Mass Spectrometric Characterization of Intact Half-mAb

separated from intact monoclonal antibody and fragments using analytical size exclusion chromatography column

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Introduction

- Monoclonal antibodies (mAbs) are multidomain proteins that are extensively used as a research tool in molecular biology and as therapeutics in medicine.
- In many cases, antibodies are engineered to contain surface cysteines for the site-specific conjugation of payloads in antibody drug conjugates.
- Recent research has shown an interest in mAb half-bodies as therapeutic vectors, as they can be further targeted for conjugation, enzyme labeling or antibody immobilization.
- Half-mAb containing an intact antigen binding site is getting attention in nano-bioengineering, for example in the development of ultra small diagnostic nano probes.
- Half antibody fragments are being used to improve biosensor sensitivity without loss of selectivity.
- Half antibodies are useful as bioligands in other type of biosensors also.

Introduction

We discuss this report in 2 sections:

- **In section A,** we report the separation of a half mAb from intact monoclonal antibody using a 2 µm TSKgel® UP-SW3000 column offering dual functionality for use in both UHPLC and HPLC systems. This column is used to monitor the separation of half mAb from intact mAb and LMW (low molecular weight) fractions by monitorig TCEP (tris(2-carboxyethyl)phosphine) digestion.
- The same separation using 4 µm and 5 µm size exclusion chromatography (SEC) columns on a conventional HPLC instrument is also reported.
- Characterization of the half mAb was done by SDS-PAGE followed by coomassie blue staining using transferrin (78 kDa) as a MW standard for the half mAb (data not shown here).
- **In Section B,** we discuss the further characterization of the half mAb by mass spectrometry.

Section A:

Separation of a half mAb from intact monoclonal antibody and LMW fractions

Materials and Methods

Instruments:

- Agilent 1200 series HPLC system, Chemstation® (ver. B.04.02)
- UltiMate® 3000 UHPLC system run by Chromeleon® (ver 7.2)

SEC Chromatographic Columns:

- The following silica-based diol coated 25 nm pore size columns with a calibration range of 10 - 500 kDa are discussed in this report:
  - TSKgel UP-SW3000, 2 µm, 4.6 mm ID × 30 cm
  - TSKgel SuperSW3000, 4 µm, 4.6 mm ID × 30 cm
  - TSKgel SuperSW mAb HR, 4 µm, 7.8 mm ID × 30 cm
  - TSKgel G3000SWXL, 5 µm, 7.8 mm ID × 30 cm

Purification of Monoclonal Antibody TBL-mAb-01

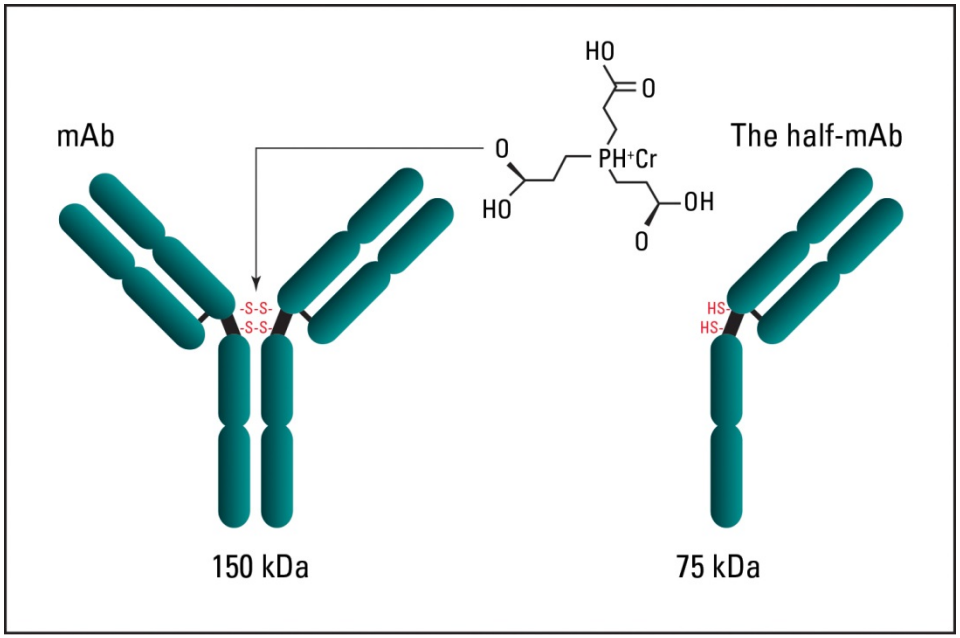
- A single clone #47 expression was grown elsewhere (not disclosed in this report) in a 50 L wave bioreactor in PowerCHO™ 2 media and harvested on day 18 at 63.7% viability.
- Media clarification was carried out by depth filtration and 0.2 µm filters.
- Supernatant feedstock was dispensed to 1.0 L bottles. This feedstock containing monoclonal antibody, henceforth referred to as TBL-mAb-01, is used for this study.
- Partially purified mAb was obtained from this feed stock using Protein A chromatography.

