. Figure 7 a), 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 5.

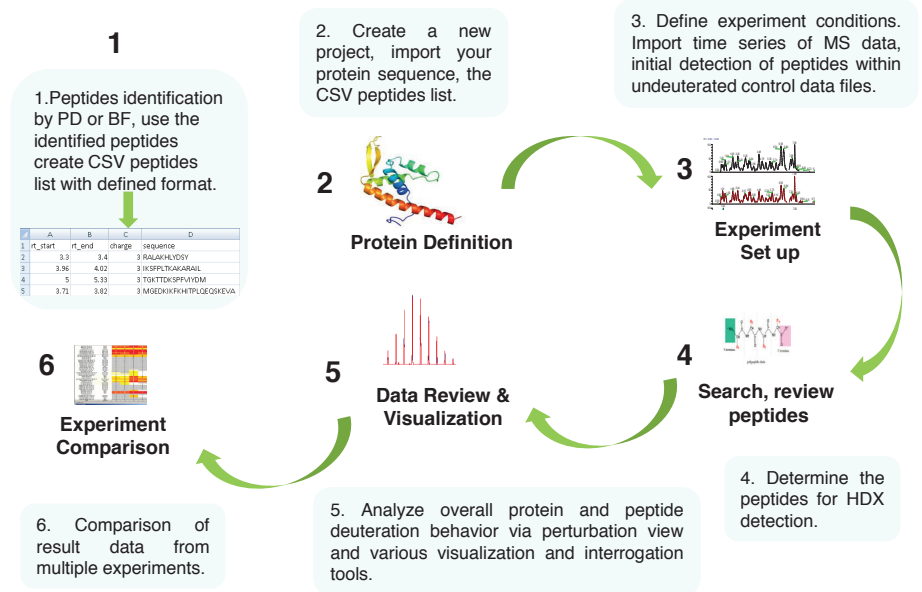
Figure 7. Herceptin and pH 0.5 stressed sample heavy chain deuterium uptake residual plots, Top left: %D Top right: #D Bottom: peptide deuterium uptake plots a), b), c)



REFERENCES

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2. T. Zhang et al, ThP180, ASMS 2016
3. Z. Zhang et al, Anal. Chem. 2012, Vol. 84, 4942-493

Figure 8. HDX WorkBench data analysis workflow



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 uptake 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 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 residual consolidated perturbation sequence coverage of the heavy chain.

Figure 9. HDX WorkBench Results Dashboard

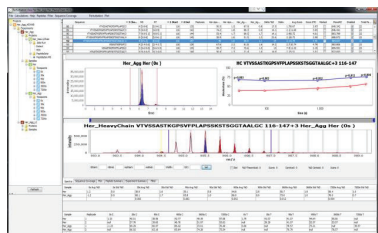


Figure 10. Her and Her pH 0.5 stressed samples heavy chain peptides deuterium uptake plots

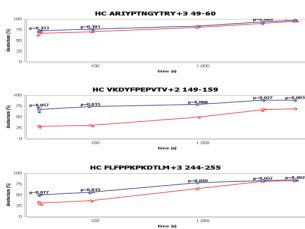
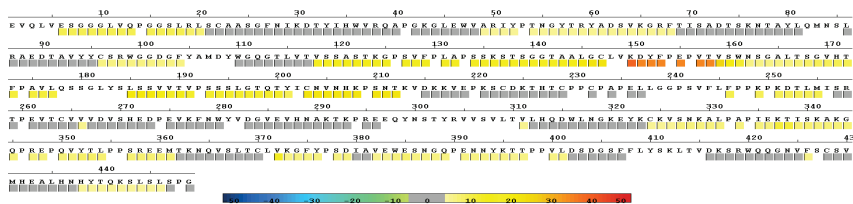


Figure 11. Herceptin and pH 0.5 stressed sample heavy chain consolidated perturbation sequence coverage



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 H/D exchange and back exchange process at the single residue level. The protection factor plot can be used to evaluate the whole protein conformational property.
- HDXaminer can directly import the .xls or csv files from a peptides identification results. The HDX data process is straight forward 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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