Application Note

Biopharma/Pharma

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Physicochemical Characterization of Coformulated Monoclonal Antibodies Using Agilent Biopharma Analytical Workflow Solutions

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Abstract

A recent trend in protein biotherapeutics development is to coformulate monoclonal antibodies (mAbs) to improve drug efficacy. This application note demonstrates the use of Agilent biopharma analytical tools, including cation-exchange chromatography (CEX), size exclusion chromatography (SEC), and peptide mapping, for the analysis of coformulated mAbs.

Introduction

Coformulated combination (mAbs) are gaining popularity in the biopharma pipeline as next-generation protein therapeutic strategies. In this approach, multiple mAbs are combined in the same drug to target multiple antigens. Coformulation is used to improve drug efficacy, and several coformulated mAbs are currently under investigation for therapeutic combinations.[1] Although this approach has many benefits, formulation scientists must consider different attributes, such as the molar ratio of active molecules and buffer composition, increasing the complexity of analytical development, and product characterization.

Differences in the complementarity-determining regions (CDRs) of coformualated mAbs tend to contribute most to differences in their physicochemical properties. The complex properties of binary mAb mixtures require new analytical methods to identify and measure their critical quality attributes (CQAs) to improve our understanding of their behavior during the manufacturing process. However, despite growing interest in coformulated mAbs, only a few reports focus on analytical characterization. This application note demonstrates charge variant, aggregation, and post-translational modifications (PTMs) analysis of a binary mAb mixture using efficient chromatographic separation techniques.

Experimental

Chemicals

Sodium phosphate dibasic, sodium phosphate monobasic, sodium chloride, Trizma base, guanidine hydrochloride, *tris*(2-carboxyethyl)phophine (TCEP), iodoacetic acid (IAA), formic acid, and LC/MS grade solvents were purchased from Sigma-Aldrich. Fresh ultrapure water was obtained from a Milli-Q Integral system, equipped with a 0.22 µm membrane.

Samples

Commercially available mAb 1 and mAb 2 formulations were used as model proteins to make coformulated mAb lot. Binary mixtures were prepared by combining mAb 1 and mAb 2 (m/m). The trypsin digestion was performed as described in Development of an LC/MS/MS peptide mapping protocol for the NISTmAb.[2]

Equipment

- Agilent 1290 Infinity II bio high-speed pump (G7132A) (Aggregation and peptide mapping)
- Agilent 1290 Infinity II bio flexible pump (G7131A) (Charge variant)
- Agilent 1290 Infinity II bio multisampler (G7137A)
- Agilent 1290 Infinity II multicolumn thermostat (G7116B)
- Agilent 1290 Infinity II DAD (G7117B)
- Agilent 6545XT AdvanceBio LC/Q-TOF

Columns

- Agilent AdvanceBio SEC 300Å, 7.8 × 150 mm, 2.7 µm (part number PL11803301)
- Agilent Bio Mab HPLC column, NP5, 2.1 × 250 mm, PEEK (part number 5190-2411)
- Agilent AdvanceBio peptide mapping, 2.1 × 150 mm, 2.7 µm, 120Å (part number 653750-902)

Software

- Agilent OpenLab CDS version 2.5
- Agilent MassHunter LC/MS Data Acquisition 11.0 software
- Agilent MassHunter BioConfirm software, version 11.0

Methods

Charge Variant Analysis (Calculated by Buffer Advisor Software)					
Parameter	**Value**				
pH Gradient, pH 7 to 8.4 in 30 mM Sodium Phosphate Buffer	Time (min)	A*	B*	C*	D*
	0.0	34.3	0	42.1	23.6
	8.6	44.9	0	22.5	32.6
	14.09	49.7	0	13.8	36.5
	21.4	53.4	0	6.8	39.8
	30	55.6	0	2.8	41.6
	30.33	44.2	9.8	5.8	40.2
	30.5	37.6	14.7	8.9	38.8
	30.67	30.2	19.6	13.6	36.6
	31	11.7	29.4	29.6	29.3
	35	11.7	29.4	29.6	29.3
	38.21	28.5	10.5	33.5	27.5
	40	34.3	0	42.1	23.6
Stop Time	40 min				
Post Time	20 min				
Flow Rate	0.2 mL/min				
Injection Volume	2 µL				
Sampler Temperature	10 °C				
Column Temperature	25 °C				
DAD	280 nm/4 nm, Ref: OFF				
Peak Width	>0.025 min (10 Hz)				

