Accelerating monoclonal antibody peptide mapping with high acquisition speed Orbitrap-based MS

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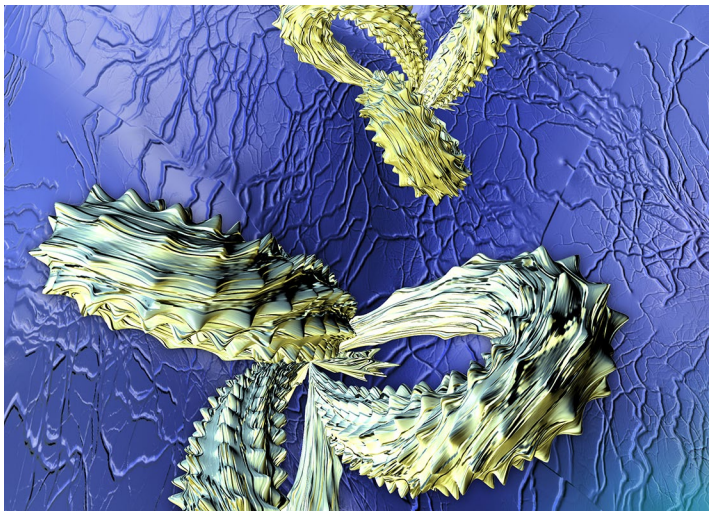


Application benefits

- Demonstrating how high resolution and fast scanning speeds can be combined for rapid peptide mapping analysis using the Thermo Scientific™ Orbitrap Exploris™ 480 mass spectrometer.
- Increasing throughput of peptide mapping while maintaining sequence coverage, post-translational modification identification, and quantification using shortened gradients.
- Highlighting the capacity for high-throughput peptide mapping to be utilized in support of process development and optimization.

Goal

To demonstrate the capability of the Orbitrap Exploris 480 mass spectrometer to confidently identify and characterize monoclonal antibody post-translational modifications (PTMs) in a high-throughput peptide mapping analysis using shortened chromatographic separations while maintaining full sequence coverage.

Introduction

The biopharmaceutical industry continues to grow at a rapid rate, so much so that by 2024 it is expected approximately 52% of sales within the top 100 selling drugs globally will be from biological products. Furthermore, monoclonal antibodies (mAbs) and related products (such as Fc-fusion proteins) are predicted to remain the dominant class of biotherapeutics.[1]

Biotherapeutics are highly heterogeneous molecules produced using living cellular expression systems, typically of hamster, mouse, or bacterial origin, which are highly sensitive to changes in their growth conditions. Such changes to the physical environment of these cells can have a marked effect on the physicochemical characteristics of the final purified drug product. As such, the large-scale processes required to manufacture these

products undergo extensive optimization throughout the development process to ensure that product quality is maintained.

The high molecular mass and structural complexity of mAbs make them particularly susceptible to PTMs at certain amino acid residues, some of which can be highly detrimental to product safety, stability, and efficacy. The relative effects of such modifications on the safety, structure, and function of the molecules are what define them as Critical Quality Attributes (CQAs). It is imperative that the analytical methods used to characterize these molecules can confirm the relative presence of these modifications both in the final drug product and throughout the manufacturing process development.

Ultra-high performance liquid chromatography coupled to high-resolution, accurate-mass mass spectrometry (UHPLC-HRAM-MS) is one of the most powerful and widely adapted tools for the characterization of complex therapeutic proteins, such as mAbs, and peptide mapping as an important analytical technique in the determination and monitoring of CQAs. Following proteolytic digestion, analysis of the resulting peptides by reversed phase (RP)-LC-MS allows a myriad of information to be gathered on the mAb, such as confirmation of amino acid sequence as well as localization and relative quantity of PTMs including, but not limited to, oxidation, deamidation, and *N*-linked glycosylation.

The versatility of peptide mapping as an analytical tool perfectly lends itself to protein characterization at multiple stages throughout the development cycle. It is typically employed as a means for in-depth characterization to gather as much detailed information about the molecule as possible. However, peptide mapping also has applicability for process support and screening studies, where the fine detail of every modification may not be required but high-level information about specific characteristics is desirable. As an example, peptide mapping can be used to determine whether a specific cell-line clone generates the desired glycosylation profile or whether an individual downstream purification step induces deamidation. These types of studies often require analysis of large numbers of samples, which can challenge the capabilities of the analytical method to generate the desired data in a much shorter timeframe than would be the case for in-depth characterization activities.

Here, we present an automated and versatile, increased throughput peptide mapping approach. Using the Orbitrap Exploris 480 mass spectrometer, we performed primary sequence determination and PTM characterization of the therapeutic monoclonal antibody, golimumab (Simponi™). To demonstrate the utility of this approach for high-throughput attribute screening, we have taken advantage of the increased acquisition rates of the Orbitrap Exploris 480 mass spectrometer for high-speed peptide mapping, even using very short chromatographic gradients, without sacrificing performance or data quality.

Experimental

Forced oxidation of golimumab

- A 1,000 ppm stock solution of hydrogen peroxide (H_2O_2, where neat 30% H_2O_2 is equivalent to 300,000 ppm) was prepared by serial dilution using water.
- Golimumab (at 100 mg/mL) was diluted 5-fold with water to a final concentration of 20 mg/mL.
- The 1,000 ppm H_2O_2 and 20 mg/mL golimumab sample were mixed in equal volumes so that the final concentrations were 500 ppm and 10 mg/mL for H_2O_2 and golimumab, respectively.
- Oxidation was induced by incubation at room temperature for 24 hours prior to tryptic digestion.

Automated protein digestion

- Untreated golimumab (at 100 mg/mL) was diluted 5-fold with water to a final concentration of 20 mg/mL.
- Both untreated and forced oxidized (see above) golimumab were digested for 45 min at 75 °C with 15 µL Thermo Scientific™ SMART Digest™ magnetic bulk resin.
- Digestion was performed using a final golimumab concentration of 0.5 mg/mL and in the presence of TCEP at a final concentration of 5 mM (Table 1).
- The digestion was automated using a Thermo Scientific™ KingFisher™ 96 Deepwell plate with the Thermo Scientific™ KingFisher™ Duo Prime purification system, controlled by Thermo Scientific™ KingFisher™ BindIt™ 4.0 software.

Table 1. Layout of KingFisher Deepwell, 96 well plate

Lane	Description	Contains	Volume per well
A	Sample	Sample (10 mg/mL)	10 µL
		SMART Digest buffer	148 µL
		Water (at least 18.2 MΩ/cm)	40 µL
		TCEP (500 mM)	2 µL
B	Tips	12-tip comb	-
C		Empty	
D	SMART Digest Trypsin	Magnetic Bead Bulk Resin	15 µL
		SMART Digest buffer	100 µL
E	Bead wash buffer	Water (at least 18.2 MΩ/cm)	160 µL
		SMART Digest buffer	40 µL
F	Waste	Water (at least 18.2 MΩ/cm)	200 µL

LC-MS

- For each analysis, 2 µL golimumab digest was injected (1 µg peptide load) for chromatographic separation using a Thermo Scientific™ Acclaim™ VANQUISH™ C18 UHPLC column, (2.2 µm, 2.1 x 250 mm), using a Thermo Scientific™ Vanquish™ Horizon UHPLC system. (Tables 2 and 3)
- Gradient durations ranged from 5 to 40 min (Table 4), all using the following:
  - Mobile phase A: 0.1% formic acid in water
  - Mobile phase B: 0.1% formic acid in acetonitrile
  - Flow rate: 0.3 mL/min
  - Column temperature: 60 °C
- Data were acquired using an Orbitrap Exploris 480 mass spectrometer equipped with BioPharma option, using a resolution setting of 60,000 (at *m/z* 200) for MS^1 and 15,000 for MS^2 (dd-MS^2, Top 15) (Table 5).
- Thermo Scientific™ Chromeleon™ 7.2.10 chromatography data system (CDS) software was used for instrument control and data acquisition.

