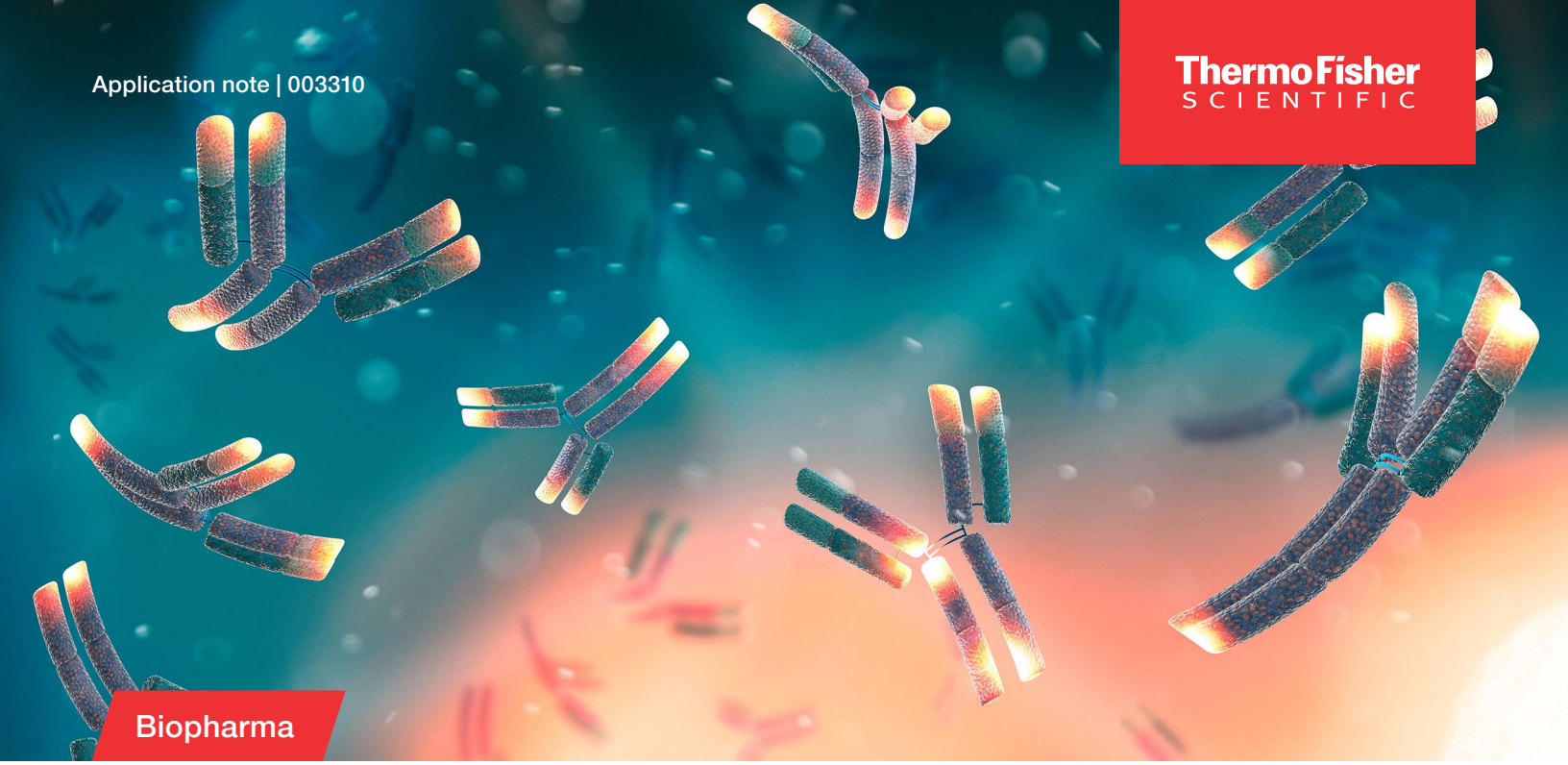

Monitoring of multiple quality attributes of intact monoclonal antibodies from bioreactors by switchable 2D-LC-MS

Authors

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Keywords

Process control, protein A, charge variants, size variants, cation exchange, size exclusion, SCX, SEC, heart-cut 2D-LC, intact protein

Application benefits

- Heart-cut 2D-LC with protein A affinity chromatography (ProA) of cell culture samples enables titer determination and purification for subsequent charge or size variant analysis.
- The Thermo Scientific™ ProPac™ 3R SCX column is a high-efficiency strong cation exchange (SCX) column and facilitates the separation of mAb charge variants by pH gradient or serves as a trap column to focus the purified mAb fraction prior to introduction into size exclusion chromatography (SEC).
- Volatile mobile phases in all dimensions enable direct coupling with mass spectrometry (MS) for mAb analysis at the intact level under native conditions, providing deep insights into glycan profiles, modifications, and aggregation throughout the production process.

Goal

Monitoring of multiple quality attributes, including mAb titer, glycation pattern, post-translational modifications (PTMs), charge variants, and aggregation of bioreactor samples

Introduction

Monoclonal antibodies (mAbs) play a key role in the biological therapeutics segment, which has become a rapidly growing portion of the pharmaceutical market. The biosynthetic production processes of biopharmaceuticals significantly differ from those of small molecule drugs and raise more complex analytical challenges, due to the inherent microheterogeneity. Amongst others, enzymatic and chemical post-translational modifications (PTMs) of the proteins lead to the presence of various co-existing molecules introduced at the different upstream and downstream stages of product development. These modifications can significantly impact the biochemical and biophysical properties of the drug product, such as activity, efficacy, and safety. Therefore, it is crucial to thoroughly characterize and monitor these variations as product quality attributes (PQAs) and indicators for process performance throughout the development and manufacturing chain, involving a broad range of analytical techniques like liquid chromatography (LC) and mass spectrometry (MS). Two of the most important PQAs of mAbs are their charge variant and aggregate profiles. Charge variants, for example, arise from PTMs such as amino acid deamidation, oxidation, glycosylation, or C-terminal lysine truncation, which are commonly analyzed by ion-exchange chromatography (e.g., SCX chromatography). Size variants of mAbs result from fragmentation or aggregation into dimers or oligomers and can be separated by SEC.

Traditionally, analytical assays for mAbs in quality and process control have relied on LC coupled to optical detection techniques, while the usage of MS was mainly reserved for research and development laboratories. These analytical LC methods commonly employed for protein characterization utilize non-volatile buffers with high salt concentrations, which are not compatible with MS detection. However, there is a growing interest and demand in direct coupling of various LC techniques with MS for deeper characterization and more comprehensive analysis to gain a better molecular understanding of the separated components.[1-3]

In this application note, we introduce a novel approach using a switchable multi-method two-dimensional LC (2D-LC) platform coupled to a high-resolution accurate mass MS (HRAM MS) approach, tailored for detailed monitoring of PQAs in the upstream processing of biotherapeutics. Figure 1 summarizes the study setup, which started with method development using NISTmAb reference material (NIST™, RM 8671; expressed in a murine cell system), followed by the analysis of a NISTmAb analogue (cNISTmAb) expressed in and harvested from Chinese hamster ovary (CHO) cell culture (HCC). Cell culture supernatants were sampled from a bioreactor over the course of 10 days and directly analyzed on a switchable multi-method 2D-LC-MS platform. Specifically, the first dimension (^{1}D) employed ProA affinity chromatography for the titer determination of the produced mAb and for its simultaneous separation from host cell and media material. The purified mAb was subsequently transferred via a heart-cut loop to a short, high-efficiency SCX column in the second dimension (^{2}D). The SCX column was either eluted by a pH gradient for charge variant analysis (CVA) or switched in-line with an SEC column for size variant analysis.