Human IgG (polyclonal) from Sigma was similarly used for digestion and SEC analysis as well.

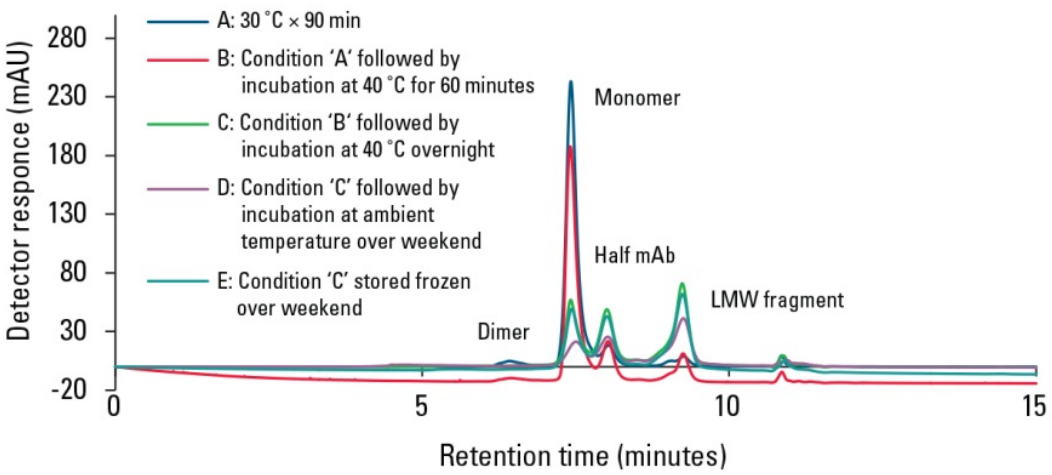
Materials and Methods

Half-mAb formation by TCEP reduction

- 500 mmol/L TCEP was used for protein reduction
- Briefly, 125 µL of protein was mixed with 325 µL SEC mobile phase
- 50 µL TCEP was then added to bring the total volume to 500 µL and the reaction mixture was incubated at 30 ºC for 90 minutes
- The reduction was further monitored at different duration of digestion period



Digestion Monitoring of a Half mAb by UHPLC using 2 µm TSKgel UP-SW3000, 30 cm Column



Column:	TSKgel UP-SW3000, 2 µm, 4.6 mm ID × 30 cm
Mobile phase:	0.1 mol/L phosphate/0.1 mol/L sulfate buffer + 0.05% NaN_3
Flow rate:	0.3 mL/min
Detection:	UV @ 280 nm
Temperature:	30 °C
Injection vol.:	10 µL
Sample:	TBL mAb 01