* Mobile phases:
(A) Water
(B) NaCl (1,700 mM)
(C) NaH_2PO_4 (32 mM)
(D) Na_2HPO_4 (70 mM)

Aggregation Analysis	
Parameter	**Value**
Mobile Phase	100 mM sodium phosphate buffer + 150 mM NaCl, pH 7.0
Flow Rate	0.8 mL/min
Stop Time	10 min
Injection Volume	5 µL
Sampler Temperature	10 °C
Column Temperature	25 °C
DAD	280 nm/4 nm, Ref:OFF
Peak Width	>0.025 min (10 Hz)

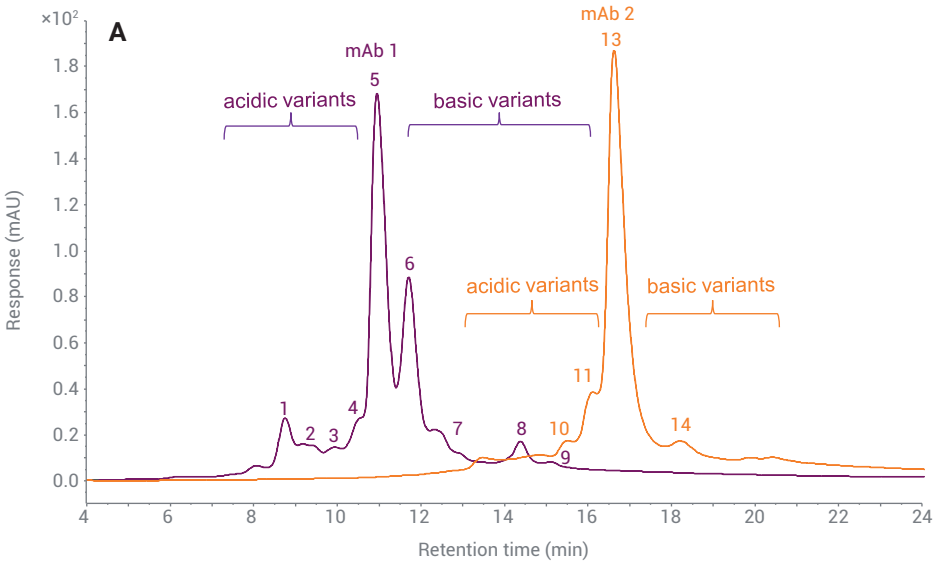
Peptide Mapping/PTM	
Parameter	**Value**
Injection Volume	4 µL
Sample Thermostat	5 °C
Mobile Phase A	0.1% formic acid in water
Mobile Phase B	0.1% formic acid in acetonitrile
Gradient	Time (min) %B 0 3 5 3 78 40 80 90 85 90
Stop Time	85 min
Column Temperature	60 °C
Flow Rate	0.25 mL/min
Mass Spectrometry Conditions	
Ion Mode	Positive ion mode, dual AJS ESI
Drying Gas Temperature	325 °C
Drying Gas Flow	13 L/min
Sheath Gas Temperature	275 °C
Sheath Gas Flow	12 L/min
Nebulizer	35 psi
Capillary Voltage	4,000 V
Nozzle Voltage	0 V
Fragmentor Voltage	175 V
Skimmer Voltage	65 V
Oct RF Vpp	750 V
Reference Mass	121.050873, 922.009798
Acquisition Mode	Data were acquired in extended dynamic range (2 GHz)
MS Mass Range	110 to 1,700 *m/z*
Acquisition Rate	8 spectra/s
Auto MS/MS Range	50 to 1,700 *m/z*
MS/MS Acquisition Rate	3 spectra/s
Isolation Width	Narrow (~*m/z* 1.3)
Precursors/Cycle	Top 10
Collision Energy	Charge state Slope Offset 2 3.1 1 3 and >3 3.6 −4.8
Threshold for MS/MS	1,000 counts and 0.001%
Dynamic Exclusion On	One repeat, then exclude for 0.1 or 0.2 minutes
Precursor Abundance-Based Scan Speed	Yes
Target	25,000 counts/spectrum
Use MS/MS Accumulation Time Limit	Yes
Purity	100% stringency, 30% cutoff
Isotope Model	Peptides
Sort precursors	By charge state then abundance; +2, +3, >+3

Results and discussion

Chromatographic techniques are at the forefront of protein biotherapeutic analytical characterization. Methods designed for individual mAbs may not be suitable for simultaneous measurement of coformulated mAbs. This study developed chromatographic methods to monitor coformulated mAb critical quality attributes (CQAs), including charge variants, aggregation, and PTMs.

