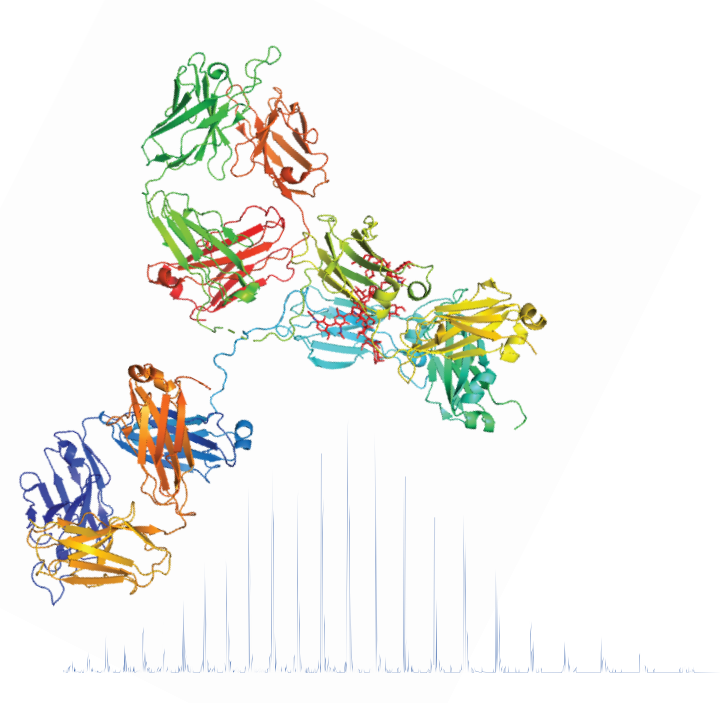
Structural characterization of the therapeutic antibody and biosimilar product with hydrogen deuterium exchange mass spectrometry



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Goal

The aim of this work is to demonstrate a fully automatic hydrogen deuterium exchange mass spectrometry workflow to study conformations of therapeutic antibodies and their biosimilars.

Introduction

Recombinant monoclonal antibodies (mAb) and their derivatives have become the fastest growing class of human therapeutics since the 1980s. The patent expiration of first generation mAb therapeutics provides an opportunity for biosimilar mAbs to enter the biotherapeutics market. The U.S. Food and Drug Administration (FDA) expects that extensive characterization of both biosimilar products and the innovator reference products, as the foundation of biosimilarity, be completed. In addition, protein secondary, tertiary, and quaternary structures should be considered. There are many analytical tools, such as X-ray crystallography, cryogenic electron microscopy (cryo-EM), and nuclear magnetic resonance (NMR) to study protein conformation, conformation dynamics, and protein-protein interactions. However, these techniques require large amounts of highly purified proteins and are limited by the size of the analyzed proteins. Recently, hydrogen deuterium exchange mass spectrometry (HDX-MS) has emerged as a powerful tool to fill the analytical gap[1,2] to investigate the conformation of intact proteins, including mAbs. In this application note, a fully automated HDX workflow station powered by the Thermo Scientific™ Orbitrap Exploris™ 480 mass spectrometer was used to characterize the conformations of trastuzumab and its biosimilar product.

Experimental

Methods and materials

Trastuzumab:	30 µm in formulation buffer
Biosimilar:	30 µm in formulation buffer
Column:	Thermo Scientific™ Acclaim™ PepMap™ 100 C18, 1.0 mm × 5 cm, 3 µm (P/N 164454)
Trapping column:	Thermo Scientific™ Acclaim™ PepMap™ 300 C18, 5 µm (P/N 163593); Holder (P/N 164650)
Digestion column:	Immobilized protease III/pepsin, NovaBioAssays (NSA2014002)
Mobile phase A:	0.1% formic acid in water
Mobile phase B:	0.1% formic acid in 80% acetonitrile, 20% water
Labeling buffer:	20 mM Hepes in deuterated water, pD 7.4
Mass spectrometer:	Orbitrap Exploris 480 mass spectrometer
Liquid chromatography:	Thermo Scientific™ UltiMate™ NCS-3500RS