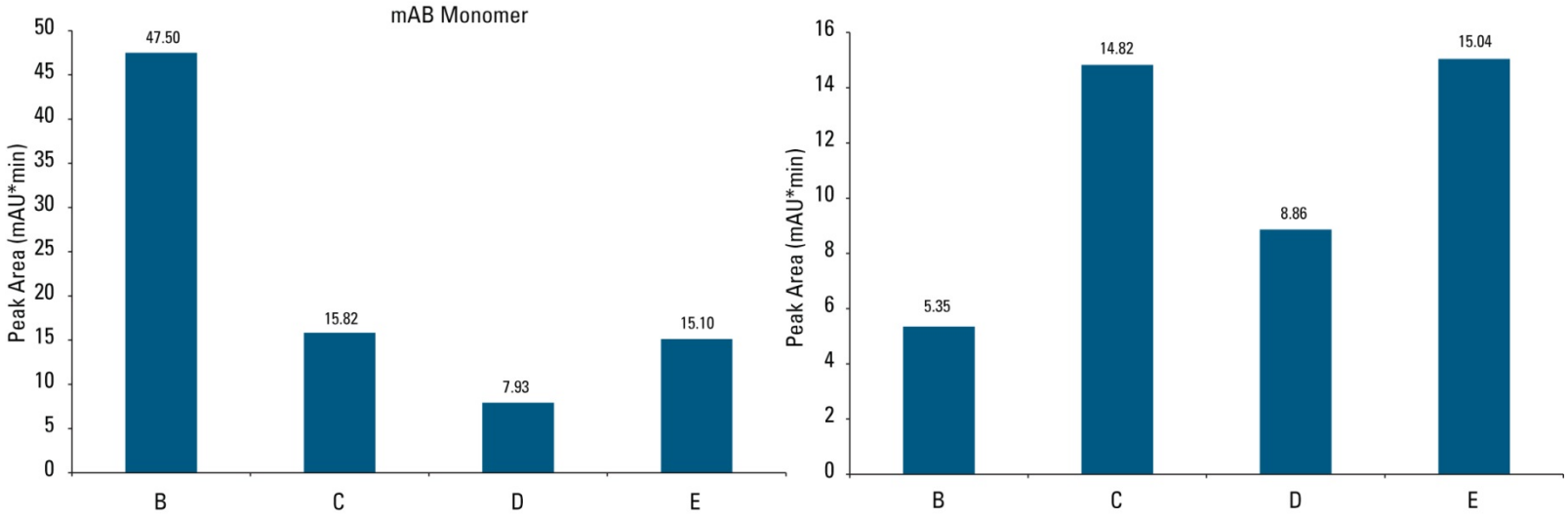
- Capping method was not used to arrest the digestion, so that the reaction could be monitored over time. Digestion for 90 minutes at 30 ºC (Condition A) did not digest the mAb and was compared here as reference to the other digestion conditions (B-E).
- The separation of the half-mAb could be monitored by a 2 µm TSKgel UP-SW3000 SEC column using a UHPLC instrument as a function of the incubation period.

Reproducibility of Peak Retention Times

TCEP Digestion conditions	Monomer RT (min)	Half mAb RT (min)	LMW Fragment RT (min)
B	7.412	8.032	9.250
C	7.418	8.007	9.235
D	7.507	8.02	9.253
E	7.435	8.01	9.242