Charge variants

Cation-exchange chromatography (CEX) is the preferred technique for charge-based separation of antibody isoforms. In this mode of separation, the main mAb isoform can be separated from acidic and basic variants using salt or pH gradients. To characterize the charge heterogeneity of coformulated mAbs, a pH-gradient method was developed on the Agilent 1290 Infinity II Bio LC quaternary LC system using an Agilent Bio Mab PEEK ion exchange column (NP5, 2.1 × 250 mm). Figures 1A and 1B show the CEX profiles of each constituent mAb by itself, and a 1:1 coformulated mAb mixture, respectively. The pH-gradient method provided high resolution separation of acidic, basic, and main peaks. The same charge variant profiles were observed in the coformulated sample as in the individual constituent mAbs. The precision RT and area RSDs for all charge variants are shown in Figure 1.



Peak	RT RSD %	Area RSD %
1	0.16	1.53
2	0.08	5.57
3	0.09	9.07
4	0.06	5.93
5	0.03	0.83
6	0.06	0.82
7	0.06	2.51
8	0.05	1.92
9	0.03	6.35
10	0.01	2.05
11	0.01	6.17
12	0.02	1.58
13	0.02	0.35
14	0.01	1.80

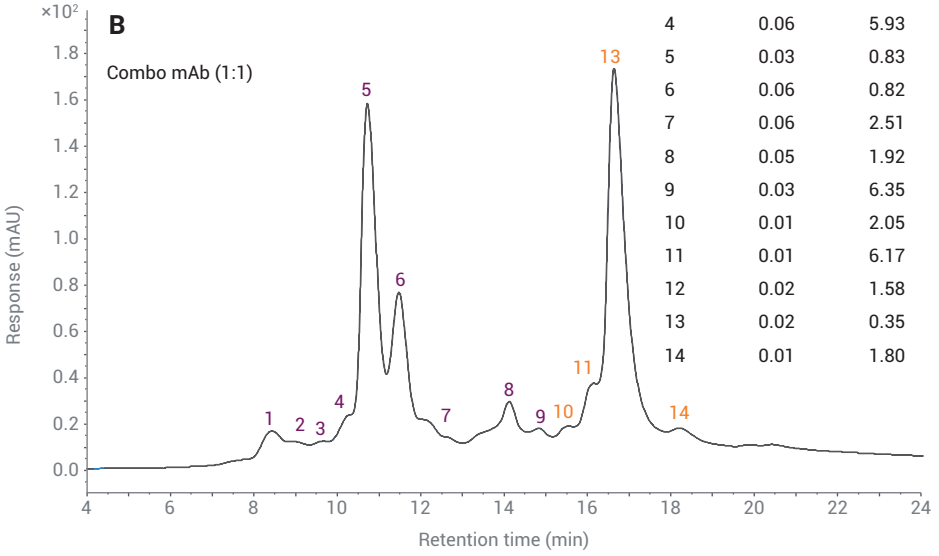


Figure 1. Cation-exchange chromatography profiles of mAb 1 and mAb 2 antibodies, individually formulated (A) and after coformulation (1:1) (B) on the Agilent Bio MAb, NP5, 2.1 × 250 mm, PEEK.

Aggregation

Size exclusion chromatography (SEC) is the gold standard technique for measuring soluble aggregates in mAb formulations. The separation mechanism is based on size/hydrodynamic volume. To characterize the aggregation of coformulated mAbs, we developed an SEC method on an Agilent 1290 Infinity II bio LC binary system using Agilent AdvanceBio SEC 300Å, 7.8 × 150 mm, 2.7 µm. Although it was not possible to separate the coformulated mAbs, based on the small differences in their molecular weights and hydrodynamic radii, the overall aggregation ratio of the mixture could be measured. Figures 2A and 2B show the SEC profiles of individual mAb and 1:1 coformulated mAbs mixture, respectively. With 100 mM sodium phosphate buffer, 150 mM NaCl, and a pH 7.0 mobile phase used, there was a marginal separation of the two monomer peaks, attributable to each individual mAb. The Figure 2B insert shows a zoomed view of the separation of aggregates from the monomer peak. The precision RT and area RSDs for the monomer and co-aggregation peaks are shown in the Figure 2.