A fully automated HDX platform based on the H/D-X PAL™ (Trajan, LEAP-HD-Automation) and the UltiMate NCS-3500RS system coupled to an Orbitrap Exploris 480 mass spectrometer was used to perform a bottom-up HDX-MS experiment for trastuzumab and its biosimilar to compare their conformations. Trastuzumab and biosimilar samples (3 µL of 30 µmol of each) were exchanged 1:20 with deuterium buffer at 10 °C at multiple time points. 40 µL of exchanged protein sample were quenched with an equal volume of quenching buffer using a cooling syringe. 50 µL of quenched protein solution, 30 pmol of exchange protein sample were injected and digested at 15 °C for three minutes. The digested peptides were trap washed for two minutes and eluted with a gradient from 10% to 35% of 80% acetonitrile in 0.1% formic acid for ten minutes. The eluted peptides were analyzed with an Orbitrap Exploris 480 mass spectrometer. A data-dependent HCD MS^2 was collected from the undeuterated protein digest to identify all detectable peptides. Triplicate MS full scan spectra were collected for HDX-MS analysis. The experimental parameters are shown in the tables on the right.

Data analysis

All data analysis, including the protection factor plots based on the experimental deuterium incorporation, was performed on Thermo Scientific™ BioPharma Finder™ 3.2 software. HDX-MS experimental data was analyzed with HDExaminer™ 2.5 software (Sierra Analytics).

HPLC

Separation conditions

Retention [min]	Flow [µL/min]	%B
0.9	40.00	3.0
1.0	40.00	10.0
11.0	40.00	35.0
12.0	40.00	95.0
14.0	40.00	95.0
15.0	40.00	3.0
16.0	40.00	95.0
17.0	40.00	95.0
18.0	40.00	3.0

Loading conditions

Retention [min]	Flow [µL/min]	%B
2.0	150	0
2.1	100	0
5.0	100	0
5.1	100	2
10.0	100	2
11.0	100	0
18.0	100	0
19.0	150	0

Mass spectrometry

ESI source conditions

Parameter	MS
Spray voltage (V)	3600
Sheath gas	20
Aux gas	2
Capillary temperature (°C)	220

MS conditions

Parameter	MS	MS/MS
MS full resolution	60K	60k
AGC	100%	100%
MS mass range (*m/z*)	300–1300	200–2000
RF lens	40	
Isolation		2
Dynamic exclusion		Top 15
Collision energy (%)		27
Charge state	2–6	2–6
Intensity threshold		5e4
Max injection time (ms)	100	150