The combination of multiple LC techniques into a single workflow enabled the simultaneous assessment of quality attributes and improved the analytical efficiency. The thorough development of LC methods fully compatible with MS across all dimensions facilitated direct hyphenation with MS, providing more comprehensive insights into glycan profiles, modifications, and aggregates throughout the biopharmaceutical production process by analyzing the intact mAb under native conditions.

Experimental

Chemicals

- Deionized water, 18.2 MΩ·cm resistivity or higher, from a Thermo Scientific™ Barnstead™ GenPure™ xCAD Plus Ultrapure Water Purification System (P/N 50136149)
- Acetic acid, Optima™ LC/MS, Fisher Chemical™ (P/N A113-50)
- Ammonium acetate (NH_4OAc), Optima™ LC/MS, Fisher Chemical™ (P/N A114-50)
- Ammonium bicarbonate (NH_4HCO_3), 99%, for analysis, Thermo Scientific Chemicals (P/N 393212500)
- Ammonium hydroxide (NH_4OH), extra pure, 5 wt%, 1 L, Thermo Scientific Chemicals (P/N 15400815)
- NISTmAb RM 8671 (P/N 8671 (NIST))

Sample handling

- Thermo Scientific™ Orion™ 3 Star pH Benchtop Meter (P/N 13-644-928)
- Thermo Scientific™ SureSTART™ 2 mL Short Thread Screw Glass Vials + Caps, Level 3, amber (P/N 6PCK575W)

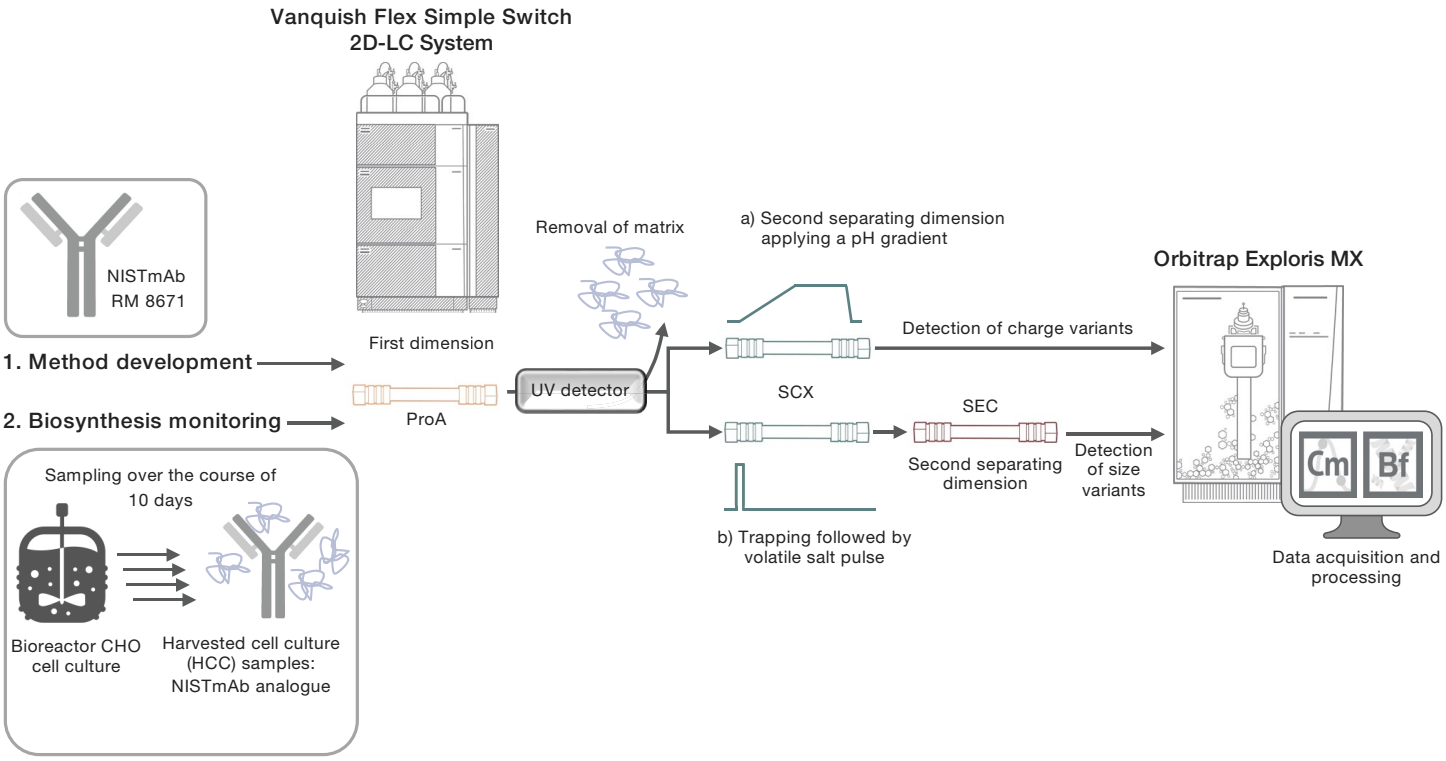


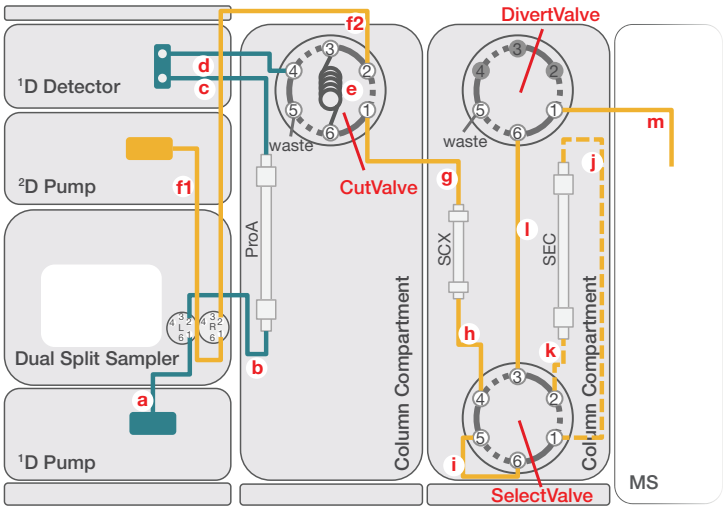
Figure 1. Overview of the experimental study design. In step 1, NISTmAb standard (reference material - RM 8671) was used for method development and optimization. In step 2, NISTmAb analogue expressed in CHO cells and sampled straight from the bioreactor over the course of 10 days was analyzed.