Table 2. Instrumentation and software

Instrumentation and software	Part number
Thermo Scientific Vanquish Horizon system consisting of:	
Vanquish System Base	VH-S01-A
Vanquish Binary Pump H	VH-P10-A
Sampler HT	VH-A10-A
Vanquish Column Compartment H	VH-C10-A-02
Active Pre-heater	6732.0110
Thermo Scientific Orbitrap Exploris 480 mass spectrometer with BioPharma option (Extended mass range to *m/z* 8000)	BRE725533 + BRE725539
Thermo Scientific™ BioPharma Finder™ 3.2 Software	OPTON-30932
Chromeleon CDS Software	CHROMELEON7

Table 3. Recommended consumables

Recommended consumables	Part number
Thermo Scientific SMART Digest Trypsin Kit, Magnetic Bulk Resin option	60109-101-MB
Thermo Scientific KingFisher Deepwell, 96 well plate	95040450
Thermo Scientific™ KingFisher™ Duo 12-tip comb	97003500
Thermo Scientific KingFisher Duo Prime purification system	5400110
Thermo Scientific™ Bond-Breaker™ TCEP Solution	77720
Hydrogen peroxide (30% in Water), Fisher BioReagents™	10386643
Thermo Scientific Acclaim VANQUISH C18 column, 2.2 µm, 2.1 × 250 mm	074812-V
Fisher Scientific™ Water, Optima™ LCMS grade	10505904
Fisher Scientific™ Water with 0.1% formic acid (v/v), Optima™ LCMS grade	10188164
Fisher Scientific™ Acetonitrile with 0.1% formic acid (v/v), Optima™ LCMS grade	10118464

Table 4. Chromatographic parameters

Gradient	Conditions
1	0–40 min: 2–40% B, 40–42 min: 40–80% B, 42–45 min: 80% B, 45–45.5 min: 80–2% B, 45.5–60 min: 2% B
2	0–30 min: 2–40% B, 30–32 min: 40–80% B, 32–35 min: 80% B, 35–35.5 min: 80–2% B, 35.5–50 min: 2% B
3	0–25 min: 2–40% B, 25–27 min: 40–80% B, 27–30 min: 80% B, 30–30.5 min: 80–2% B, 30.5–45 min: 2% B
4	0–20 min: 2–40% B, 20–22 min: 40–80% B, 22–25 min: 80% B, 25–25.5 min: 80–2% B, 25.5–40 min: 2% B
5	0–15 min: 2–40% B, 15–17 min: 40–80% B, 17–20 min: 80% B, 20–20.5 min: 80–2% B, 20.5–35 min: 2% B
6	0–10 min: 2–40% B, 10–10.5 min: 40–80% B, 10.5–13.5 min: 80% B, 13.5–14 min: 80–2% B, 14–28.5 min: 2% B
7	0–5 min: 2–40% B, 5–5.5 min: 40–80% B, 5.5–8.5 min: 80% B, 8.5–9 min: 80–2% B, 9–23.5 min: 2% B

Table 5. MS parameters

Parameter	Value
Sheath gas	25
Auxiliary gas	10
Ion transfer tube temp. (°C)	320
Vaporizer temp. (°C)	150
RF lens (%)	40
Spray voltage (kV)	3.7
Polarity	Positive
Application mode	Peptide
Pressure mode	Standard
Full MS parameters	
Mass range (*m/z*)	200–2000
Resolution (at *m/z* 200)	60,000
AGC target (%)	300
Max. injection time (ms)	100
In-source fragmentation (eV)	0
MS^2 parameters	
Resolution (at *m/z* 200)	15,000
AGC target (%)	50
Isolation width (*m/z*)	2
Min. threshold	5e4
Normalized collision energy (NCE)	28
TopN MS^2	15
Max. injection time (ms)	200
Dynamic exclusion (s)	10
Include charge states	2–6

Data analysis

- Data were analyzed using Thermo Scientific BioPharma Finder 3.2 software.
- For all analyses, default peptide mapping parameters were used with the exception of "Absolute MS Signal Threshold" at a setting of 5.00E+5 (with MS Noise Level at 2,500 and S/N Threshold at 200).
- Golimumab sequence was searched with the following modifications included:
  - Gln → pyro-Glu at the *N*-terminus
  - Lysine loss at the *C*-terminus
  - NH_3 loss, oxidation (M, W) and deamidation (N, Q) on side chains
  - CHO glycosylation
  - Maximum of three modifications per peptide
- Visual data was generated using both Thermo Scientific™ FreeStyle™ 1.7 data visualization software and Chromeleon CDS.

Results and discussion

Chromatography and sequence coverage

Peptides from tryptic digestion of golimumab were analyzed using seven different gradients, of decreasing length, from 40 minutes to 5 minutes, and for all gradients, very good separation was achieved (Figure 1).

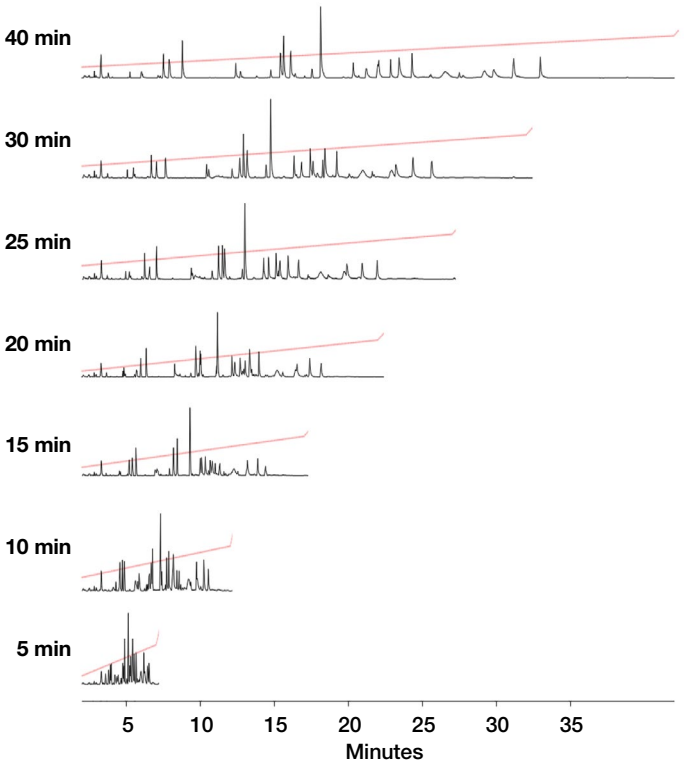


Figure 1. Base peak chromatograms following peptide mapping experiments of golimumab using a series of different gradient lengths (40, 30, 25, 20, 15, 10, and 5 minutes). Gradient ramps (2–40% solvent B) are shown in red for each gradient. The illustrated gradient ramps all include a 2-minute dwell time to account for the delayed elution of the peptides at the experimental flow rate.

Despite the high number of tryptic peptides generated during mAb digestion, the combination of excellent chromatographic performance and the fast MS acquisition rates of the Orbitrap Exploris 480 mass spectrometer, enabled 100% sequence coverage of both the light chain (LC) and heavy chain (HC) achieved in all experiments (Table 6), even when using the shortest 5-minute gradient (Figure 2). The different colored bars on the sequence coverage map reflect the number of mis-cleaved peptides, which proves advantageous for obtaining full sequence coverage as a more complete digest would produce a number of tryptic peptides too small to be efficiently captured during LC-MS analysis.

