Optimization of a Fully Automated Hydrogen/Deuterium Exchange Mass Spectrometry Platform for Probe Protein Conformation/Conformation Dynamics

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Poster Note 64466

Overview

Purpose: Development and optimization of a fully automatic hydrogen/deuterium exchange mass spectrometry (HDX) workflow.

Methods: An HDX platform based on the H/D-X PAL™ (LEAP Technology) and Thermo Scientific™ Dionex™ UltiMate™ 3000 nano pump system coupled online with an Orbitrap mass spectrometer operated with software in a fully automated fashion was employed.

Results: Optimized experimental conditions were obtained and two lots of a monoclonal antibody (mAb) were analyzed using the optimized experimental conditions. Both lots showed very similar deuterium uptake profiles confirming there are no significant conformational differences for the two analyzed mAb lots.

Introduction

There are many analytical tools, such as X-ray and NMR, to study protein conformation, conformation dynamics and protein-protein interactions. Each method faces different challenges and limitations depending on the protein studied. Recently, hydrogen/deuterium exchange mass spectrometry (HDX) has emerged as a powerful tool to fill the analytical gap[1,2]. In this study, a fully automated HDX workflow was developed and optimized.

Experimental Set Up and Methods

An HDX platform based on the H/D-X PAL and the Ultimate 3000 pump system coupled online with an Orbitrap mass spectrometer was developed which is software operated in a fully automated fashion. Figure 1 shows the HDX work station. Figure 2 details the components of the H/D-X PAL system. 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. Figure 3 a) describes the three valve configurations at various stages of the experiment inside the cooling chamber. The flexibility allows for maximized productivity. Figure 3 b) shows the dual heads on single high pressure mixing pump on the left and ternary loading pump on the right. Flow rates and solvents can be changed on the fly during the experiment. The execution of the whole experiment was completely automated and controlled by Chronos software. The Chronos software is fully integrated with Xcalibur, the user interface is shown in Figure 4.

Cytochrome c was used to evaluate and optimize the HDX system performance. Samples were diluted (1 to 9 ratio) with labeling buffer and incubated for multiple time points. The samples were then quenched and subjected to online pepsin digest (Life Technogies, pepsin column, 2.1 x 3mm). The digested peptides were injected into a Thermo Scientific™ Dionex™ PepMap™ trapping column (0.5mm x 15mm) and eluted to a Thermo Scientific™ Hypersil™ Gold C18 reversed phase column (0.5mm x 100mm). The separated peptides were analyzed with a Thermo Scientific™ Orbitrap™ Fusion™ Tribrid™ mass spectrometer. In a first step, data dependent MS/MS HCD spectra were collected from the undeuterated protein digest to identify all detectable peptides. For HDX analysis MS full scan spectra at a resolution setting of 60K were collected.

Experimental conditions such as deuterium labeling, quenching, and digestion via pepsin column were systematically investigated. Different trapping and analytical reversed phase columns were used to analyze the digested peptides. Several gradient conditions were performed to assess optimal separation, while keeping the deuterium back exchange to a minimum. Different MS parameters were applied to evaluate the effect of deuterium uptake measurement. In a more advanced study, two different lots of mAbs were analyzed and compared.

Data Analysis

Data analysis was performed with Thermo Scientific™ Proteome Discoverer™ 1.4 software for peptide identification. HDX experimental data were analyzed with HDExaminer (Sierra Analytics).

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

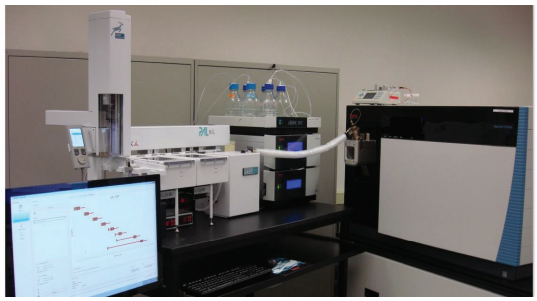


FIGURE 2. Components of H/D-X PAL

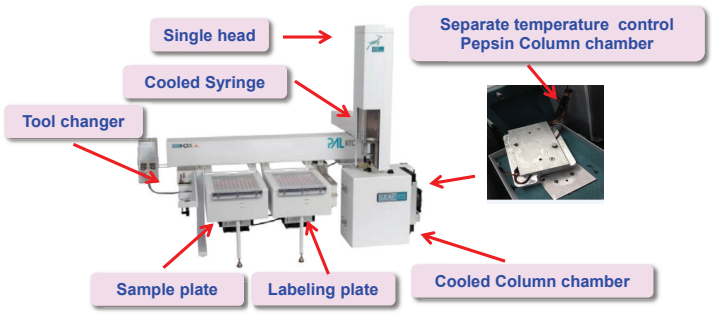
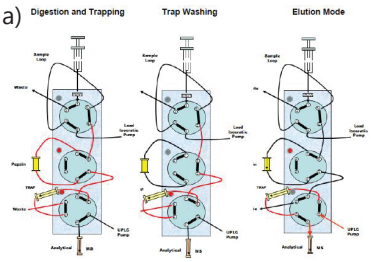