Instrumentation

A Thermo Scientific™ Vanquish™ Flex Simple Switch™ 2D-LC system for loop heart-cutting was used, enabling the transfer of a fraction from a ^{1}D separation to a ^{2}D separation. In the ^{2}D, two separation mechanisms were selectable. The system consisted of:

- Vanquish System Base Horizon/Flex (P/N VF-S01-A-02)
- Vanquish Binary Pump F (^{2}D pump) (P/N VF-P10-A-01)
- Vanquish Quaternary Pump F (^{1}D pump) (P/N VF-P20-A)
- Vanquish Dual Split Sampler FT (P/N VF-A40-A-02)
- Sample loop, biocompatible, MP35N, 1,000 µL (left injection unit, ^{1}D) (P/N 6850.1980)
- Sample loop, biocompatible, MP35N, 100 µL (right injection unit, ^{2}D) (P/N 6850.1918)
- 2x Vanquish Column Compartment H (P/N VH-C10-A-03)
- 3x VH-C Valve 2-p 6-p 150 MPa bio (P/N 6036.2520)
- Sample loop, MP35N, 250 µL (P/N 6823.0030)
- Vanquish Diode Array Detector FG (^{1}D detector) (P/N VF-D11-A-01)
- Flow cell, semi-micro biocompatible, 7 mm, 2.5 µL (P/N 6083.0550)
- Thermo Scientific™ Orbitrap Exploris™ MX Mass Detector (P/N BRE725536) with BioPharma option (P/N BRE725539)

The setup, including fluidic connections, is depicted in Figure 2. In case a direct injection to the ^{2}D is not required and the system is solely used in 2D-LC-MS mode, the installation of a Vanquish Split Sampler FT (P/N VF-A-10-A-02) might be preferred.



#	Connection	Part Number
a	Thermo Scientific™ Viper™ Capillary, ID × L 0.10 × 550 mm, MP35N	6042.2360
b	Active Pre-heater, ID × L 0.1 × 380 mm, MP35N*	6732.0110
c	Viper Capillary, ID × L 0.10 × 450 mm, MP35N	6042.2350
d	Viper Capillary, ID × L 0.10 × 350 mm, MP35N*	6042.2340
e	Loop 250 µL, MP35N	6823.0030
f	Viper Capillary, ID × L 0.1 × 450 mm, MP35N**	6042.2350
f1	Viper Capillary, ID × L 0.1 × 550 mm, MP35N	6042.2360
f2	Viper Capillary, ID × L 0.1 × 550 mm, MP35N	6042.2360
g	Active Pre-heater, ID × L 0.1 × 380 mm, MP35N	6732.0110
h	Viper Capillary, ID × L 0.10 × 250 mm, MP35N	6042.2330
i	Viper Capillary, ID × L 0.10 × 65 mm, MP35N	6042.2306
j	Viper Capillary, ID × L 0.10 × 550 mm, MP35N	6042.2360
k	Viper Capillary, ID × L 0.10 × 150 mm, MP35N	6042.2320
l	Viper Capillary, ID × L 0.1 × 650 mm, MP35N	6042.2370
m	Viper Capillary, ID × L 0.1 × 350 mm, MP35N	6042.2340
	2x Waste line, VH-D1	6083.2425

Figure 2. Fluidic scheme of the 2D-LC-MS setup for ProA-SCX-MS and ProA-SCX-SEC-MS analysis. Blue and yellow lines mark the ^{1}D and ^{2}D flow paths, respectively. Solid yellow lines indicate the ^{2}D flow path for ProA-SCX-MS runs, while the dotted yellow lines add to the ^{2}D flow path for ProA-SCX-SEC-MS runs.

* Included in System Base Ship Kit VF-S01-A-02

** Replaces f1 and f2 if Split Sampler is installed instead of Dual Split Sampler

Samples

NISTmAb RM 8671 was used for method development and titer calibration. Calibration standards were prepared with concentrations ranging from 0.1 mg/mL to 1.0 mg/mL by diluting a 10 mg/mL stock solution with fresh culture media.

The target mAb was a NISTmAb analogue expressed by a new CHO cell line (NISTCHO, NIST Research Grade Test Material (RGTM) 10197), here referred to as cNISTmAb.[4] HCC samples and corresponding reference culture media were provided by the National Institute for Bioprocessing Research and Training (NIBRT), Ireland. One 10 mL sample per day was collected from each of two lab-scale bioreactors operated in parallel over the course of 10 days, filtered using 0.45 µm and 0.2 µm PVDF filters and stored at -20 °C until analysis.

Chromatographic conditions

Table 1. Chromatographic conditions of ^{1}D: ProA purification for matrix removal

Parameter	Value
Column	Thermo Scientific™ MAbPac™ Protein A Antibody Analysis and Purification HPLC Column, 12 µm, 4 × 35 mm (P/N 082539)
Mobile phase	A: 50 mM NH_4OAc in water, pH 7.0 B: Acetic acid in water, pH 2.5
Flow rate	0.5 mL/min (0.1 mL/min from 14.0 min)
Injection volume	10–1,000 µL
Gradient	Time (min) % B 0.0 0 3.5 0 3.5 100 8.0 100 8.0 0 26.0/31.0 0
Column temperature	25 °C (still air)
Autosampler temperature	5 °C
UV detection	280 nm, 5 Hz, 1 s response time

Table 2. Chromatographic conditions of ^{2}D SCX: separation of charge variants using a pH gradient (path a in Figure 1)

Parameter	Value
Column	Thermo Scientific™ ProPac™ 3R SCX, 2 × 50 mm, 3 µm (P/N 43103-052068) with Viper inline filter (P/N 6036.1045)
Mobile phase	A1: 25 mM NH_4HCO_3 + 30 mM acetic acid in water, pH 5.3 B1: 10 mM NH_4OH in water, pH 10.9
Flow rate	0.2 mL/min
Injection volume	^{1}D fraction volume
Gradient	Time (min) % B1 0.0 82 11.0 82 21.0 95 21.0 100 23.0 100 24.0 82 26.0 82
Column temperature	30 °C (still air incl. active eluent preheating)

Table 3. Chromatographic conditions of ^{2}D SCX-SEC: SCX trapping, transfer to and separation of size variants by SEC (path b in Figure 1)

Parameter	Value
Column	Thermo Scientific™ MAbPac™ SEC-1, 5 µm, 4 × 300 mm (P/N 074696)
Trap column	Thermo Scientific™ ProPac™ 3R SCX, 2 × 50 mm, 3 µm (P/N 43103-052068)
Mobile phase	A2: 150 mM NH_4OAc in water, pH 5.5 B2: 500 mM NH_4OAc in water
Flow rate	0.2 mL/min
Injection volume	^{1}D fraction volume
Gradient	Time (min) % B2 0.0 0 8.0 0 8.0 100 9.0 100 9.0 0 31.0 0
Column temperature	30 °C (still air incl. active eluent preheating)

MS settings

Table 4. Parameter settings of the Orbitrap Exploris MX mass detector equipped with the BioPharma option

Parameter	Value
Method type	Full MS
Scan range (*m/z*)	2,500–8,000
Orbitrap resolution	60,000
RF lens (%)	175
Normalized AGC target (%)	25
Maximum injection time	200
Microscans	10
Source fragmentation energy (V)	120
Source settings	
Spray voltage (+)	4,200
Polarity	Positive
Ion transfer tube temperature (°C)	275
Sheath gas (Arb)	25
Aux gas (Arb)	10
Sweep gas (Arb)	0
Vaporizer temperature (°C)	175

To decrease the overall cycle time of the 2D-LC runs, overlapping injection preparation was applied by the *PrepareNextInjection* command in the Chromeleon script editor. The configuration file, instrument methods, and ePanel are available in the download section of the Thermo Scientific™ AppsLab™ Library of Analytical Applications entry.