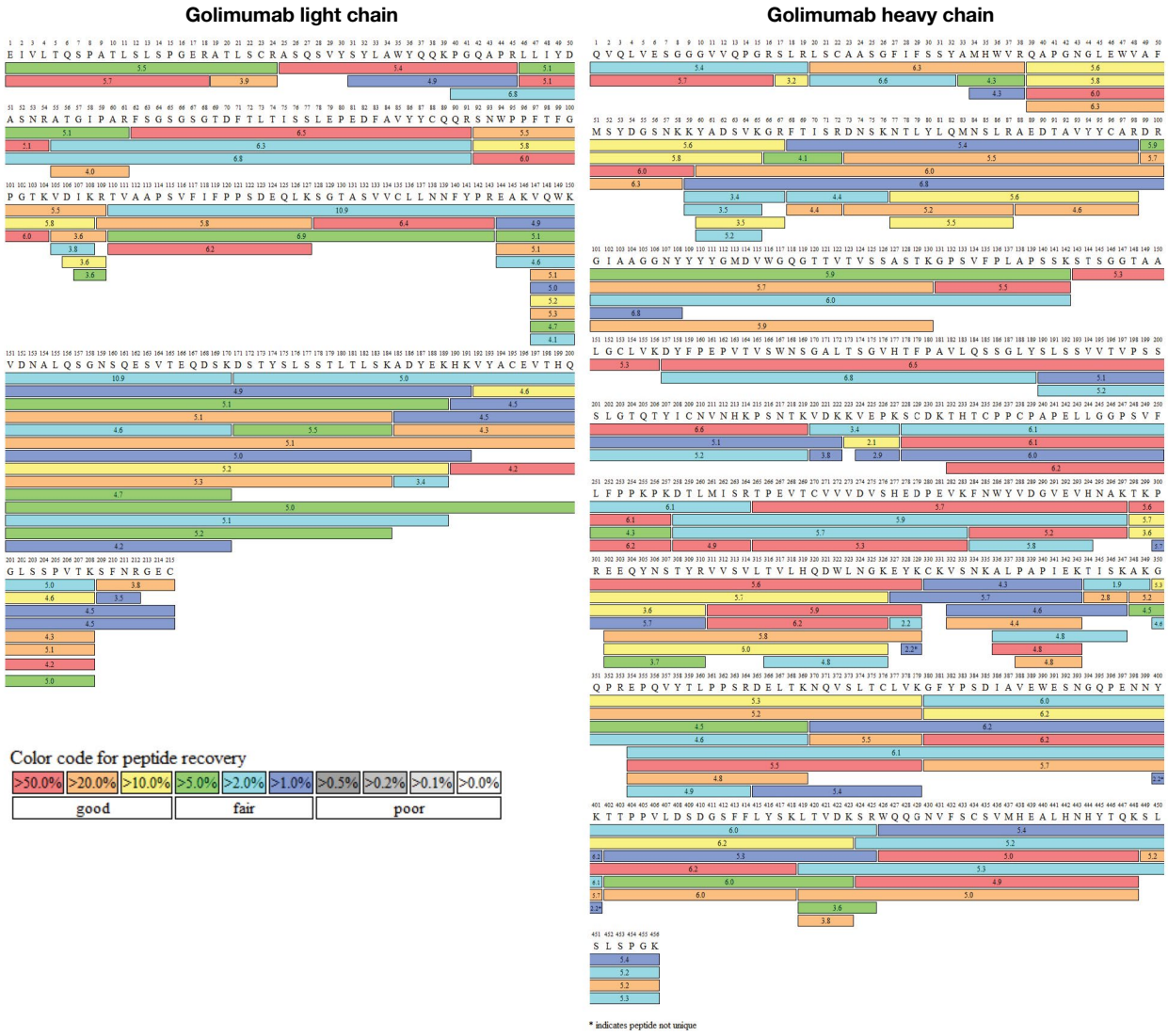


Figure 2. Sequence coverage map of golimumab LC (left) and HC (right) using the 5-minute gradient for peptide separation. The different colored bars represent the peptide recovery of the MS^1 scan with red, orange, and yellow being "good recovery" and green, blue, and cyan being "fair recovery", i.e., less intense. The numbers on the colored bars represent the retention time of the respective peptides.

Table 6. Golimumab HC and LC sequence coverage obtained from peptide separation using different length gradients

Gradient length (min)	HC coverage (%)	LC coverage (%)
40	100	100
30	100	100
25	100	100
20	100	100
15	100	100
10	100	100
5	100	100

Oxidation

Oxidation primarily occurs at methionine and tryptophan residues, with cysteine and histidine residues also susceptible. Aberrant oxidation within biotherapeutics can have a detrimental effect on protein structure and function: oxidation at specific methionine residues of circulating IgG can decrease binding to the neonatal Fc receptor (FcRn) and subsequently shorten the in vivo half-life of the molecule[2]. Oxidation can be induced during product processing, and so it is important to monitor this during manufacturing process optimization. Similarly, traditional peptide mapping sample preparation approaches can also artificially induce oxidation, however, an advantage of the SMART Digest approach is the shortened digestion times reduce the likelihood of this sample-preparation induced oxidation.

Oxidation of golimumab using the Orbitrap Exploris 480 mass spectrometer was assessed by forced oxidization, with H_2O_2, and direct comparison to the unmodified sample. This ensured that oxidized peptides could be adequately detected by LC-MS analysis, as the native sample contained very low oxidation levels.

Four sites of oxidation, all within the HC of golimumab, were observed following H_2O_2 treatment. All four sites were at methionine residues, where addition of a single oxygen causes conversion to methionine-sulfoxide (Figure 3A). The level of oxidation at all four sites was consistently calculated across all gradients, even for sites M51 and M113 where only low levels of oxidation were determined (Figure 3B). It is noteworthy some sites were not fully oxidized following incubation with 500 ppm H_2O_2, indicating differential susceptibility of these residues to modification in the native mAb structure.

Methionine 261 (M261) was determined to be near 100% oxidized following 500 ppm H_2O_2 treatment. This residue sits within the "DTLMISR" peptide, which is a commonly monitored peptide for mAb oxidation. Even when using the 5-minute gradient, there is clear chromatographic separation between the unmodified and the oxidized peptide (Figure 4A).

The full MS spectra were acquired with excellent mass accuracy (<1.5 ppm), and the observed mass shift between unmodified and oxidized peptides was exceptionally close to the expected +15.9949 Da (Figure 4B). The presence of

