HPLC Columns

LC-MS analysis of intact and subunit-level mAb enabled by a novel monodisperse supermacroporous reversed-phase platform

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Abstract

Purpose: To achieve high-resolution chromatographic separation of monoclonal antibody fragments while maintaining MS-compatible conditions that enable high-quality spectra for confident analyte identification.

Methods: Reversed-phase separation of intact mAb and mAb subunits using a Thermo Scientific™ SurePac™ Protein RP MDi™ column coupled with a high-resolution mass spectrometer.

Results: Intact mAb, LC, HC, scFc and F(ab′)$_2$ are successfully analyzed using a 10-min gradient under LC-MS compatible conditions, with accurate mass determination for all species.

Introduction

Monoclonal antibodies (mAbs) are structurally complex biotherapeutics that require multi-level analytical characterization to ensure product quality, safety, and efficacy. LC-MS analysis is routinely applied at the intact protein and subunit levels to assess molecular mass, heterogeneity, and structural integrity. Enzymatic subunit generation, such as IdeS digestion, enables targeted domain-level characterization while reducing analytical complexity compared to full peptide mapping. Achieving high-performance reversed-phase separations across intact and subunit-level workflows remains challenging, as chromatographic columns optimized for intact proteins often compromise performance of smaller subunits. Consequently, multiple columns are required to support comprehensive mAb characterization.

In this study, we evaluate a reversed-phase separation column based on a novel 2.5 µm monodisperse supermacroporous (SMP) polymeric stationary phase for unified LC-MS analysis of monoclonal antibodies under intact, reduced, and IdeS-digested conditions. The SMP resin's uniform particle size and highly accessible pore structure enable efficient separation of both small and large analytes, including intact mAbs, reduced subunits (LC and HC), and IdeS-generated fragments such as scFc and F(ab′)$_2$. Using the SurePac Protein RP MDi column, baseline separation of these species was achieved within a 10-minute gradient, providing a fast, all-in-one LC-MS approach for mAb variant characterization.

Materials and methods

Chemicals and reagents

IdeS protease was purchased from Promega, DTT from Thermo Fisher Scientific, NISTmAb from Sigma-Aldrich, 0.1% FA in water LC-MS grade and 0.1% FA in MeCN LC-MS grade from Fisher Scientific™ channel.

Sample reduction

Reduction of inter-chain disulfides in a mAb (4 mg/mL) was achieved by incubation of mAb with 20 mM DTT on incubator at 37 °C and 300 rpm for 30 min.

Sample IdeS digestion

IdeS protease was added at 1 unit enzyme per 1 µg of mAb ratio. The digestion was carried out on incubator at 37 °C and 300 rpm for 30 min.

Column

SurePac Protein RP MDi, 2.1 x 50 mm (P/N: 43722-052132)

Mobile phase

Eluent A: H2O/FA (99.9:0.1 v/v)

Eluent B: MeCN/H2O/FA (90:9.9:0.1 v/v/v)

Instruments

Thermo Scientific™ Vanquish™ Horizon UHPLC system;

Thermo Scientific™ Orbitrap Exploris™ 480 mass spectrometer with Thermo Scientific™ OptaMax™ NG ion source.

Mass Spectrometry conditions

The protein mode with positive ion polarity and low-pressure settings was used for this study. Intact mAb and mAb fragments were analyzed by HESI-MS. Detailed conditions are provided in Tables 1 and 2.

Data analysis

The UHPLC system with mass spectrometer were operated with Thermo Scientific™ Xcalibur™ 4.7 software. Full MS spectra of intact mAbs and mAb fragments were analyzed using Thermo Scientific™ Freestyle™ 1.8 and BioPharma Finder™ 5.4 software that utilizes the ReSpect and Xtract algorithms for molecular mass determination.

Table 1. Summary of MS parameters

MS method settings	Settings for intact NISTmAb analysis	Settings for DTT-reduced NISTmAb analysis	Settings for IdeS digested NISTmAb analysis
Scan type	Full MS	Full MS [two segments]	Full MS [two segments]
Total run time, min	18	0 to 8 8 to 18	0 to 8 8 to 18
Full MS mass range, m/z	2200-5500	800-2600	800-2600 1400-3500
Resolution setting	30 000	240 000 15 000	240 000 15 000
AGC target value, %	100	300	300
Maximum injection time, ms	200	200	200
In-source CID, V	80	N/A	N/A
Microscans	10	10	5 10

Table 2. Summary of tune parameters

MS ion source settings	Settings for intact NISTmAb analysis	Settings for DTT-reduced NISTmAb analysis	Settings for IdeS digested NISTmAb analysis
Sheath gas, a.u.	40	25	25
Auxiliary gas, a.u.	10	10	10
Vaporizer temperature, °C	250	150	150
Source voltage, kV	3.5	3.8	3.8
Ion transfer tube temperature, °C	325	320	320

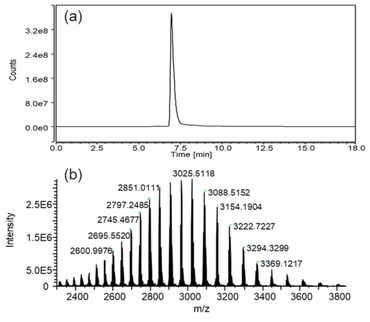
Results

Intact monoclonal antibody analysis

Monoclonal antibodies are complex heterogenous molecules with a huge variety of post-translational modifications, such as C-terminal lysine truncation, N-terminal pyro-glutamate formation, methionine oxidation, and glycosylation.