Valve configurations

Table 5. ProA-SCX valve configuration

Time [min]		CutValve*	SelectValve**	DivertValve†
0.0	^{1}D ProA separation, no heart-cutting	6_1	1_2	1_2
7.6	^{1}D ProA separation, fraction heart-cut	1_2		
8.0	^{2}D SCX separation to waste	6_1		
12.0	^{2}D SCX separation to MS			6_1
26.0	^{2}D SCX separation to waste			1_2

Table 6. ProA-SCX-SEC valve configuration

Time [min]		CutValve*	SelectValve**	DivertValve†
0.0	^{1}D ProA separation, no heart-cutting	6_1	6_1	1_2
7.6	^{1}D ProA separation, fraction heart-cut	1_2		
8.0	^{2}D SCX-SEC separation to waste	6_1		
19.0	^{2}D SCX-SEC separation to MS			6_1
31.0	^{2}D SCX-SEC separation to waste			1_2

*Upper valve/left column compartment, **lower valve/right column compartment, †upper valve/right column compartment

Chromatography and MS data system

The Orbitrap Exploris mass detector was controlled by the Thermo Scientific™ Orbitrap Exploris Series 4.2 SP4 Instrument Control Software. The Thermo Scientific™ Chromeleon™ 7.3.2 Chromatography Data System (CDS) was used for data acquisition, deconvolution, and data analysis. Thermo Scientific™ BioPharma Finder™ software version 5.2 was used for MS data deconvolution and evaluation.

Data processing

For intact protein deconvolution and protein sequence matching in BioPharma Finder software, MS raw data were exported from Chromeleon CDS. Protein sequences were set up based on the NISTmAb amino acid sequence, with fixed modifications for conversion of N-terminal glutamine to pyro-glutamic acid, loss of C-terminal lysine for the heavy chain, and presence of 16 disulfide bridges. Five sequences were created for the main glycoforms observed on the NISTmAb standard with fixed glycan modifications of G0F, G1F, and G2F and up to two variable modifications (Lys, glycans).

After deconvolution and sequence matching, an Intact Protein Workbook containing the major glycoforms was exported from BioPharma Finder software to a Chromeleon CDS processing method. Target quantification was achieved considering the six most abundant charge states for each component, characterized by relative *m/z,* retention time (RT), and a defined RT window.

A manually defined mass tolerance of 0.3 AMU was used for each *m/z*, and a smoothing of 7 point moving average was applied.

Additional charge variants were detected by multiconsensus deconvolution. For quantification the respective components were conveniently transferred from the intact protein deconvolution results in Chromeleon CDS to the processing method.

Table 7. Deconvolution parameter settings used in Chromeleon CDS and BioPharma Finder software

Parameter	Value
Algorithms	ReSpect™ (for isotopically unresolved spectra); Sliding Windows
m/z range	2,500–8,000
RT range	18.0–26.0 min
Target avg. spectrum width	0.5 min
Target avg. spectrum offset	25%
Merge tolerance	10 ppm
Max. RT gap	0.5 min
Min. number of detected intervals	2
Output mass range	140,000–160,000 Da
Model mass range	10,000–160,000 Da
Deconvolution mass tolerance	20 ppm
Multiconsensus component mass tolerance	20 ppm
Multiconsensus component RT tolerance	0.5 min

Results and discussion

The objective of this study was the development of a 2D-LC-MS method setup suitable for monitoring of quality attributes of an antibody drawn straight from a bioreactor. 2D-LC was utilized for separation followed by MS detection for quantitative monitoring of individual glycoforms. For method development, the well characterized NISTmAb reference standard was used before applying the optimized setup for the analysis of a NISTmAb analogue (cNISTmAb) expressed in and harvested from two CHO cell cultures grown in parallel over the course of a 10-day period.

2D-LC-MS hardware setup, method development, and fraction transfer

The development of the 2D-LC-MS methods involved the utilization of a Vanquish 2D-LC system, which allowed for the alternative operation of two standard 1D-LC/1D-LC-MS separations in the dual fluidic paths. The Vanquish Dual Sampler allows for the direct sample injection into the ^{2}D fluidic path, enabling the application of both 2D-LC-MS and 1D-LC-MS without replumbing, which facilitated straightforward method optimization. Three columns were applied in this study: MAbPac Protein A column for sample cleanup and removal of host cell material, ProPac 3R SCX column for separation of charge variants, and MAbPac SEC-1 column for the separation of size variants with mobile phase systems based on recent publications with minor modifications.[1,2,5] The buffer systems preserved the protein´s near-native state, resulting in a series of lower charge states upon ionization in the MS ion source compared to analyses under denaturing conditions. The Orbitrap Exploris MX mass detector equipped with the BioPharma option provided the mass range required for mAb analysis under native conditions (up to *m/z* 8,000), along with robust and reliable performance. NISTmAb RM was used for the development and optimization of all LC and MS method parameters.

Titer calibration curve: For the titer calibration of the ProA dimension, a linear calibration curve was prepared by injecting 1–20 µg of NISTmAb RM onto the column. Excellent linearity with a coefficient of determination R^2=0.99913 was obtained (data not shown).

Heart-cut fractionation: The mAb fraction purified by ProA in the ^{1}D was transferred via the 250 µL heart-cut loop to the ^{2}D by switching the CutValve after UV detection (time-triggered). To reduce sample breakthrough and sample loss due to the parabolic concentration profile of the fraction,[6] the maximum collected volume was set to 175 µL in the Chromeleon Fraction Collection parameters, and the ProA column was run at its lowest recommended flow rate (0.5 mL/min). The transfer of a 175 µL fraction resulted in an effective transfer of around 85% of mAb material to the ^{2}D compared to only 60% material when 250 µL were transferred.

Switchable ^{2}D separation: The mAb contained in the heart-cut fraction was subsequently captured by the short, high-efficiency ProPac 3R SCX column. As depicted in Figure 1, the SCX column was used for both charge and size variant analysis. When a CVA was run in the ^{2}D (Figure 1, path a), the SelectValve was in position 1_2, so that only the SCX column was in the ^{2}D pump flow (solid yellow lines in Figure 2), and a pH gradient was applied to separate acidic variants, main peak, and basic variants of the mAb. When conducting a size variant analysis in the ^{2}D (Figure 1, path b), the SelectValve was in position 6_1 to switch the ProPac 3R SCX and the MAbPac SEC-1 column in-line (solid and dotted yellow lines in Figure 2). Here, the mAb trapped on the SCX column was eluted by a volatile salt pulse in a narrow band to the SEC column. Since SEC sample bands cannot be re-focused at the column head, low band dispersion is extremely critical for adequate separation. The trap/elute procedure, with the SCX column positioned in front of the SEC column (ProA-SCX-SEC), significantly improved both peak shape and peak width compared to a direct fraction transfer to the SEC column (ProA-SEC), as shown in Figure 3.