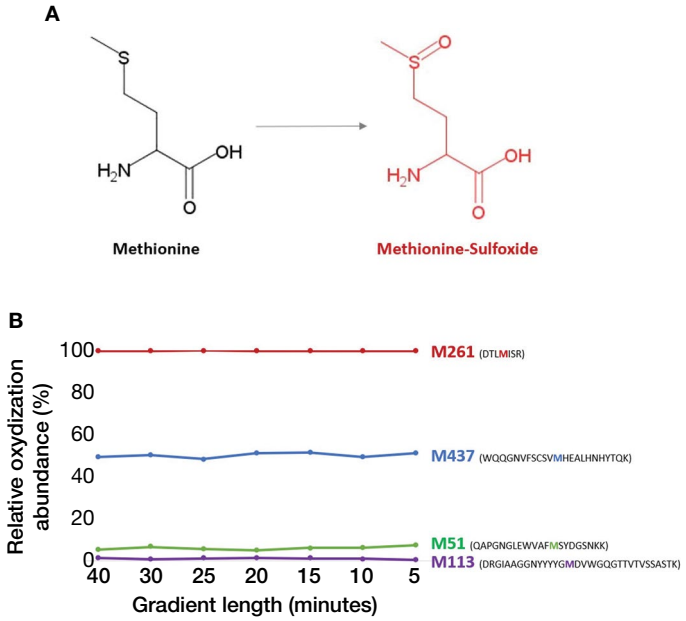
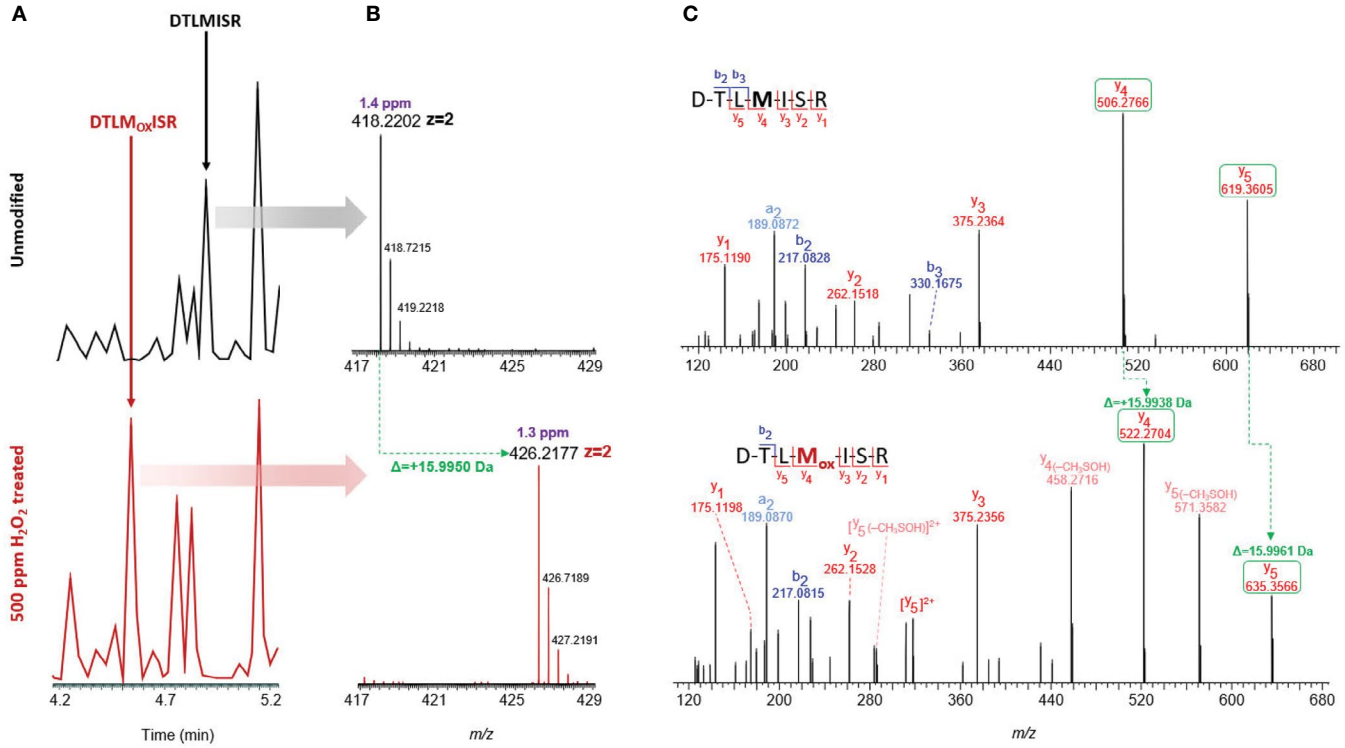


Figure 3. Comparison of relative oxidation abundance in golimumab treated with 500 ppm H_2O_2. A) Methionine-sulfoxide formation following addition of oxygen to side chain of methionine residue. B) Relative oxidation levels at four methionine residues, detected in the HC of golimumab following H_2O_2 incubation. Relative levels of oxidation were determined using BioPharma Finder software.



of the unmodified DTLMISR peptide (black, upper section) and the peptide from the sample treated with 500 ppm H_2O_2 (red, lower section) A) Zoomed section of the base peak chromatogram; B) Zoomed sections of the full MS spectra; C) MS^2 HCD fragment ion spectra with coverage of b- and y-ion fragments showing in blue and red respectively. Note the diagnostic neutral loss (~64 Da) of methane sulfenic acid (CH_3SOH) from y_4 and y_5 fragment ions that contain the oxidized methionine residue.

methionine-sulfoxide was further confirmed using HCD MS^2 fragmentation, with methionine-containing fragment ions (y_4 and y_5) also exhibiting the expected mass shift (Figure 4C); a characteristic loss of methane sulfenic acid (CH_3SOH) was also observed in these ions[3]. The high quality MS^1 and MS^2 spectral data obtained for these peptides highlights the exceptional performance of the Orbitrap Exploris 480 mass spectrometer and showcases its utility in the determination of oxidation determination, even when using short chromatographic separations.

Deamidation

Deamidation is the chemical conversion of amine groups, primarily within the side chains of asparagine and glutamine residues, to acidic groups. Asparagine (Asn) residues are by far the most commonly modified and are converted to either aspartic acid (Asp) or isoaspartic acid (*iso*-Asp) via a succinimide (Asu) intermediate (Figure 5A). Depending on the specific location of the modification, deamidation can have a significant effect on protein structure, potentially impacting both product safety and efficacy.[4]

Deamidation kinetics are heavily dependent on a multitude of factors, one of which is the identity of residues adjacent to the site of deamidation. The majority of potential deamidation sites are stabilized by the surrounding higher order structure; however, asparagine residues with neighboring glycine residues, referred to as Asn-Gly (or 'NG'), are particularly susceptible to deamidation due primarily to the increased flexibility of this structure. Golimumab contains an asparagine, located at position 43 within the HC (N43), which resides between two glycine residues on each side, in a "GNG" moiety (Figure 5B).

Identifying deamidation is particularly challenging due to the small mass shift (Δmass) associated with the modification (+0.9840 Da), even when using mass spectrometry, primarily due to the overlapping isotopic envelopes of the unmodified and deamidated peptides. The use of HRAM-MS overcomes this challenge, as it allows for robust and confident detection of deamidation, which has been demonstrated here for golimumab (Figure 6A).

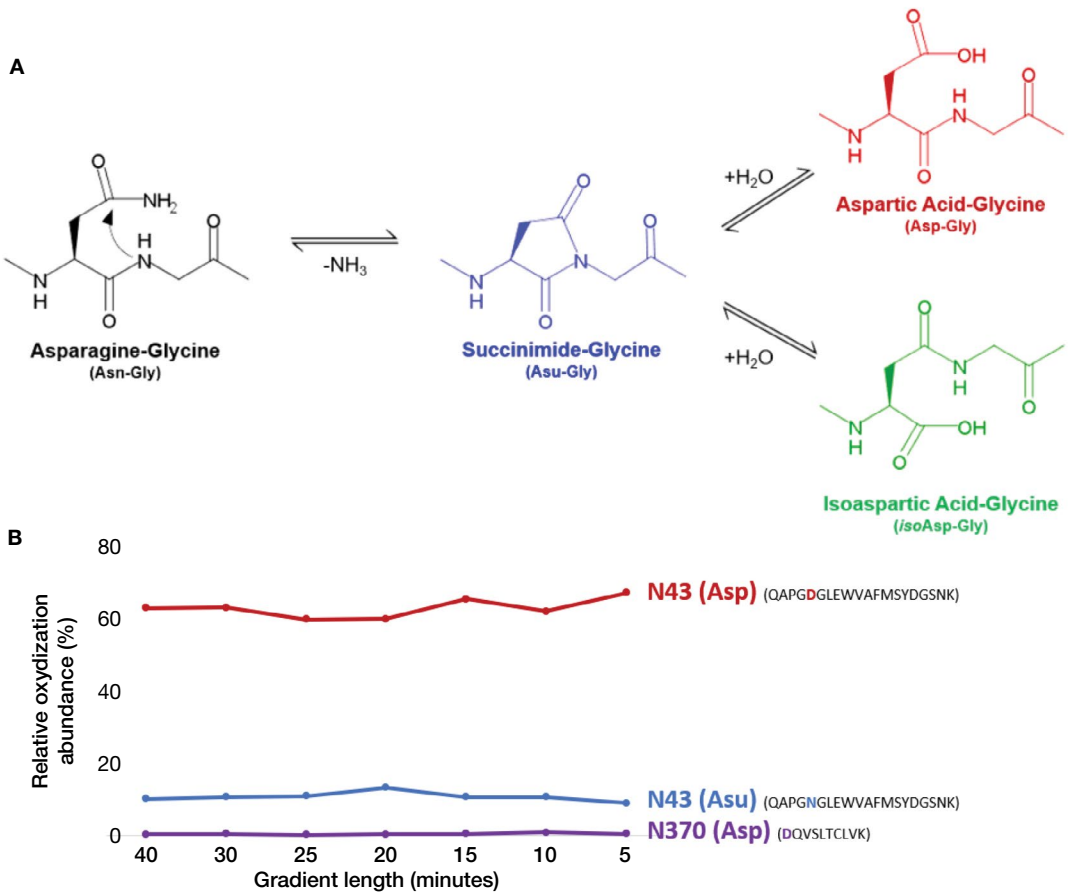


Figure 5. Comparison of relative deamidation abundance in golimumab. A) Process of deamidation at an asparagine residue *N*-terminal of a glycine. The asparagine residue (Asn) is converted to aspartic acid (Asp) or isoaspartic acid (isoAsp) via a succinimide intermediate (Asu) through chemical conversion of the amine group in its side chain. B) Relative deamidation levels at two asparagine residues in the golimumab HC, in one of which, N43, both deamidation through to aspartic acid and succinimide intermediates are detected. Relative levels of deamidation were determined using BioPharma Finder software.