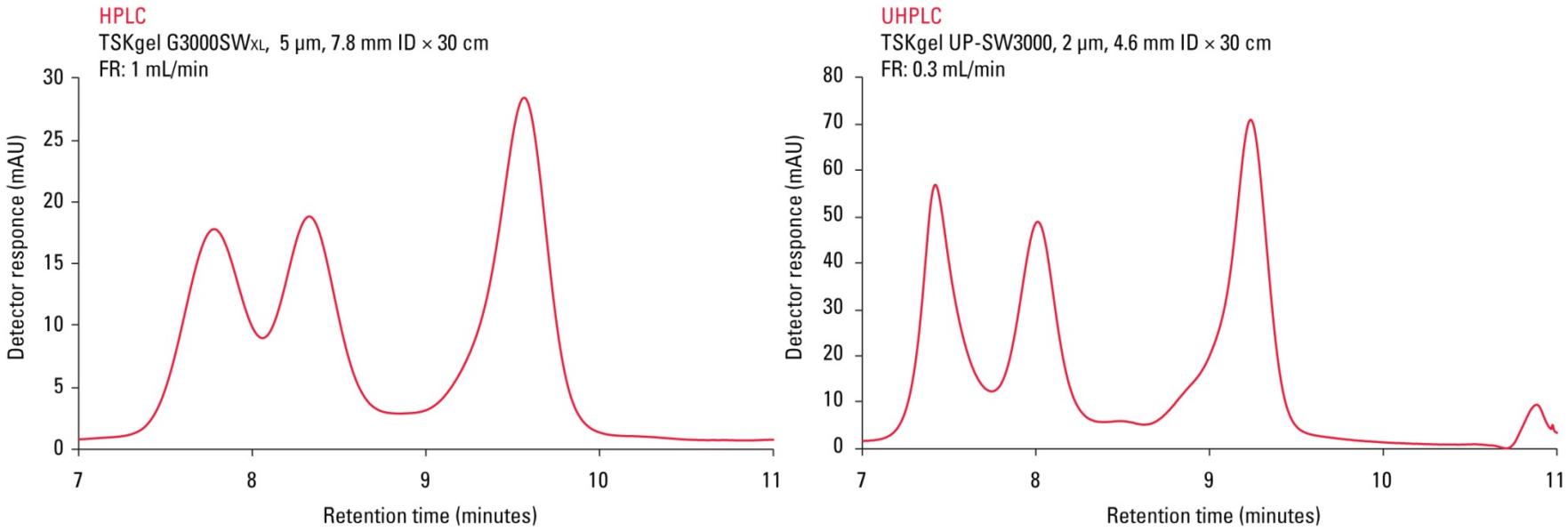
- A TSKgel UP-SW3000 column could separate the half mAb from the intact mAb and other LMW fragments reproducibly.
- The correlation of the peak areas of monomer peak, half-mAb peak and the LMW fragment are explained in the next slide.

Correlation of the Peak Areas of Monomer Peak, Half-mAb Peak and the LMW Fragment



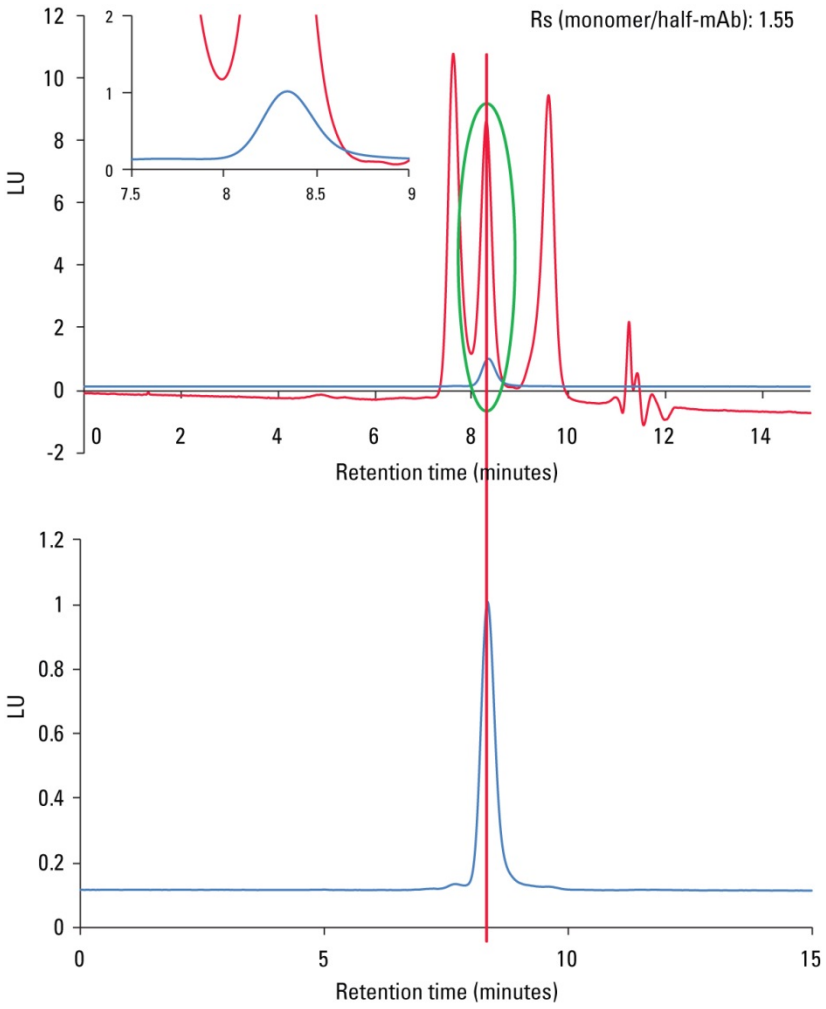
- Monomer peak area decreased linearly over time under the conditions B - D.
- Half mAb peak area increased under conditions B - C. Under overnight incubation (D) at ambient temperature over weekend, half mAb peak area decreased due to further digestion.
- Peak area (C) did not change when the sample was stored under frozen conditions (E) instead of storing at ambient condition (D).
- Please note that a capping method was not used in this study to monitor the digestion over the duration of the digestion period. Uncapped reaction mixture can be best stored at -20 ºC to arrest further digestion.
- The results show that the TSKgel UP-SW3000 column could be used to monitor the reaction successfully.