FIGURE 3. a). Flexible three valve configuration for carrying out different experimental tasks simultaneously in the cooling chamber, b). NCS-3500RS with binary rapid separation micro flow pump with ternary loading pump

a)



b)

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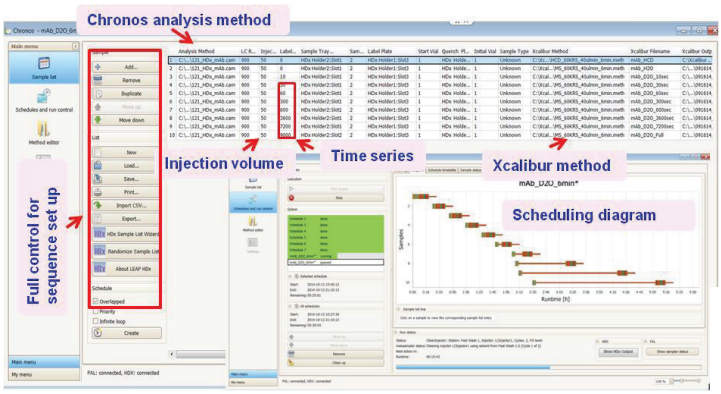
FIGURE 4. Chronos software interface, integrated with Thermo Scientific™ Xcalibur™ software



Results

HDX workflow experimental optimization

The described workflow was applied to the analysis of Cytochrome c. It is highly desirable to have as many as possible sequentially overlapping peptides identified for unlabeled protein, in order to achieve high structural resolution in HDX experiments[3]. The extraordinary sensitivity of the Orbitrap mass spectrometer is ideal for this purpose. Figure 5 shows many sequentially overlapping peptides identified in Cytochrome c. To demonstrate the system performance, HDX experiments of Cytochrome c were performed in triplicate. The results were highly reproducible as shown in Figure 6. Both labeling temperature and labeling buffer play very important roles in determining the deuterium exchange rate. The higher the labeling temperature the faster the deuterium exchange (data not shown). It is important to keep the labeling buffer consistent with the protein formulation buffer to maintain the protein native states. Labeling with D_2O or formulation buffer was investigated with mAb. The results showed that the protein labeled with D_2O has lower deuterium uptake rate when compared to labeling with formulation buffer for all of the peptides monitored, as shown in Figure 7. Moreover, the difference in deuterium uptake rate is peptide specific, i.e., for the peptide shown in Figure 7a) the difference is approximately a factor of 3, while for the peptide in Figure 7b) the difference is much greater at almost a factor of 8. Deuterium back exchange of labeled peptides is a persistent problem for HDX[4]. A set of fully deuterated peptide mixture was used to measure the deuterium back exchange. The results showed that quenching has a greater effect than using longer analytical separation gradient time (data not shown), consistent with what has been reported in the literature.[4] For some proteins, elevated digestion temperature is necessary to improve the digest efficiency and the protein sequence coverage. The mass spectrometer's resolution and number of microscans have minimal effect on deuterium uptake measurement. Very consistent deuterium uptake results were also obtained from different charge states of the same peptides even when up to 5 different charge states were measured (see Figure 8).

FIGURE 5. Identification of overlapping peptides and heat map of Cytochrome c

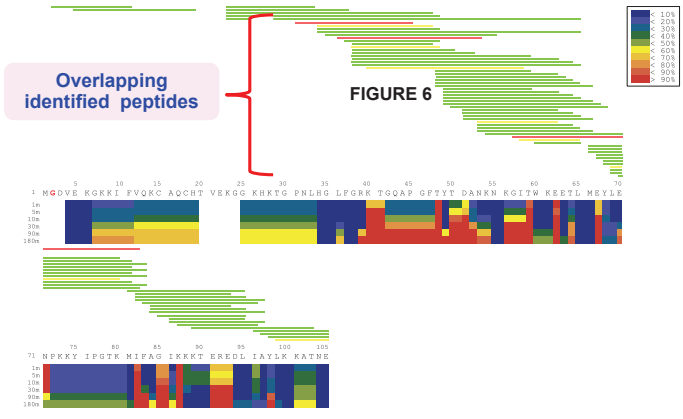


FIGURE 6. System reproducibility test. Less than 4% of the measured peptides with less than 3% deuterium uptake variability.