Significant levels of deamidation were detected at position N43, which is not unexpected considering that the modification site is within a “GNG” sequence moiety. In addition, a considerable amount (~10%) of the succinimide intermediate containing peptide, signified by a Δmass of -17.0266 Da, was also detected. Even when using the 5-minute gradient, the Asn, Asp, and Asu containing peptides were all chromatographically separated and detected with exceptional mass accuracy (≤0.6 ppm) (Figure 6B). The chromatographic separation and fast scan speed of the Orbitrap Exploris 480 mass spectrometer allowed for high quality MS^2 spectra to be collected for each species and the site of modification was unequivocally confirmed (Figure 6C).

The relative abundances of both deamidation and succinimide formation at N43 were calculated using BioPharma Finder software, and these modifications were detected at a consistent level across all chromatographic gradients. (Figure 5B). This was also the case for a secondary low-abundant site of deamidation (site N370), located in the HC, highlighting that even for a challenging modification such as deamidation—where both the mass shift and chromatographic separation are minimal—the Orbitrap Exploris 480 mass spectrometer excels in characterizing these modifications with exceptional mass accuracy.

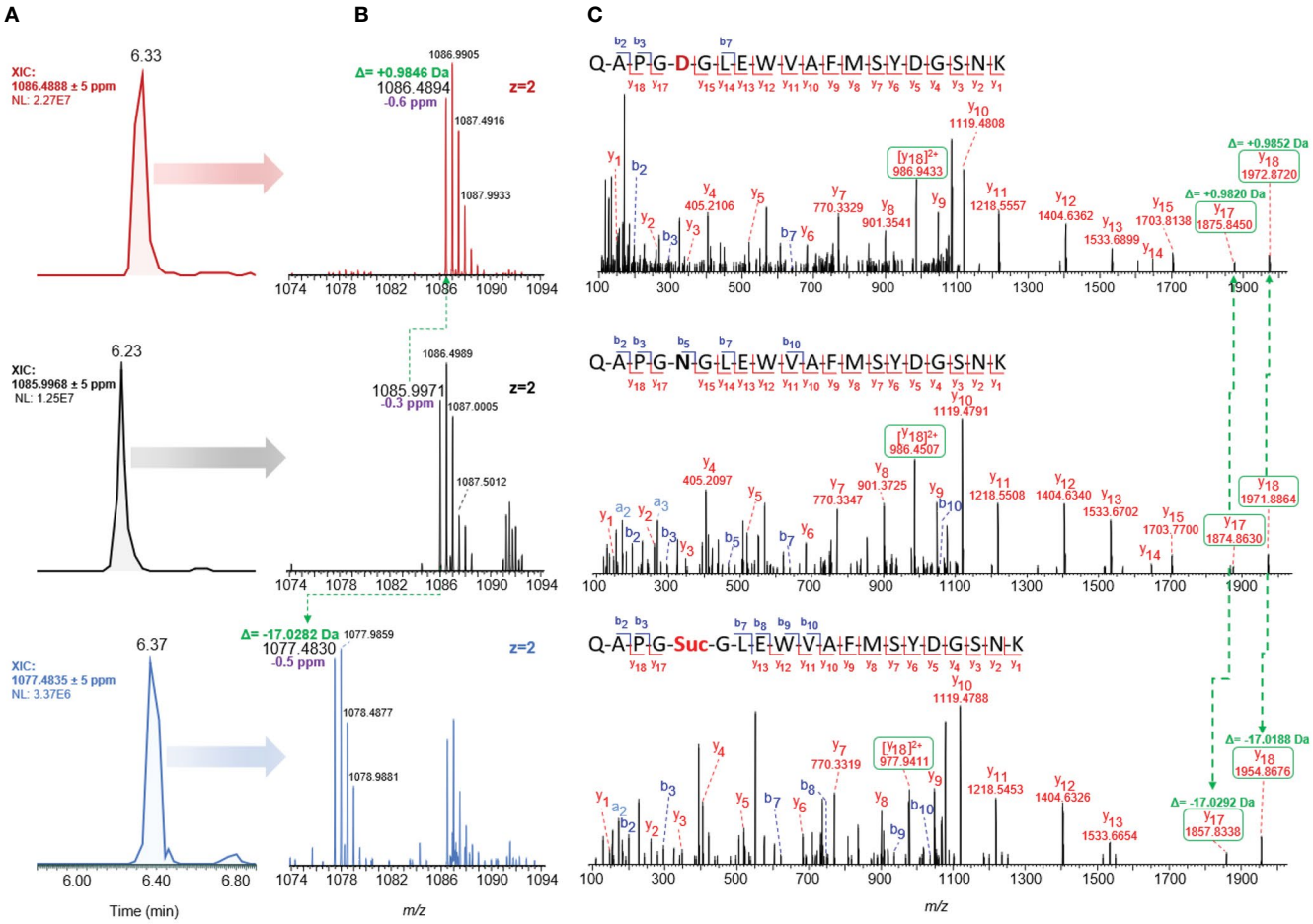


Figure 6. Detection of golimumab deamidation at N43 in the HC, using the 5-minute gradient. Comparison of the unmodified peptide (black, middle section), the deamidated peptide (red, upper section), and the succinimide containing peptide (blue, lower section). A) XICs for the three versions of the peptide; B) Zoomed sections of the full MS spectra, with mass accuracy and determined mass shifts of the deamidated and succinimide containing peptides; C) MS^2 HCD fragmentation spectra with coverage of b- and y-ion fragments in blue and red, respectively. The key fragment ions (y_{17} and y_{18}) for confirmation of the location of the modification are highlighted with green boxes, along with the determined mass shifts.

N-linked glycosylation

N-linked glycosylation is a key modification impacting both the safety and efficacy of the therapeutic. mAbs typically contain a single site of glycosylation within each HC (two glycosylation sites in total). In golimumab, this site is located at asparagine 306 (N306). Due to the nature of glycan addition within the cell, *N*-linked glycosylation is a highly heterogeneous modification, meaning that glycan analysis is inherently a profiling method—where the relative abundance of each glycan is calculated and must remain as consistent as possible between manufacturing batches. For example, a batch of mAb, which contains noticeably more "high mannose" type glycans, is likely to be removed from the body quicker, affecting the overall activity of the biotherapeutic.[5]

Although released glycan analysis is the primary method for in-depth glycan characterization, the relative simplicity of mAb glycosylation makes glycopeptide mapping a viable alternative—at least for the purposes of a high-level comparison of the overall glycan profile. Because of the potential impact of glycosylation on mAb structure and function, and the tendency for alterations in the glycan profile to occur during the fermentation process,[6] it can be desirable to monitor glycosylation at an early stage of the development process. Glycosylation assessment may have utility within cell-line development and cell-screening activities to determine the relative ability of the screened cell lines to produce the desired glycan profile. Therefore, high-throughput glycan profiling methods are highly desirable to support such activities.