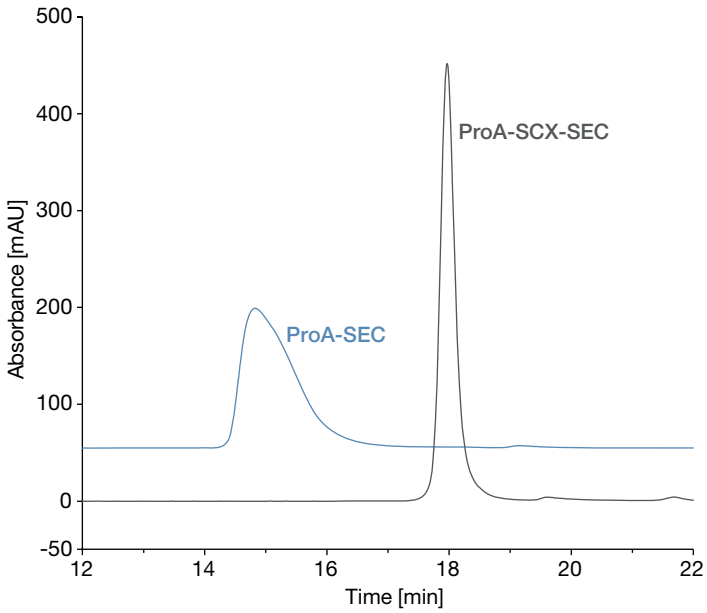


Figure 3. UV chromatograms obtained from ProA-SEC (blue) and ProA-SCX-SEC (black) setups, demonstrating significant improvement of peak shape and width by the ProA-SCX-SEC procedure

[1]D affinity separation of HCC samples

The strong affinity of protein A from *Staphylococcus aureus* for immunoglobulin G antibodies makes it the most commonly applied method for mAb purification. At neutral pH, the mAb binds to the [1]D MAbPac Protein A column, while matrix components are flushed out. A step gradient to acidic conditions elutes the mAb for UV detection. Figure 4 shows a representative [1]D chromatogram obtained from an HCC sample indicating the collected heart-cut fraction for the transfer to the [2]D.

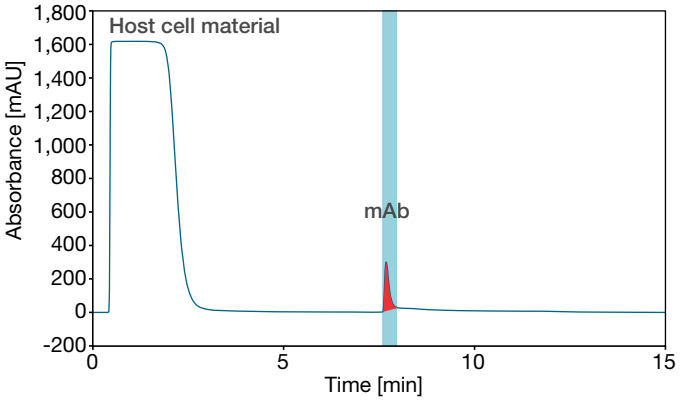


Figure 4. [1]D ProA-UV chromatogram obtained from the HCC sample drawn on day 5 with heart-cut fraction indicated (blue shading)

As the mAb concentrations of the HCC samples were very low, volumes of 250 µL (late days) to 1,000 µL (early days) had to be injected to obtain proper UV signals within the calibrated range (1–20 µg). The highly reproducible RT of the ProA separation with a standard deviation of only 0.008 min over 20 samples fulfilled the prerequisite for a reliable and reproducible time-triggered heart-cutting procedure. The cNISTmAb titer results of the HCC samples over the 10-day period are summarized in Figure 5, starting at 7 µg/mL on day 1 and increasing up to 52 µg/mL on day 10.

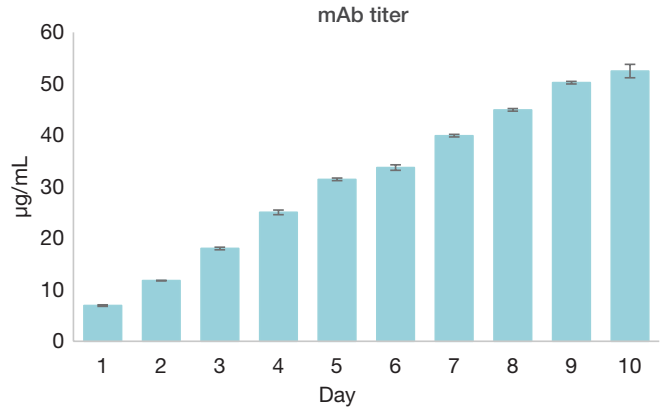


Figure 5. mAb titer determination of HCC samples from [1]D ProA-UV. Samples were drawn from the bioreactors over the course of 10 days and analyzed via ProA-SCX separation. Bars represent the titer averaged over the two samples drawn per day from each of the two cultures, with error bars indicating the maximum and minimum value.

[2]D charge variant analysis of HCC samples via ProA-SCX

As outlined before, our setup facilitated two options for the downstream analysis of the ProA-purified mAb obtained from the [1]D heart-cut, with the first one being a CVA using a SCX column. A pH gradient was applied for the separation of charge variants and subsequent mass detection of the intact protein species, providing chromatograms and mass spectral raw data to derive the intact masses via deconvolution.

The total ion chromatograms (TIC) obtained from the HCC samples show a broad elution and a complex mass profile, typical for samples drawn straight from a bioreactor followed only by ProA purification. Figure 6 displays the comparison of the chromatograms and deconvoluted mass spectra obtained from the bioreactor sample on day 6 (Figure 6 C, D) versus the pre-purified, clean NISTmAb standard (Figure 6 A, B). The spectra were obtained from the RT range 18–26 min, respectively, using the ReSpect algorithm in combination with the sliding windows feature in BioPharma Finder software and illustrate the considerably higher complexity of the HCC derived sample.

The spectrum representing NISTmAb RM is dominated by the five major glycoforms in addition to some lower abundant basic variants with C-terminal lysine residues,[7] while in contrast the HCC cNISTmAb sample spectrum shows many more high and medium abundant variants. The presence and highest abundance of the glycoforms G0F_G0F, G0F_G1F, G0F_G2F/G1F_G1F, and G1F_G2F under the main peak was also confirmed in the bioreactor mAb. (Note: G1F_G1F cannot be distinguished from G0F_G2F at the intact protein level as the masses are identical.) A match for the mass of the G2F_G2F glycoform was consistently present, but the apexes of the deconvoluted peaks appeared at slightly acidic RTs, indicating a different identity (e.g., a glycation variant). A mass shift of ~2–3 Da of the masses obtained upon deconvolution of the HCC sample derived spectra compared to the NISTmAb data may be attributed to the complexity and significant overlap of different variants or may also indicate some alteration of the cNISTmAb product, for example by deamidation, partial reduction or presence of trisulfide bonds. For reliable identification and assignment of additional glycoforms, peptide mapping experiments from the sample are indispensable.