Method Transfer from HPLC to UHPLC



- The method of TCEP digestion already developed could be transferred very easily from 5 µm SEC Column using a HPLC instrument to a 2 µm SEC Column using a UHPLC instrument.
- The same method was used, except adjustment in the flow rate.
- The 2 µm SEC column yielded higher sensitivity and sharper peaks.
- There was a slight difference in digestion time between analysis on each column.
- This method can be similarly used with a TSKgel UP-SW3000, 15 cm column for faster separation within half the run time shown here.

Separation of Half mAb from mAb Monomer and LMW Fragment by 4 µm SEC Column



Column:	TSKgel SuperSW3000, 4 µm, 4.6 mm ID × 30 cm
Mobile phase:	100 mmol/L sodium phosphate/ 100 mmol/L sodium sulfate, pH 6.7 + NaN_3
Gradient:	Isocratic
Flow rate:	0.35 mL/min
Detection:	FLD (λex: 280 nm, λem 350nm)
Temperature:	30 °C
Injection vol.:	10 µL
Sample:	TBL mAb 1 (4 mg/mL)

- Note: Detection of the peaks was carried out using FLD (λex: 280 nm, λem 350nm) for better sensitivity of the SEC purified dilute half mAb sample.
- Intrinsic tryptophan fluorophore was used for this study.
- Overlay of reduced TBL mAb 1 (red) purified half-mAb (blue) are shown here.
- Resolution of >1.5 could be achieved.
- Half mAb sample could be successfully purified as a single pure peak from the digestion.
- The purified peak was confirmed by SDS-PAGE with coomassie blue staining using transferrin (78 kDa) as MW standard.