The major golimumab glycoforms (Figure 7A) were detected, and their relative abundance consistently determined, across all gradient lengths (Figure 7B). There were several minor glycoforms identified by BioPharma Finder software, but their identification was more variable due to their relative low amounts and the propensity for all glycopeptides to elute in a relatively small retention time window when separated by RP-LC (Figure 8A).

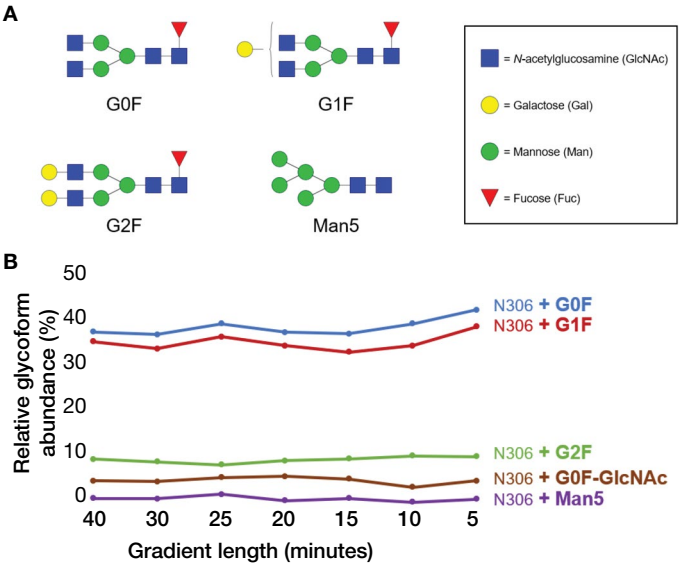


Figure 7. Comparison of relative *N*-linked glycosylation abundance within golimumab. A) The major glycoforms associated with golimumab and the majority of therapeutic mAbs. B) Relative *N*-glycosylation levels for the major glycoforms detected at N306, across all chromatographic gradients. Relative levels of *N*-glycosylation were determined using BioPharma Finder software.

Due to the inherent missed cleavages, the golimumab glycosylation site is represented by multiple peptide peaks, which complicates quantification of this PTM. The glycopeptide most abundantly observed in golimumab are the base glycopeptide (EEQYNSTYR) and the mis-cleaved TKPREEQYNSTYR peptide. While the different glycopeptides are chromatographically distinguishable even with a 5-minute gradient, the various glycoforms associated with each form of the glycopeptide are not readily separated from each other (Figure 8A). Informative MS^2 spectra were acquired from the more abundant species (Figures 8B and 8C), which produce characteristic fragment ion spectra, dominated at the low *m/z* range by glycan oxonium ions and by glycopeptide fragments at high *m/z* (Figure 8C). For a more thorough quantitative characterization of glycosylation, alternatives such as released glycan analysis or using a hydrophilic-based chromatographic separation is recommended. However, the approach demonstrated here is perfectly suitable for initial assessments of the more abundant glycopeptides.

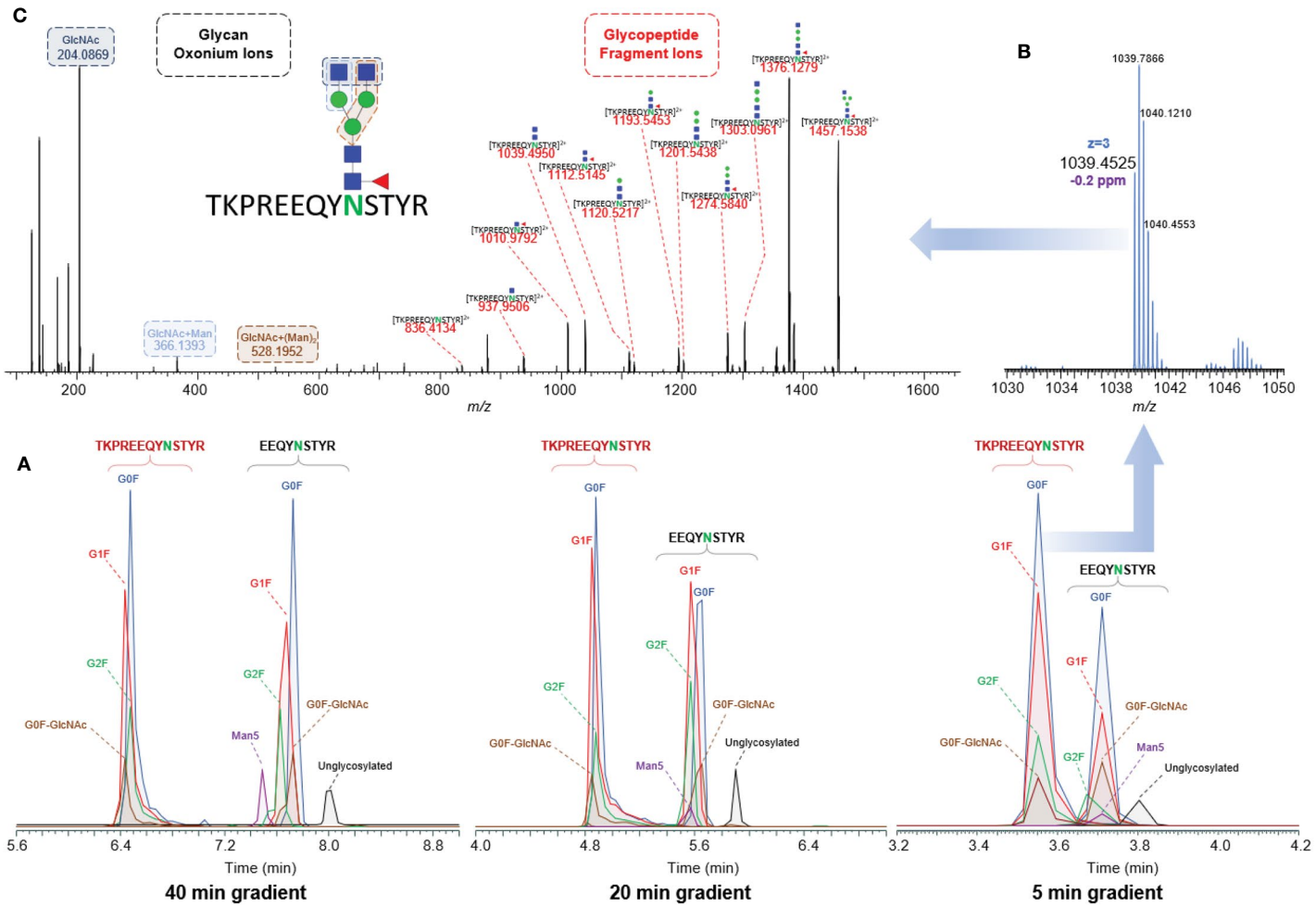


Figure 8. Detection of golimumab *N*-linked glycosylation at site N306. A) XICs for the major glycoforms detected on the two most abundant glycopeptides for the 40 minutes gradient (left), the 20 minute gradient (middle), and the 5-minute gradient (right); B) Zoomed sections of the full MS spectra, with mass accuracy for the glycopeptide; TKPREEQYNSTYR + G0F detected using the 5-minute gradient; C) MS^2 HCD fragment ion spectra of the TKPREEQYNSTYR + G0F glycopeptide highlighting the glycan oxonium ions that dominate the low *m/z* region and the glycopeptide fragments that dominate the higher *m/z* region.