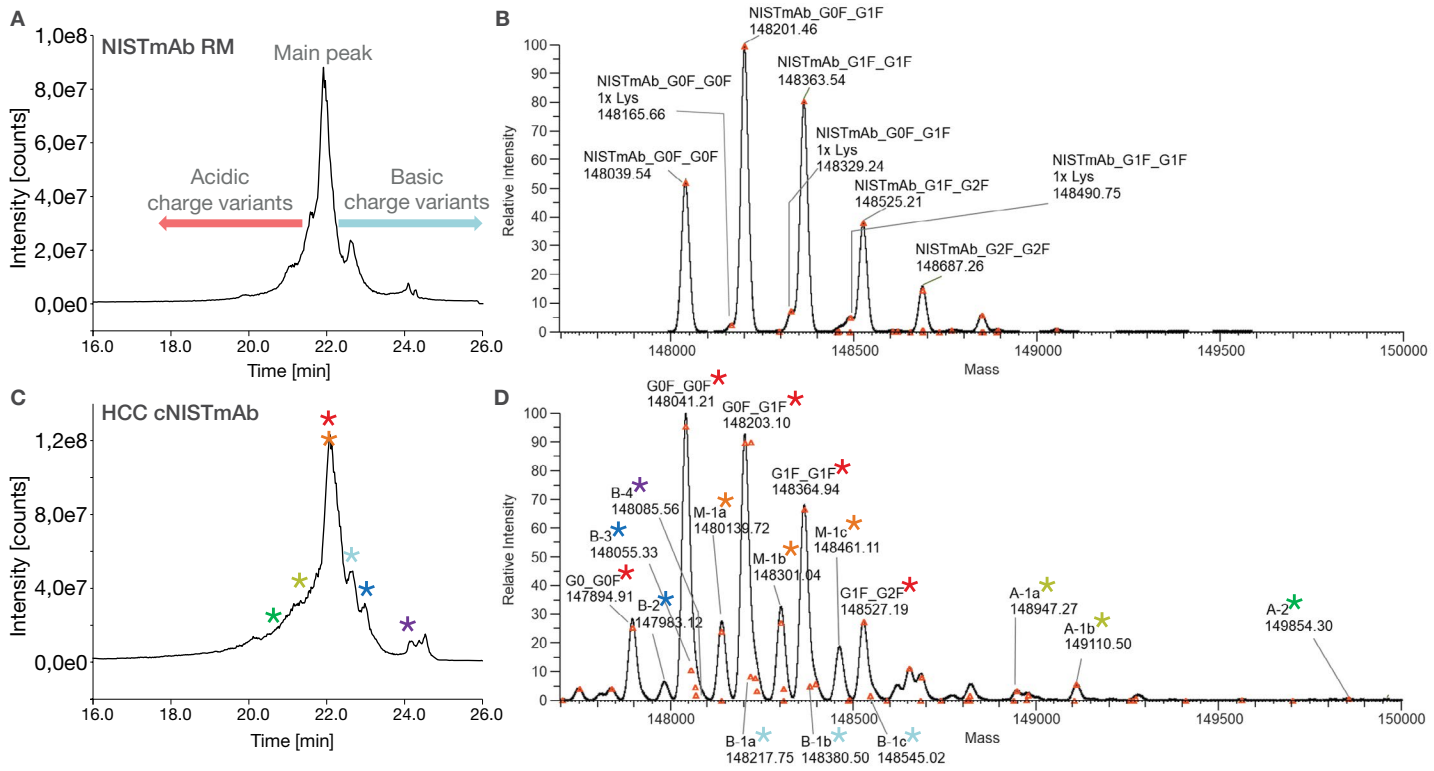


Figure 6. Comparison of TIC and deconvoluted mass spectra obtained from NISTmAb RM (A, B) vs. HCC cNISTmAb sample on day 6 (C, D). Asterisks in panel C indicate respective retention of species in correlation to deconvoluted intact masses in panel D

Targeted quantitation of glycoforms: The quantitation of the four major glycoforms in the HCC samples was performed with Chromeleon CDS based on extracted ion chromatograms of the six most abundant charge states of each compound. As the sample injection volume was modified over the 10 days, results in Figure 7 are presented in relative ratios. G0F_G1F was the most abundant form over the first eight days making up for 35–40%.

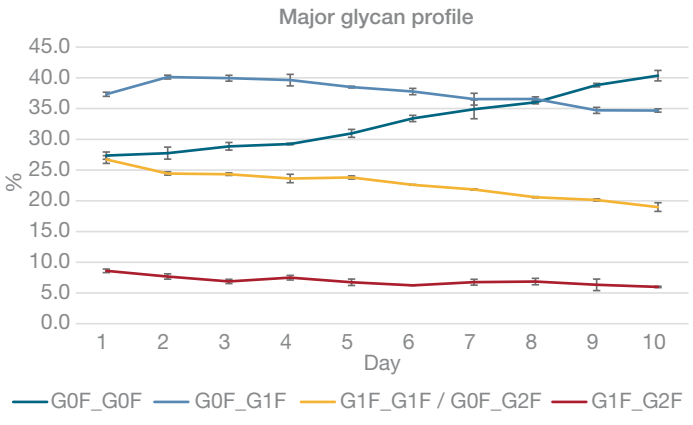


Figure 7. Development of main glycoform ratio over 10 days, determined with Chromeleon CDS

On day 1 and 2, the G0F_G0F and G1F_G1F glycoforms were on an equivalent level of around 27%. While the abundance of the G1F_G1F isoform steadily decreased down to 19% by day 10, the abundance of G0F_G0F increased to 40%, emerging as the most abundant one on day 9 and 10. The abundance of the G1F_G2F form decreased from 9% to 6%.

Additional charge variants: Multiconsensus processing of the CVA sample data using all data files collected over the time course revealed several other abundant masses of produced mAb variants, as also apparent from Figure 6D. For abundance trend analysis over the bioproduction process, quantification of some prominent unknown isoforms was conducted in Chromeleon CDS by conveniently transferring the new components from the intact protein deconvolution results into the processing method. Figure 8 displays the Chromeleon quantitation panels with all variants considered in this study. The variants area ratios relative to the generally highest abundant glycoform G0F_G1F are showcased in Figure 9, along with a summary of tentative identifications as discussed in the following. The corresponding deconvoluted intact protein masses and RTs are indicated in Figures 6C and 6D.