Ref: TP 207

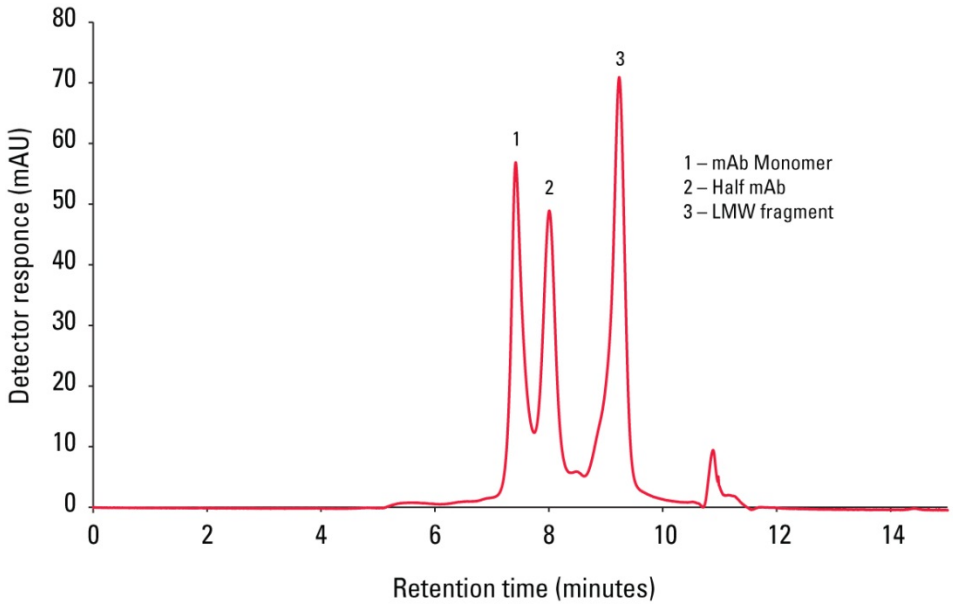
Section B:

MALDI-TOF mass spectrometry – offline SEC MS analysis of half mAb

Method

- Samples were concentrated, desalted, and spotted using sinapinic acid as the matrix.
- All spectra were collected on a Bruker microflex® MALDI-TOF mass spectrometer.
- Spectra were analyzed using the Bruker flexanalysis software.

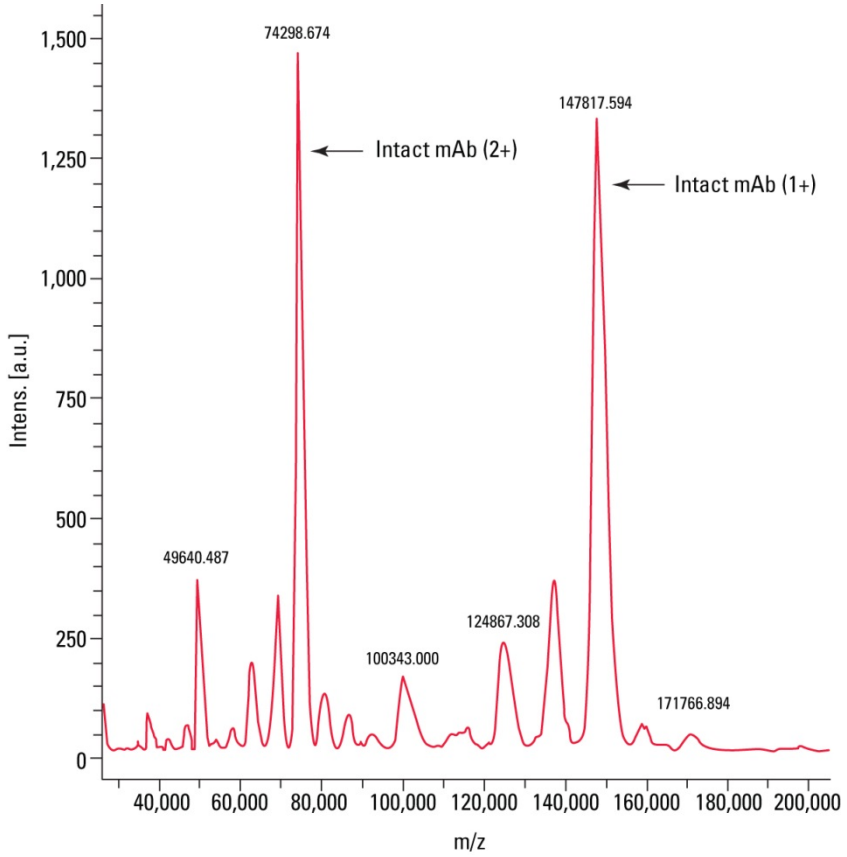
Separation of Half mAb by UHPLC using a 2 µm TSKgel UP-SW3000, 30 cm Column