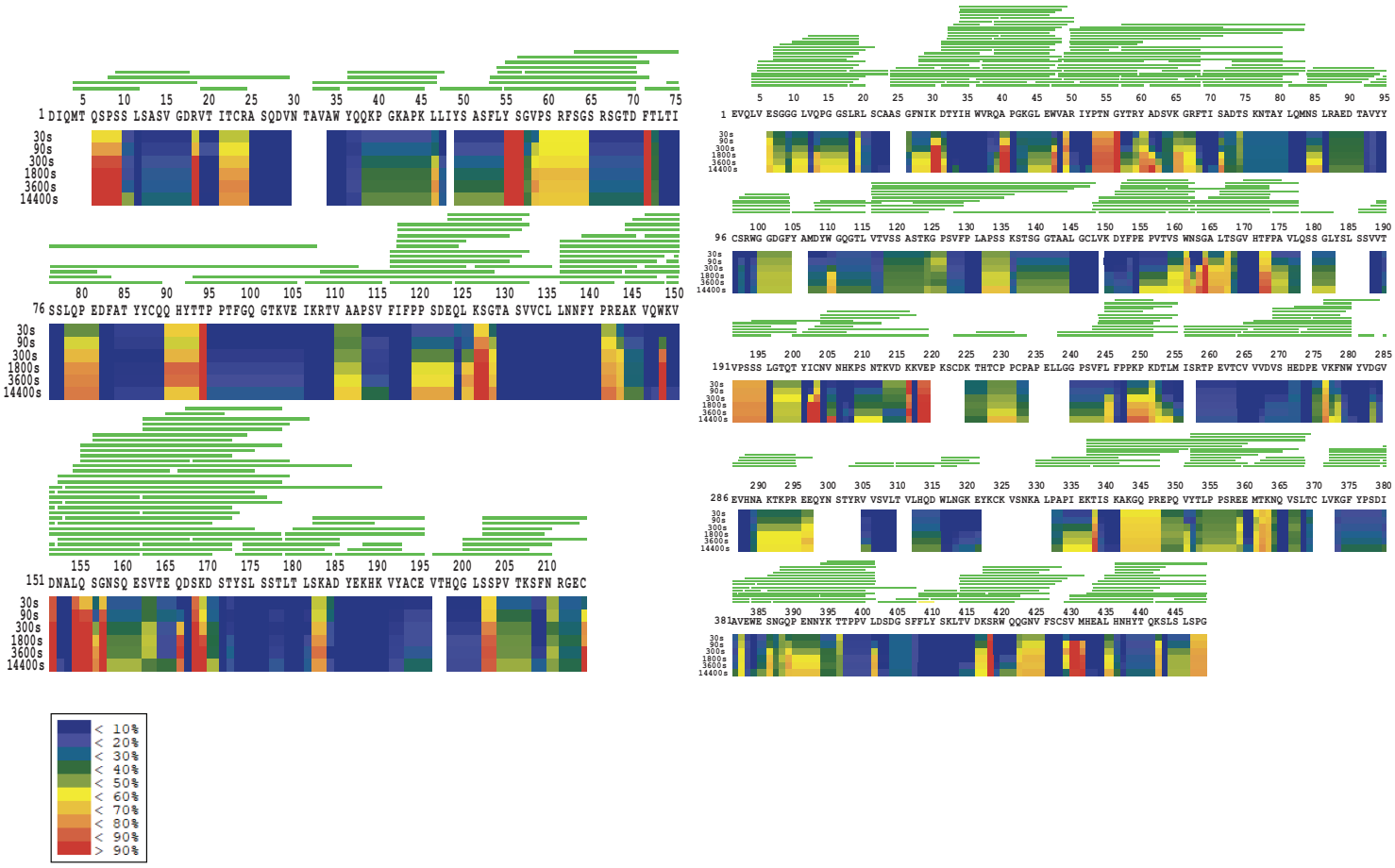

Figure 1. Deuterium uptake heat map. Left panel: light chain, right panel: heavy chain

Results and discussion

More than 800 peptides were identified with sequence coverage of over 98% for both light and heavy chains. For the deuterium incorporation calculation for light and heavy chains, 86 and 264 peptides were used, respectively. The average mass differences of non-deuterium exchange peptides to deuterated peptides were calculated and used to characterize the protein conformation. Figure 1 shows the deuterium uptake heat map for the light and heavy chains. The deuterium uptake increased from low (dark blue) to high (bright red) with the deuterium exchange time, 30 second to 4 hours (six points total). By using the deuterium uptake value of overlapped peptides, single residue resolution was obtained in multiple regions along the protein sequence.

The peptide's deuterium uptake results showed that trastuzumab and its biosimilar have very similar deuterium uptake profiles for both light and heavy chains, indicating that the two proteins have very similar solvent accessibility. To compare the difference between the two proteins, the residual plots were built by subtracting the peptide deuterium uptake of the biosimilar from Trastuzumab, as shown in Figures 2 and 3. The deuterium uptake residual plots showed that most of the peptide deuterium uptake differences were less than 4% (marked by red lines), except for few points in both light and heavy chains. In the bottom panel of Figure 2, two examples of light chain peptide's deuterium uptake plots are shown. On the left side of the figure, peptide position 136–145, the deuterium uptake difference was less than 2%; on the right side of the figure, peptide position 154–161, the difference was slightly higher than 4%. Similar to the light chain, the residual plot for the heavy chain was mostly within 4% difference range. However, there were multiple peptides from 244 to 255 that had approximately a 4% difference in deuterium uptake, which is different from the light chain. The deuterium uptake dynamics were also different. The deuterium exchange rate was different across all the exchange time points except for the last point as shown in Figure 3, in the bottom right three panels. In this area, glycan modifications are located; therefore, it may be possible that different glycoforms lead to small conformational differences between the two samples.