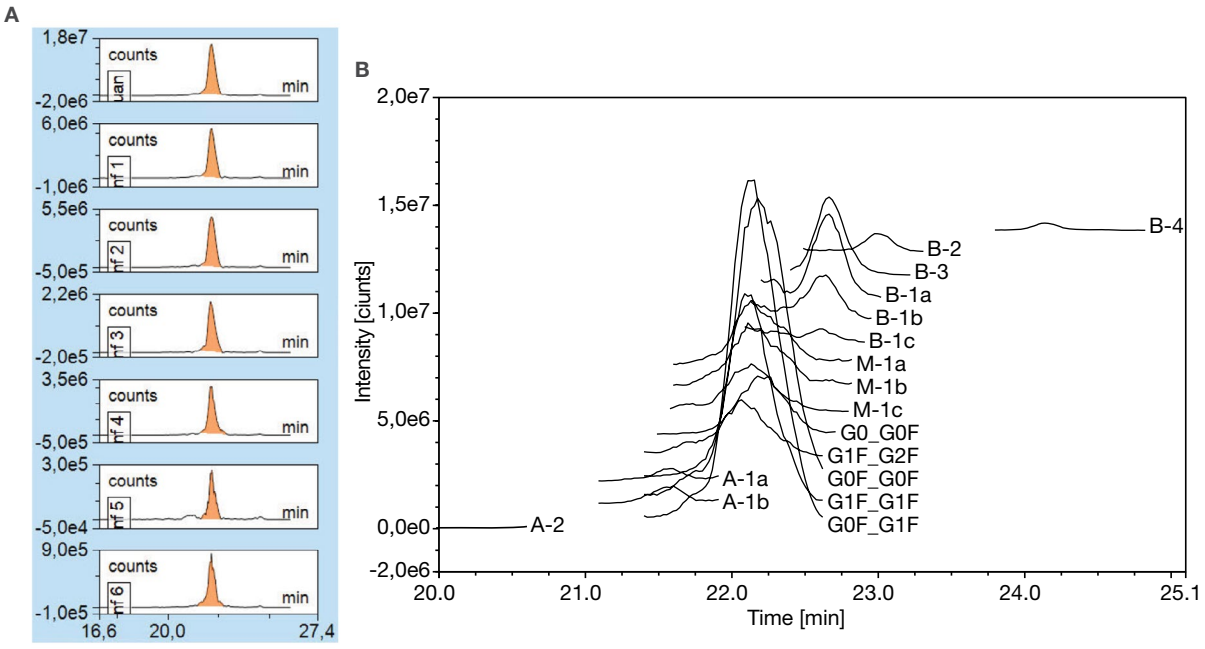
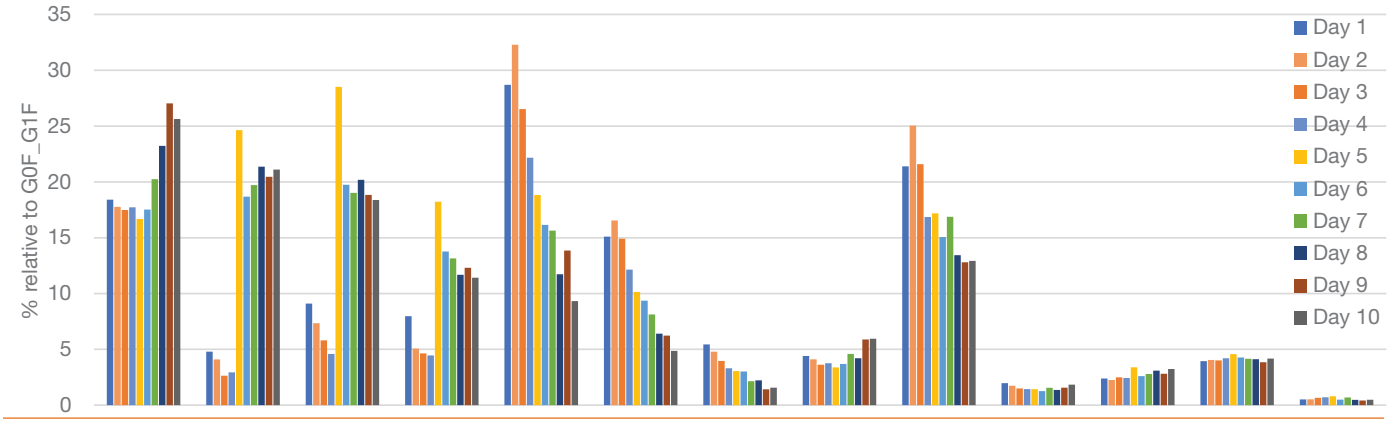


Figure 8. Quantitation processing of charge variants in Chromeleon CDS. Left panel: MS component view of the extracted ion chromatograms for G0F_G1F (six most abundant charge states (confirmation 1 to 6) and their sum in the top trace (quantifier)). Right panel: MS quantitation channel with all variants added to the processing method applied.



Variant	G0_G0F	M-1a G0F_G0F	M-1b G0F_G1F	M-1c G1F_G1F	B-1a G0F_G0F	B-1b G0F_G1F	B-1c G1F_G1F	B-2	B-3	B-4	A-1a	A-1b	A-2
Mass Δ	G0F_G0F - 146 Da	G0F_G0F + 98 Da	G0F_G1F + 98 Da	G1F_G1F + 98 Da	G0F_G0F + 177 Da	G0F_G1F + 177 Da	G1F_G1F + 177 Da	G0F_G0F - 58 Da		G0F_G1F - 116 Da	G0F_G0F + 906 Da	G0F_G1F + 906 Da	G0F_G0F + 1813 Da
Tentative identity	G0_G0F	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	1x glycine loss and proline amidation	Unknown	2x glycine loss and proline amidation	G0F_G2S 2F, G1S1F_G 1S1F	G1F_G2S 2F; G1S1F_G 2S1F	G2S2F_G 2S2F

Figure 9. Abundance trends of unknown isoforms obtained with Chromeleon CDS from CVA monitored over 10 days of cNISTmAb production and appended summary of mass deltas and tentative identifications. Font colors of table header correlate with the asterisk colors in Figure 6.

A mass with considerably high abundance and a mass delta of -146 Da compared to the G0F_G0F variant was observed under the main peak and tentatively identified as the G0_G0F glycoform. Its level relative to G0F_G1F was constant from day 1 to 6 (≈18%) but increased afterwards up to 27%. Also eluting within the main peak, three unknown variants M-1a, M-1b, and M-1c (orange asterisks in Figure 6) with an average mass delta of +98 Da with respect to the G0F_G0F, G0F_G1F, and G1F_G1F forms were detected. Very similar abundance trends were seen for all of them, with low ratios (3–9%) in the first four days, followed by an increase on day 5, and levelling at around 20%/12% in the second half of the process.

Three variants B-1a, B-1b, and B-1c with an average delta mass of +177 Da with respect to the G0F_G0F, G0F_G1F, and G1F_G1F isoforms eluted within the first basic peak (light blue asterisks in Figure 6). They all showed a similar trend over the bioproduction process, each decreasing to around one third of their initial abundance on day 10 (B-1a from 30% to 9%, B-1b from 15% to 5%, B-1c from 5% to 1.5%). No distinct identity could be proposed at this point for M-1a, M-1b, M-1c, B-1a, B-1b, and B-1c. The mass patterns suggested one neutral (+98 Da) and one basic (+177 Da) PTM of the G0F_G0F, G0F_G1F, and G1F_G1F glycoforms. Within the second basic peak, two unknown variants B-2 and B-3 were found (blue asterisks in Figure 6), B-2 with a constant amount of 4–6% and B-3 decreasing from more than 20% down to 13%. With a mass delta of -58 Da with respect to the G0F_G0F form, B-2 might be tentatively identified as a proteoform with C-terminal glycine loss and proline amidation. Variant B-4 (purple asterisk in Figure 6) showed a constant abundance of around 1.6%. The very late elution as well as a mass delta of -116 Da with respect to the G0F_G1F glycoform indicate glycine loss/proline amidations at both heavy chains.[8]

Interestingly, no matches for C-terminal lysine variants were found in the basic RTs of the HCC samples, which is most probably due to the different expression systems (CHO cells for cNISTmAb vs. murine cells for NISTmAb RM).