Column:	TSKgel UP-SW3000, 2 µm, 4.6 mm ID × 30 cm
Mobile phase:	0.1 mol/L phosphate/0.1 mol/L sulfate buffer + 0.05% NaN_3
Flow rate:	0.3 mL/min
Detection:	UV @ 280 nm
Temperature:	40 °C overnight + frozen (Condition E – as in first figure)
Injection vol.:	10 µL
Sample:	TBL mAb 01

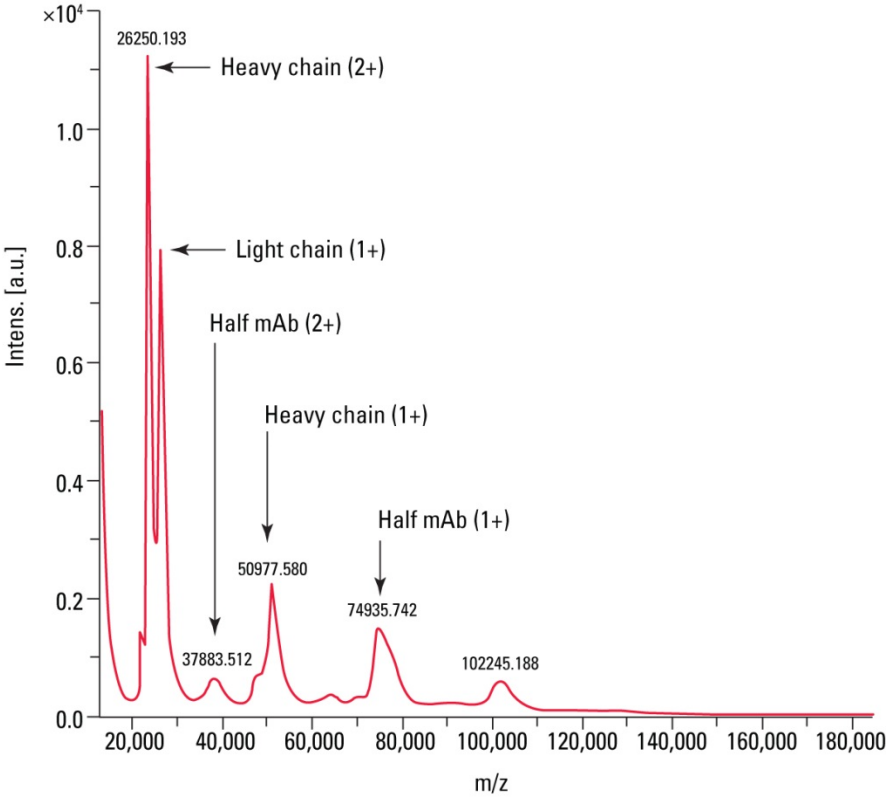
- The chromatogram shows that a 2 µm particle size TSKgel UP-SW3000 column with dual functionality is able to separate the half-mAb from the intact mAb using a UHPLC instrument.
- **The peaks 1, 2 and 3 are collected and further characterized by mass spectrometric methods as discussed in the next slides.**