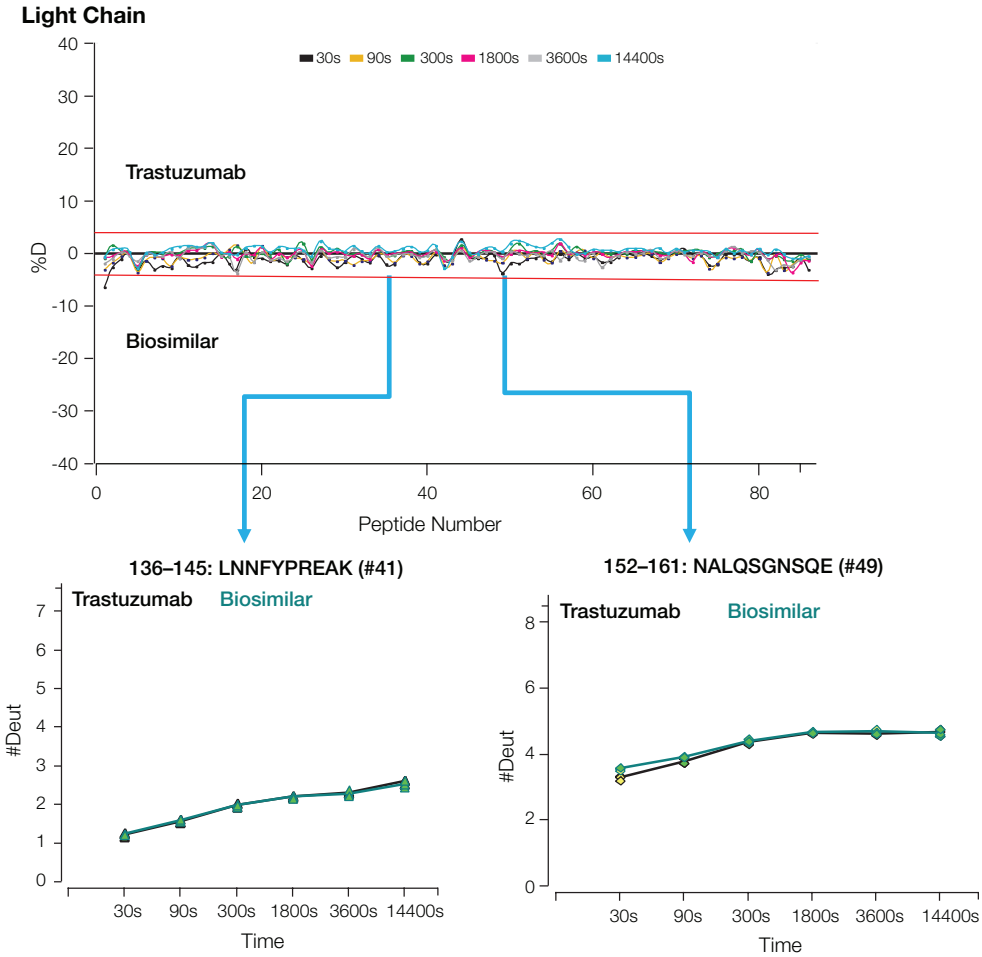

Figure 2. Trastuzumab and its biosimilar light chain deuterium uptake residual plot. X-axis: number of peptides, Y-axis: deuterium uptake percent difference per peptide. Bottom left panel: deuterium uptake plots for the peptide with less than 4% difference. Bottom right panel: deuterium uptake plots of the peptide with slightly higher than 4% difference.