In the acidic retention range, two variants, A-1a and A-1b (light green asterisks in Figure 6), were found with a mass delta of +906 Da with respect to the G0F_G0F and G0F_G1F glycoforms. Ratios were constant over the 10 days with around 2.5% for A-1a and 4% for A-1b. The retention and masses suggest that they may be sialylated glycoforms with two sialic acid residues (N-acetylneuraminic acid, Neu5Ac) in the molecule (i.e., A-1a G0F_G2S2F and G1S1F_G1S1F, A-1b G1F_G2S2F, and G1S1F_G2S1F).

A very acidic variant A-2 (green asterisk in Figure 6) was detected in constantly low amounts around 0.6%. The molecule mass matched a glycoform with four Neu5Ac residues (G2S2F_G2S2F).

^{2}D aggregate analysis of HCC samples via ProA-SCX-SEC

The second available option for the downstream analysis of the mAb isolated in the ^{1}D, was an aggregate analysis using the MAbPac SEC column in combination with the ProA-SCX setup (Figure 1, path b). The TIC of the SEC analysis obtained from the HCC sample drawn on day 9 is shown in Figure 10A. The insert presents the magnification of a very low signal of the high molecular weight (HMW) fraction of the mAb product. The mass spectrum of the main peak (Figure 10B) shows the charge envelope of the native mAb monomer (*m/z* 5,000–6,200), while the spectrum over the HMW peak (Figure 10C) is shifted to higher *m/z* values (*m/z* 6,800–8,000). This measurement requires the standard mass range (up to *m/z* 6,000) to be extended for mass detection up to *m/z* 8,000 provided by the BioPharma option. The deconvolution of the monomer peak confirmed the previous major glycoform mass matches, and their monitoring would also be possible from the SEC data. Deconvolution of the spectra from the HMW region did not provide any useful result due to the very low signal intensity in combination with a higher complexity of the spectra.

To estimate the mAb aggregation level in the samples, an MS channel for the *m/z* 6,500–8,000 was extracted from the full MS scan data in Chromeleon CDS. The area of the small peak at the HMW elution time was compared to the area of the main peak in the TIC. Figure 11 illustrates the increasing trend of the HMW fraction over the course of the 10 days of production. The ratio of HMW peak to TIC main peak was <0.01% on the first day and did not exceed 0.12% at later stages.

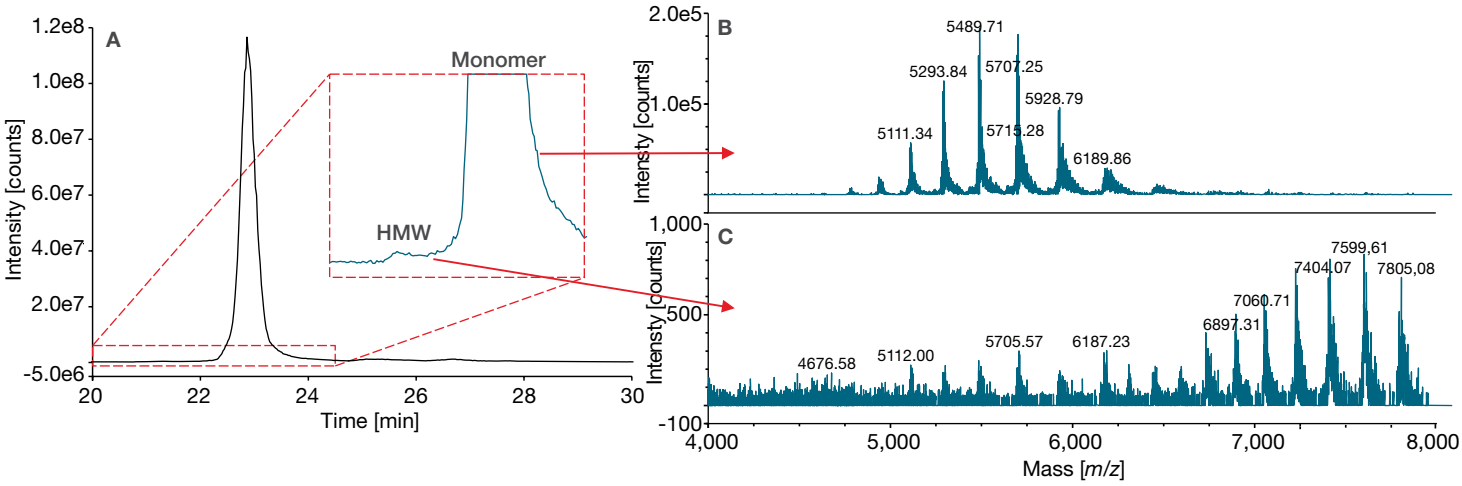


Figure 10. (A) ^{2}D TIC of ProA-SCX-SEC analysis of HCC sample drawn on day 9; (B) mass spectrum of the monomer peak obtained upon averaging across all MS scans over the monomer peak; (C) mass spectrum representing an average across all MS scans of the HMW peak

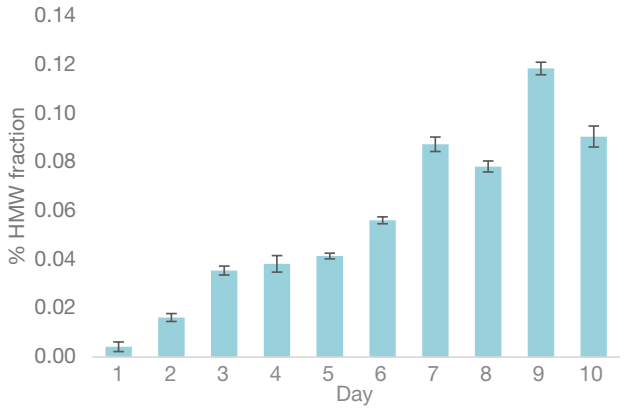


Figure 11. HMW fraction relative to the monomer peak monitored over the 10-day cell culture production period. Data derived from extracted MS channel in Chromeleon CDS from ProA-SCX-SEC aggregate analysis of HCC samples.

In summary, the 2D-LC-MS system was configured and optimized for fast and efficient monitoring of the biosynthesis of cNISTmAb, to provide a rapid and detailed insight into some trends over the production process at the intact protein level. However, due to the complex nature of the sample confirmation of tentative identifications would require additional experiments involving different structural levels, such as the peptide level and/or released glycan analysis.[9]

Conclusion

- The switchable heart-cut 2D-LC-MS approach, which allows the selection of the ^{2}D LC separation method, facilitated the upstream monitoring of multiple PQAs over a 10-day production cycle of cNISTmAb (a not fully characterized NISTmAb analogue) directly from cell culture samples.
- UV-based titer determination upon ProA affinity purification revealed mAb concentrations between 7 and 52 µg/mL.
- Charge variant analysis of the purified intact native mAb allowed for the detection and abundance monitoring of the major glycoforms, monitoring of several unknown charge variants, and some tentative charge variant identifications.
- Size variant analysis using an SCX-SEC column setup allowed for assessment of the aggregation level which was found to be low (≤0.01%) in the early growth phase and increasing over the 10-day biosynthetic process.
- The developed approach presented in this study is not only suited for the close process monitoring of new or uncharacterized modalities, but especially promises straightforward applicability for process analysis and multi-attribute monitoring at the intact protein level (iMAM) for well-known mAb products.
- The speed of the analysis allows the derivation of a set of titer, charge, and size variant information from bioreactor samples within as short as a one-hour time window.

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