Characterization of Intact mAb using MALDI-TOF Mass Spectrometry



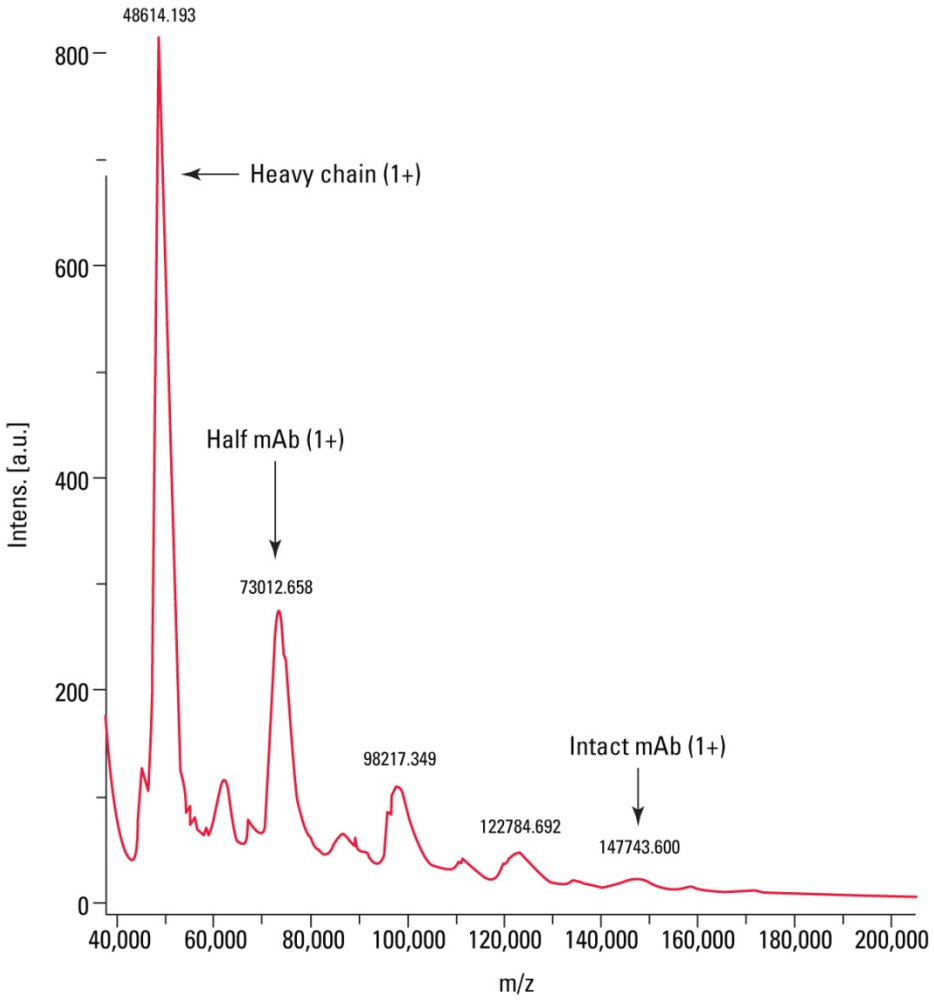
- This shows the mass spectrometric characterization of the intact mAb. As the ionization cannot be controlled in MS, the same molecule shows up as 1+ (singly charged ion) or 2+ (doubly charged ion).
- The intact mAb peak eluting from different SEC columns was previously characterized by SDS-PAGE analysis followed by coomassie blue (data not shown) .

Characterization of Half-mAb using MALDI-TOF Mass Spectrometry



- Peak 2 was confirmed by SDS-PAGE analysis followed by coomassie blue staining using transferrin (77 kDa) as reference standard for half-mAb analysis. Peak 2 MW could be verified.
- As the ionization cannot be controlled in MS, the same molecule shows up as 1+ (singly charged ion) or 2+ (doubly charged ion).
- This mass spectrometric analysis further confirms that peak 2 was a half mAb.

Characterization of LMW Fragment by MALDI-TOF Mass Spectrometry



Conclusions

- A half-mAb was generated from TBL mAb 01 and human IgG by selective reduction of the disulfide bonds present in the hinge-region of the monoclonal antibody using TCEP.
- This half mAb was separated using a variety of TSKgel SEC columns of different particle size and dimensions for seamless transfer of methods.
- A 2 µm TSKgel UP-SW3000 was used to monitor the digestion of the half mAb using a HPLC or UHPLC system.
- FLD was successfully used with SEC for the detection of the dilute purified half mAb.
- Intact mAb, half mAb and the LMW fragment was successfully characterized by mass spectrometry.
- The results are useful for the separation of half mAb for further applications in the fields of biotherapeutics and biosensors.