Modification of the *N*- and *C*-termini

N-terminal pyroglutamate formation

Typically, the amino acids present at the *N*-termini of the HC and LC are either glutamine (Gln, Q) or glutamic acid (Glu, E). This is true for golimumab, which contains *N*-terminal Q and E residues on the HC and LC, respectively. Both of these residues may be susceptible to conversion to pyroglutamate (pyroGlu), resulting from cyclization of the amine in the side chain of Q and E residues (Figure 9A)—a process which may be enzymatically driven (catalyzed by glutaminyl cyclase)[7] or non-enzymatic in nature. The spontaneous non-enzymatic cyclization in recombinant protein production is believed to occur predominantly during the cell culture stage of the process, where the typical environment within bioreactors (primarily temperature and buffer conditions) facilitates the reaction. Up to 95% of this modification is thought to occur here.[8]

Golimumab contains near 100% pyroGlu at the *N*-terminus of the HC (Table 7), with approximately 0.1% remaining unconverted (Figure 9B). MS^1 and MS^2 spectra confirm the *N*-terminus with a Δmass of -17.0266 Da, indicating the loss of NH_3 from cyclization of the glutamine residue (Figures 9B and 9C). There was no detectable glutamic acid conversion to pyroGlu at position 1 of the LC (E1). This could be explained by the conversion to pyroglutamate from glutamic acid residues occurring at a much slower rate than for glutamine conversion.[9]

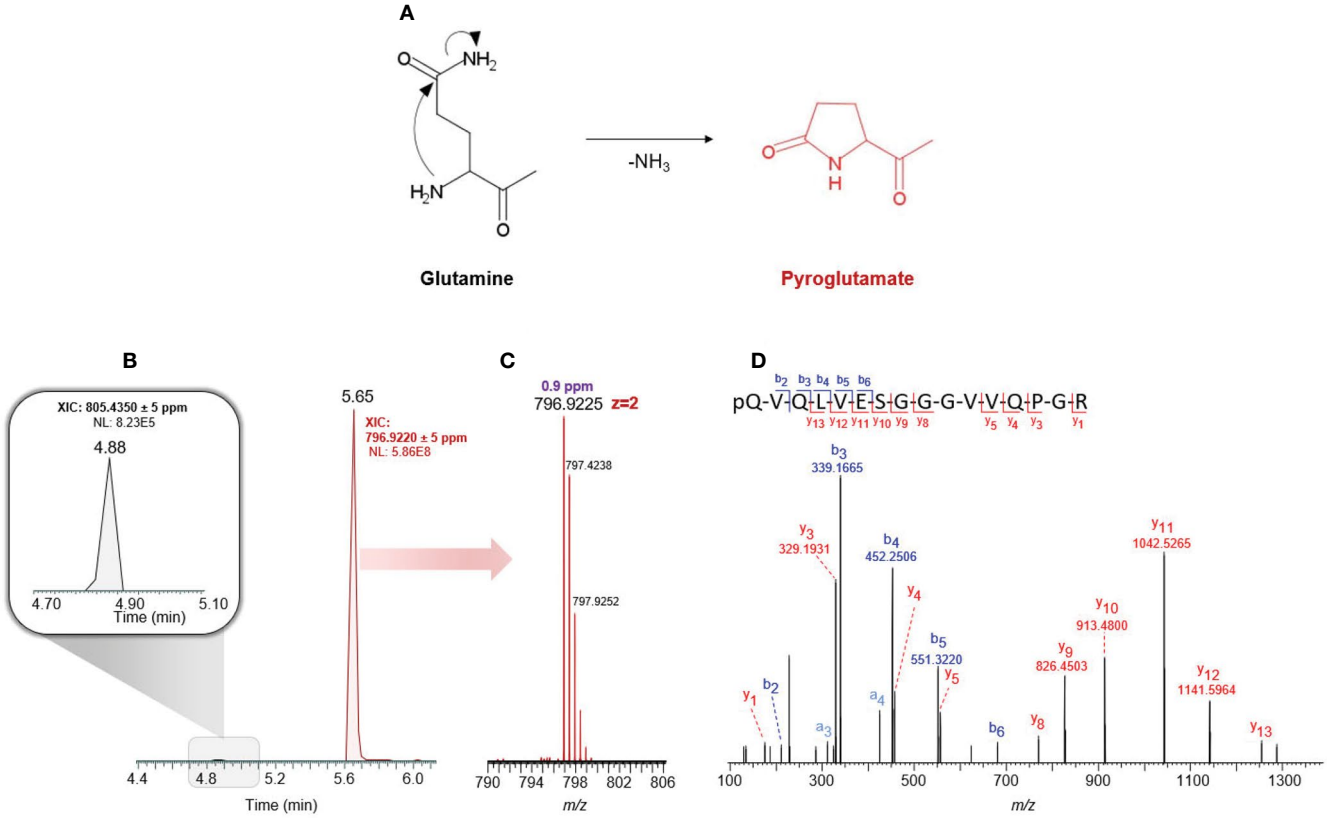


Figure 9. Confirmation of near 100% conversion of golimumab HC Q1 to pyroGlu. A) Process of glutamine conversion to pyroglutamate via a spontaneous, non-enzymatic driven cyclisation of the side chain amine group; resulting in a loss of NH_3; B) XICs for the glutamine containing (black) and pyroglutamate containing (red) *N*-terminal peptide of the golimumab heavy chain; C) Zoomed in region of the MS^1 spectrum of the pyroGlu containing peptide; D) MS^2 HCD fragment ion spectrum confirming presence of the *N*-terminal pyroGlu with excellent fragment ion coverage.

C-terminal lysine processing

Golimumab, like many other IgGs, is produced with a lysine residue at the C-terminus of both HC in the amino acid sequence Pro-Gly-Lys. Heterogeneity at the *C*-terminus occurs due to cleavage of the terminal lysine residue by intracellular carboxypeptidases.[10] Carboxypeptidase enzymes are present in Chinese hamster ovary (CHO) cells, and likewise with *N*-terminal pyroGlu formation, it is proposed that the majority of *C*-terminal lysine processing occurs within the bioreactor.

The degree of *C*-terminal clipping was consistently detected across all gradients in this study with an RSD ≤20% (Table 7). *C*-terminal lysine processing was even confirmed using the 5-minute gradient, where the characteristic shift in retention time between the *C*-terminal peptides, with and without lysine, was observed (Figure 10A). The relative abundance of each peptide can be inferred from the normalized intensity of the respective extracted ion chromatograms (XICs).

The lysine containing peptide exhibited a doubly charged MS^1 precursor, whereas the *C*-terminal clipped peptide was present as a singly charged precursor (Figure 10B). This is expected, as the absence of a charged amino acid at the *C*-terminal position of tryptic peptides often drives formation of a singly charged peptide ion. For this reason, the *C*-terminal clipped peptide was not selected for MS^2 fragmentation, as singly charged precursors were excluded from selection for fragmentation. This is an important point to consider when defining MS parameters should full characterization, including identification of the truncated *C*-terminus by MS^2, be required (Figure 10C).

Table 7. Summary of golimumab PTMs and their relative quantification across the different length chromatographic gradients

Golimumab chain	Modification	Position	Modification relative abundance (%) Gradient length (min.) 40	30	25	20	15	10	5	RSD
HC	*N*-terminal pyroGlu	Q1	100.0	99.7	100.0	100.0	99.8	99.5	99.9	0.2%
HC	Deamidation	N43 (Asp)	63.2	63.3	60.0	60.1	65.6	62.3	67.3	4.3%
HC		N370 (Asp)	0.5	0.6	0.4	0.4	0.5	1.0	0.5	38.3%
HC	Succinimide	N43 (Asu)	10.2	10.8	11.0	13.4	10.8	10.8	9.1	11.8%
HC	Oxidation	M51	5.1	6.5	5.6	4.9	6.1	6.2	7.2	13.6%
HC		M113	1.2	0.8	1.1	1.2	1.1	1.0	0.4	27.9%
HC		M261	99.9	99.9	100.0	99.9	99.9	99.9	99.9	<0.1%
HC		M437	49.4	50.3	48.4	51.1	51.5	49.4	51.1	2.3%
HC	*N*-glycosylation	N306 + G0F	38.5	37.9	40.4	38.4	38.1	40.4	43.4	4.9%
HC		N306 + G0F-GlcNac	5.9	5.8	6.6	6.9	6.3	4.5	5.9	12.9%
HC		N306 + G1F	36.4	34.8	37.5	35.5	34.1	35.5	39.7	5.2%
HC		N306 + G2F	10.7	10.1	9.4	10.3	10.7	11.4	11.2	6.4%
HC		N306 + Man5	2.1	2.0	3.0	1.6	2.0	1.3	1.9	26.6%
HC		Unglycosylated N306	2.3	2.7	-	2.7	3.0	-	-	n/a
HC	*C*-terminal lysine loss	K456	19.3	15.4	20.1	15.0	13.4	13.8	16.5	15.9%