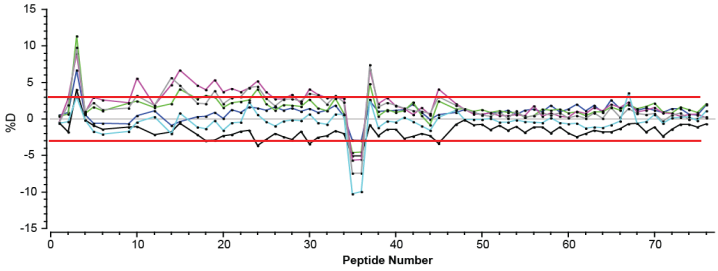
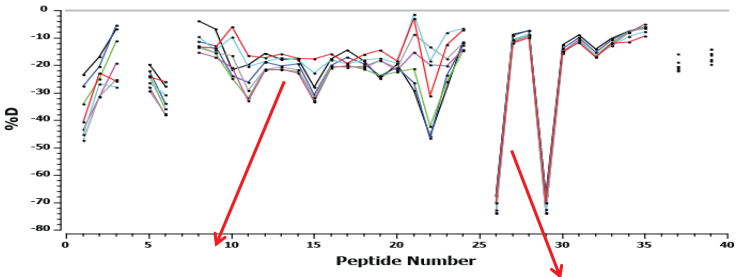


FIGURE 7. Effect of labeling solution. Deuterium uptake residual plot of two different labeling conditions. Condition two showed more deuterium uptake than labeling condition one for all of the peptides measured.



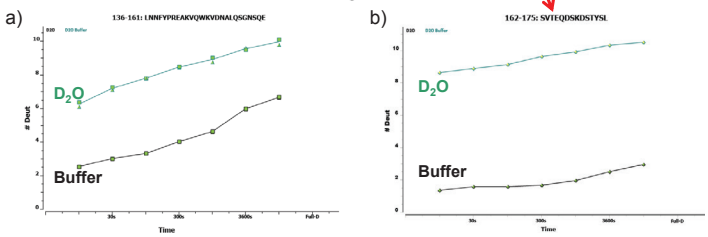
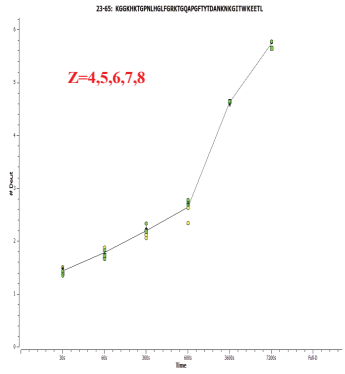


FIGURE 8. Deuterium uptake plot with five charge states measured

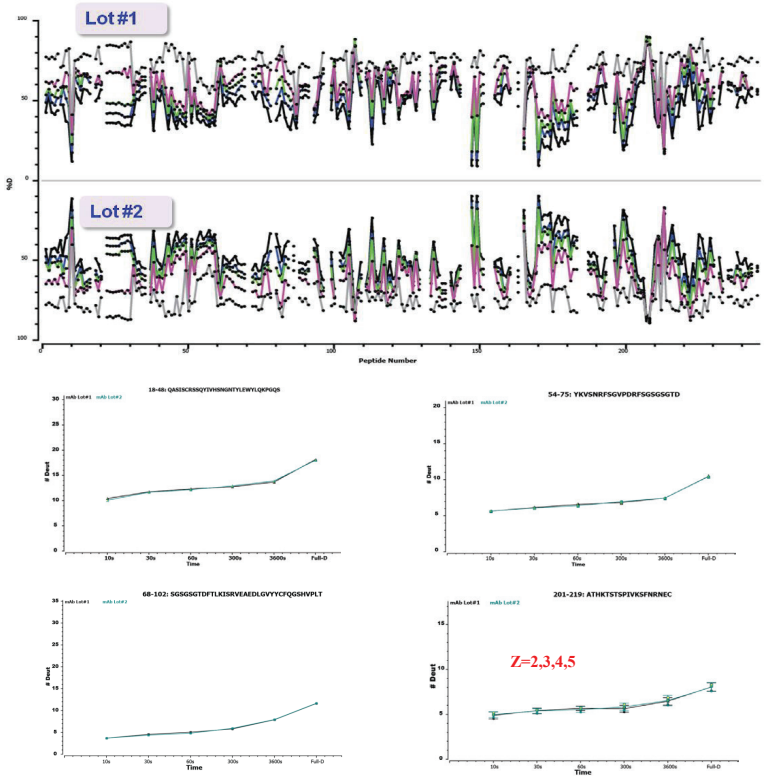


Probing mAb conformation

Two lots of a mAb were analyzed using the optimized experimental conditions. Both lots showed very similar deuterium uptake profiles, confirming that there are no significant conformational differences for these two lots as well as good performance reproducibility. Figure 9 displays the residual heat map between these two lots, which is quite homogeneous. Figure 10 shows the excellent reproducibility between these two lots when butterfly plots of deuterium uptake (top) and specific peptides (bottom) were compared.

FIGURE 9. Residual heap map of analyzed two lots of mAbs.

FIGURE 10. (top): Comparison of butterfly plots of deuterium uptake of these two lots of mAb. (bottom): Comparison of specific peptides' uptake



Conclusions

•A fully automated HDX workflow was developed and successfully applied to the study of conformational changes of proteins.

•The experimental conditions such as labeling temperature, buffer, digestion parameters, and analytical separation gradient were optimized.

•Optimized experimental conditions were applied to study the conformation of mAb from two different lots. Excellent reproducibility was obtained.

References

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