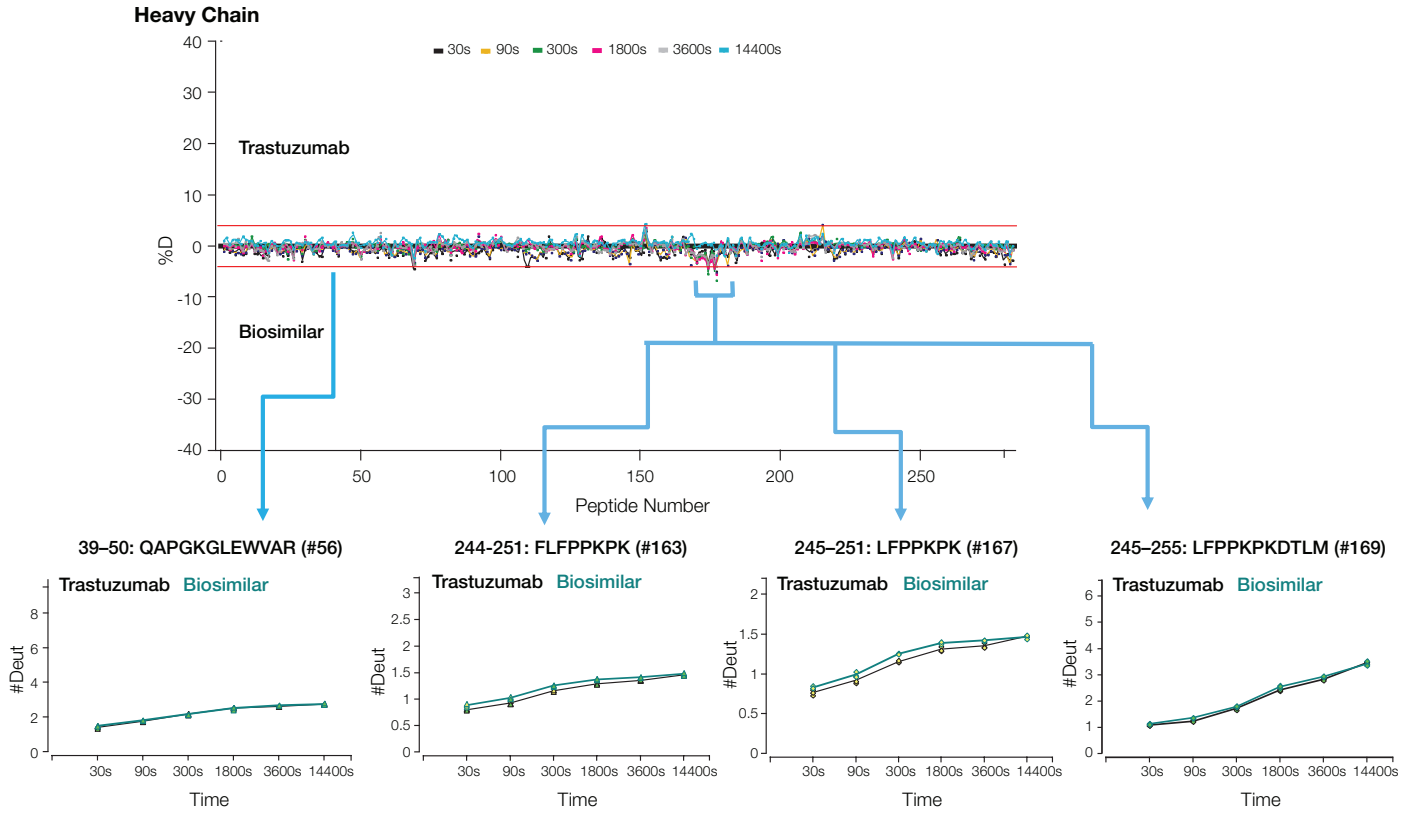

Figure 3. Trastuzumab and its biosimilar heavy chain deuterium uptake residual plots. Bottom left panel: example of 100% match of peptide's deuterium uptake plots. Right bottom three panels: multiple peptides showed slightly different deuterium uptake plots.