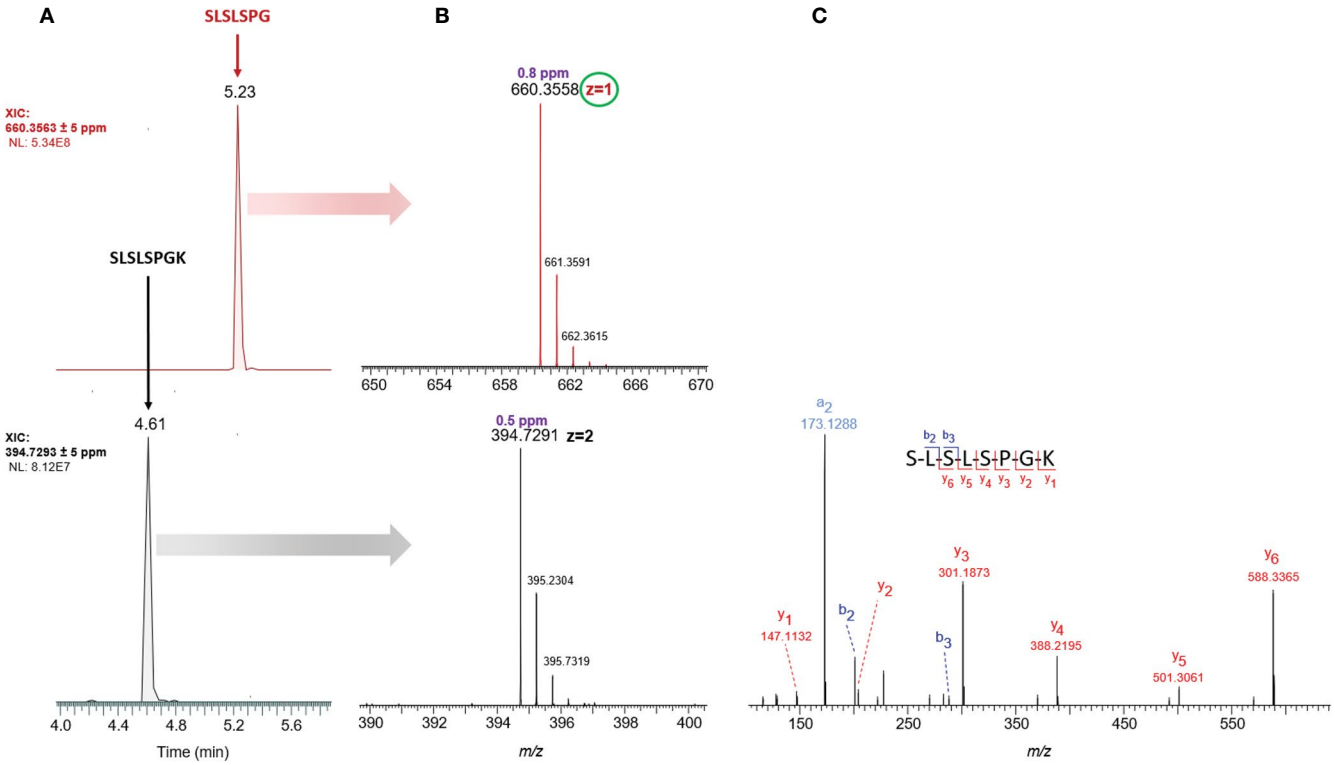


Figure 10. Confirmation of *C*-terminal lysine processing on golimumab HC. Comparison of lysine containing (bottom, black) and lysine-removed (top, red) *C*-terminal peptide of golimumab HC. A) Zoomed section showing XICs for the peptides; B) Zoomed in region of the MS^1 spectra. Note that the peptide where the *C*-terminal lysine is removed is exhibited as a singly charged precursor whereas the peptide containing the lysine is doubly charged; C) MS^2 HCD fragment ion spectrum of the lysine containing *C*-terminal peptide. The singly charged precursor for the peptide missing the terminal lysine was not selected for fragmentation as the method was set to only consider charge states 2–6.

Modifications of the *N*- and *C*-termini of therapeutic mAbs are commonplace and easily detectable by HRAM-MS, and this was confirmed in this study even using the 5-minute gradient. There is little evidence that either *N*-terminal pyroglutamate or *C*-terminal lysine heterogeneity have any significant clinical impact, and this is primarily due to their positioning within the molecule being spatially distant from functionally important regions.[11]

It is often the case that these terminal modifications are not necessarily deemed to be CQAs with respect to safety and functionality; near 100% conversion of the *N*-terminal Gln (or Glu) to pyroGlu is apparent in many clinically utilized mAbs with no reports of detrimental consequences, and what *C*-terminal heterogeneity is present on a recombinantly produced mAb is quickly removed following infusion into the patient by serum carboxypeptidase activity[11]. However, from a manufacturing perspective, they are still important modifications to consider, particularly if charge-based approaches are employed for analysis, as both will impact the overall charge profile of the product.

Any modification that occurs during biotherapeutic manufacturing is important and should be monitored. The quality of the final product is a direct indicator of the quality of the manufacturing process. Consistency in the impurity (both product- and process-related) profile of the final product highlights good manufacturing process control, which, in the production of recombinant biotherapeutics, is paramount. This is also particularly important for biosimilar manufacturers, where a principal aim is for the product be as "analytically indistinguishable" from the innovator as possible; in these instances, modifications such as *N*-terminal pyroGlu and *C*-terminal lysine heterogeneity may well be CQAs, so that regulatory justifications for analytical differences between product and innovator are less likely to be required.

Conclusion

- The combination of SMART Digest and KingFisher platforms allowed for fast, easy, and automatable protein digestion
- Excellent chromatographic performance of the Vanquish Horizon UHPLC system in combination with the Acclaim VANQUISH C18 (2.1 × 250 mm) UHPLC column provides robust and reproducible peptide mapping separations even with very short gradients.
- The increased scan speed of the Orbitrap Exploris 480 mass spectrometer facilitates data acquisition of fast UHPLC separations and is highly compatible with high-throughput peptide mapping.
- 100% sequence coverage of both golimumab HC and LC was achieved for gradients ranging from 5 to 40 minutes.
- Confident identification and relative quantification of commonly occurring PTMs even on the shortest gradients were achieved with consistent quantification of all detectable modifications across all gradients.
- The fast scan rate of the Orbitrap Exploris 480 mass spectrometer enabled acquisition and fragmentation of key PTMs, even for the challenging Δmass of 0.9848 Da for deamidation. The UHPLC separation of the deamidated and unmodified peptides in the 5-minute gradient was less than 6 s, but both were collected in separate MS scans for fragmentation, so the position of deamidation could be unequivocally determined and comfortably quantified.
- Glycosylation was the most challenging PTM, with low abundant species not consistently or confidently determined. Longer gradients offer little advantage in this case, and for in-depth glycan analysis, the methodology described here would not be used. However, this approach is still useful for high level glycoform profiling, which is desirable for process support.
- Using compliance-ready Chromeleon CDS software for secure data acquisition and storage and Biopharma Finder software for data analysis facilitates fast, comprehensive, and confident data processing along with clear, easy-to-understand data visualizations.

This work showcases the applicability of the Orbitrap Exploris 480 mass spectrometer in combination with the Vanquish UHPLC system and Acclaim VANQUISH C18 column for high-throughput peptide mapping analysis. This method can be used to support process development, due to its ability to identify and quantify key PTMs consistently. The level of detail attained for glycosylation is likely sufficient for clone selection and/or column screening activities.

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