Intact NISTmAb analysis under MS-compatible conditions resulted in a dominant chromatographic peak with minor contributions from other proteoforms (Figure 1). While the chromatogram reflects limited resolution of structural heterogeneity at the intact level, it provides robust, MS-compatible separation that enables acquisition of high-quality spectra for accurate mass determination and deconvolution analysis (Table 3).

Figure 1. Analysis of NISTmAb using SurePac Protein RP. (a)The total ion chromatogram; (b) Mass spectra, while the conditions are described on the right



Column	SurePac Protein RP MDi
Format	2.1X50mm
Eluent A	H_2O/FA (99.9:0.1 v/v)
Eluent B	MeCN/H_2O/FA (90:9.9:0.1 v/v/v)

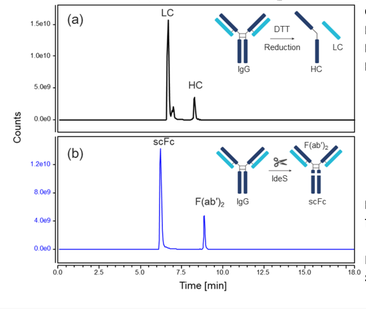
Time	%A	%B
0.0	70.0	30.0
1.0	70.0	30.0
11.0	60.0	40.0
13.0	60.0	40.0
13.0	70.0	30.0
18.0	70.0	30.0

Flow rate	0.4 mL/min
Temperature	80 °C Active pre-heater 80 °C Column chamber
Inj. Volume	0.5 µL
Sample	NISTmAb 1 mg/mL

Subunit-level analysis of monoclonal antibody

Thorough analysis of post-translational modifications requires "peptide mapping", which is time consuming. A simpler and direct option to analyse the mAb variants and locate the modifications is to measure the subunits of it. LC and HC are generated by DTT reduction of mAb; scFc and F(ab′)$_2$ - by IdeS digestion. Figure 2 shows the analysis of these subunits.

Figure 2. Subunit analysis of NISTmAb using SurePac Protein RP. (a) LC and HC of reduced NISTmAb; (b) scFc and F(ab′)$_2$ of IdeS digested NISTmAb.



Column	SurePac Protein RP MDi
Format	2.1X50mm
Eluent A	H_2O/FA (99.9:0.1 v/v)
Eluent B	MeCN/H_2O/FA (90:9.9:0.1 v/v/v)

Time	%A	%B
0.0	85.0	15.0
1.0	85.0	15.0
11.0	55.0	45.0
13.0	55.0	45.0
13.0	85.0	15.0
18.0	85.0	15.0

Flow rate	0.4 mL/min
Temperature	80 °C Active pre-heater 80 °C Column chamber
Inj. Volume	A - 0.25 µL; B - 0.5 µL
Sample	A - DTT red. mAb 4 mg/mL B - IdeS dig. mAb 2 mg/mL

The acquired mass spectra were analyzed using BioPharma Finder software, applying the Xtract algorithm for high-resolution data and the ReSpect algorithm for lower-resolution data. The resulting combinations of modifications are summarized in Table 3.

Table 3. Summary of most abundant proteoforms

	Chain	Proposed modifications	Average mass (Da)	Theoretical mass (Da)	Δ ppm
Intact mAb	Intact	1xG0F_G0F	148 036.69	148037.15	3.2
	Intact	1xG0F_G1F	148 198.64	148199.30	4.4
	Intact	1xG1F_G1F	148 360.78	148361.44	4.4
	Intact	1xG1F_G2F	148 523.04	148523.58	3.6
Red. mAb	HC	1xA2G0F	50 898.98	50 899.02	0.7
	HC	1xA2G1F	51 061.28	51 061.16	-2.5
	HC	1xA2G2F	51 223.32	51 223.30	-0.5
IdeS digested mAb	F(ab′)$_2$	N/A	97 608.67	97 609.04	3.8
	Chain	**Proposed modifications**	**Monoisotopic mass (Da)**	**Theoretical mass (Da)**	**Δ ppm**
	scFc	1xA2G0F	25 216.460	25 216.432	-1.1
	scFc	1xA2G1F	25 378.516	25 378.485	-1.2
	scFc	1xA2G2F	25 540.566	25 540.538	-1.1
Red. mAb	LC	N/A	23 109.310	23 109.273	1.6

The dominant proteoforms of NISTmAb include C-terminal lysine truncation of the heavy chains and N-terminal pyro-glutamate formation. Disulfide bonds between cysteine residues are assumed to be fully formed. These modifications were not explicitly included in Table 3 to maintain clarity, as they are consistently present across all species.
The reported species in Table 3 are consistent with known NISTmAb glycoforms and common post-translational modifications, including scFc glycosylation (A2G0F, A2G1F, A2G2F). Proteoform assignments were based on accurate mass agreement and represent the most likely glycoform compositions. These assignments should be considered putative unless confirmed by orthogonal methods such as LC–MS/MS.

Conclusions

- A SurePac Protein RP column enabled a rapid (10 min) separation of mAb LC, HC, scFc, and F(ab′)$_2$ fragments, with major proteoforms identified by mass agreement.
- The SurePac Protein RP column supports comprehensive intact and subunit-level mAb characterization in a single workflow, with strong separation performance across mAb fragments.

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