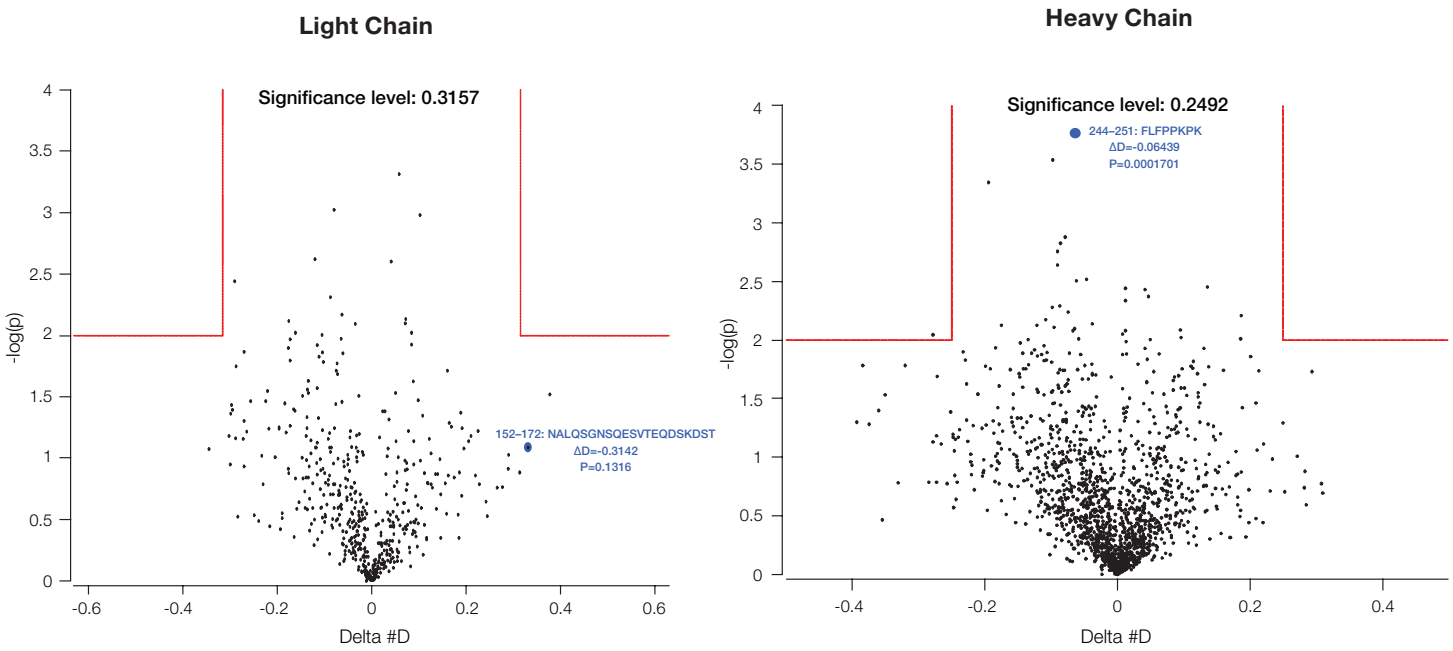

Figure 4. Light and heavy chain peptide's deuterium uptake difference; shown as volcano plots of two analyzed protein samples

To statistically evaluate whether the two proteins conformations were different, volcano plots were generated by HDExaminer™ software for both the light and heavy chains (Figure 4). In the volcano plot, each point represents a single peptide at a single time point.[5] The x-axis of the volcano plot shows the change in the measured number of deuterons between the two protein states. The red vertical lines on either side of 0 indicates significance level calculated by considering the variance in the replicate experiments. Measurements between these two vertical red lines are not significant because the measured difference between the two protein states did not exceed the replicate variance. The y-axis of the volcano plot is the negative log of the p-value for that measurement (a point high on the y-axis has a very low, and therefore significant, p-value). The horizontal red line at y=2 represents a 99% confidence interval. Measurements below the horizontal red line are considered not significant. The volcano plots were built with 86 peptides, six time points using triplicates data, 1548 data points for the light chain and 4752 data points for the heavy chain. The significant level was 0.32 and 0.25 for the light and heavy chains, respectively. There were no data points that fell into the significant difference region. The peptides with approximately 4% differences from the residual plots in Figure 3 were labeled in the volcano plots in blue; the differences were close to the outer distribution area but still not statistically significant.

Based on the experimental results, the protection factor on the single amino acid resolution, a unique feature of BioPharma Finder software's HDX-MS data analysis, was calculated as shown in Figure 5. The protection factor plots could reveal differences on the single residue level for the whole protein. The protection factor plots showed no difference between the two samples which was consistent with results from the HDExaminer analysis.

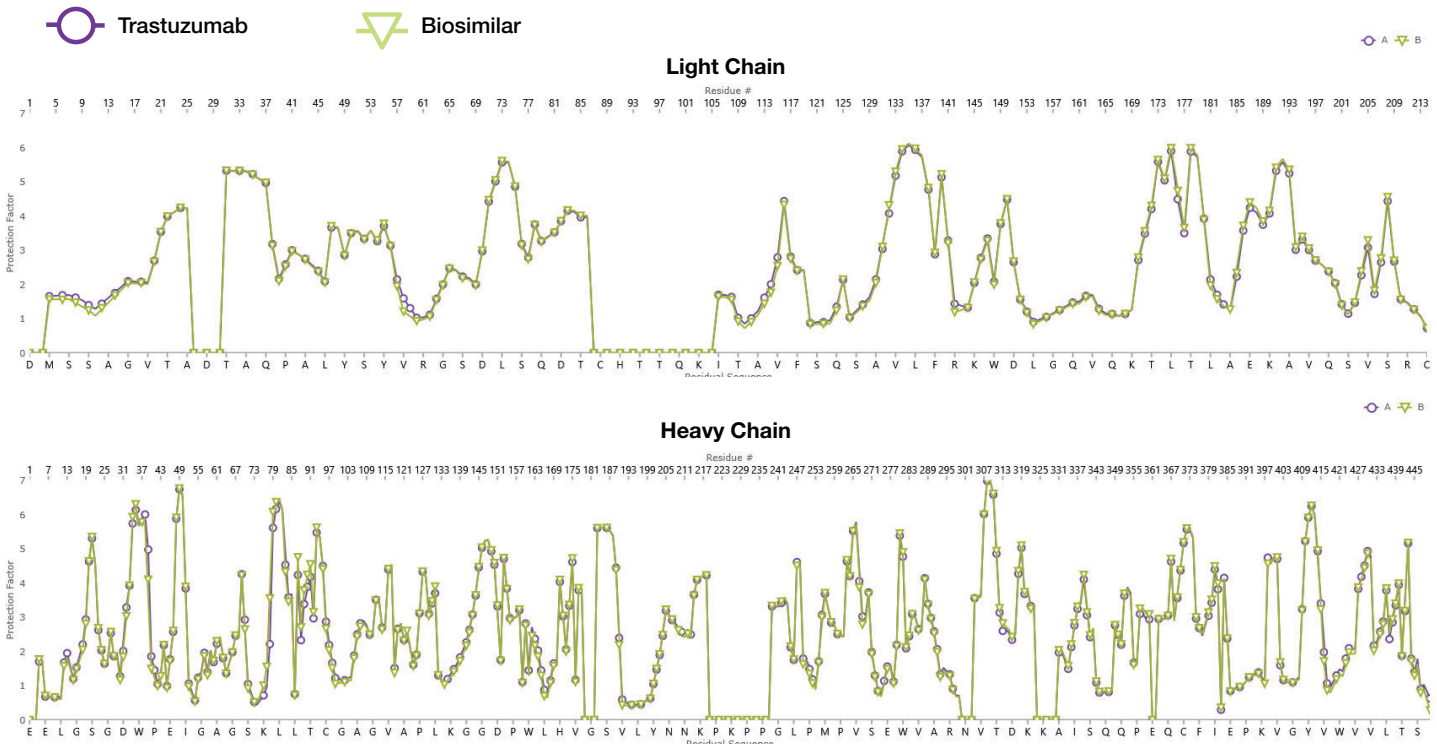

Figure 5. Protection factor plots of the light and heavy chain. The purple circle represents trastuzumab and the green triangle represents the biosimilar. Top panel: light chain, bottom panel: heavy chain.

Conclusion

Trastuzumab and its biosimilar conformation comparison was successfully performed by using a fully automated Orbitrap Exploris 480 MS-based HDX-MS workflow. Close to 100% sequence coverage and more than 800 peptides were obtained for both light and heavy chains. Altogether 355 peptide's deuterium uptake values were calculated by HDExaminer and subsequently used to characterize the conformation of trastuzumab and its biosimilar. Trastuzumab and its biosimilar demonstrated very similar solvent accessibility with deuterium uptake profiles that were within 4%, indicating very similar conformations. Statistical analysis via volcano plots using more than 6000 data points confirmed that there were no significant differences between the two protein samples. The whole protein conformation comparison based on the single amino acid level protection factor was calculated with BioPharma Finder software. The protection factor plots of trastuzumab and its biosimilar overlapped very well, indicating their conformations were almost identical.

References

1. Houde, D. et al., Analysis of Protein Conformation and Dynamics by Hydrogen/Deuterium Exchange MS, *Anal. Chem.* **2009**, *81*, 2644–51.
2. Rose, R. J. et al., Mutation of Y407 in the CH3 domain dramatically alters glycosylation and structure of human IgG, *mAbs* **2013**, *5*, 219–228.
3. Mayne, L. et al., Many Overlapping Peptides for Protein Hydrogen Exchange Experiments by the Fragment Separation-Mass Spectrometry Method, *J. Am. Soc. Mass Spectrom.* **2011**, *22*, 1898–1905.
4. Hamuro, Y. et al., High-Resolution HDX-MS of Cytochrome c Using Pepsin/Fungal Protease Type XIII Mixed Bed Column, *J. Am. Soc. Mass Spectrom.* **2019**, *30*, 227–234.
5. Hageman, T. et al., Reliable Identification of Significant Differences in Differential Hydrogen Exchange-Mass Spectrometry Measurements Using a Hybrid Significance Testing Approach, *Anal. Chem.* **2019**, *91*, 8008–8016.
6. Zhang, Z.; Zhang, A.; Xiao, G.; Improved Protein Hydrogen/Deuterium Exchange Mass Spectrometry Platform with Fully Automated Data Processing, *Anal Chem.,* **2012**, *84* (11), 4942–4949.

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