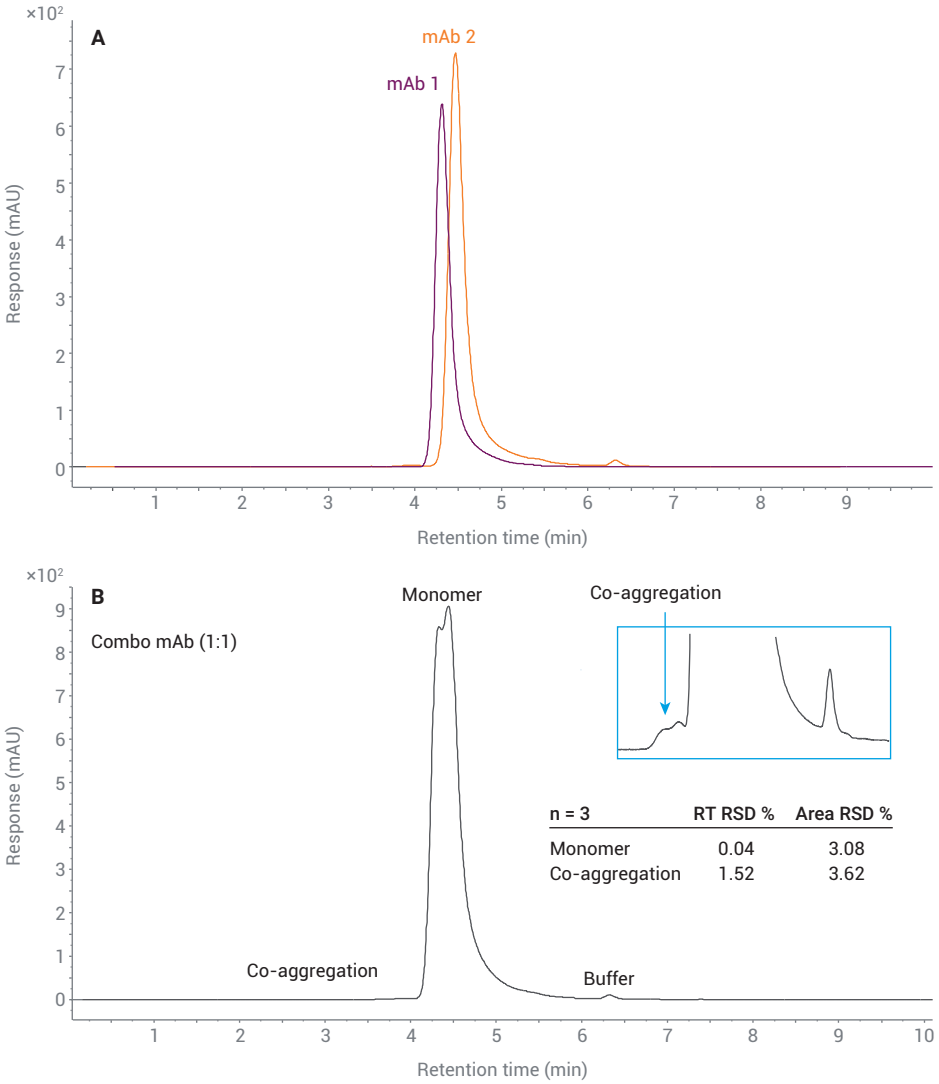


Figure 2. Size exclusion chromatography profiles of mAb 1 and mAb 2 antibodies, individually formulated (top, A) and after coformulation (1:1) (bottom, B) on the AdvanceBio SEC 300Å, 7.8 × 300 mm, 2.7 µm.

Peptide mapping/PTMs

Peptide mapping is often used to establish protein identity and to detect PTMs. It is particularly important to characterize CDR peptides, which are typically unique to each mAb. In the present study, we performed LC/MS peptide mapping analysis for coformulated mAbs with an Agilent 1290 Infinity II bio LC system coupled to an Agilent 6545XT AdvanceBio LC/Q-TOF. Figure 3A shows the LC/MS of tryptic peptide map of individual mAbs and the 1:1 coformulated mAb mixture. High-resolution peptide separation was achieved using an AdvanceBio peptide mapping column and sequence coverage was >95%. Figure 3B depicts the extracted ion chromatograms of CDR peptides in the coformulated mAb sample, which confirms the identity of mAb 1 and mAb 2. PTM analysis shows comparable levels of oxidation, deamidation, and lys-truncation across all three samples.

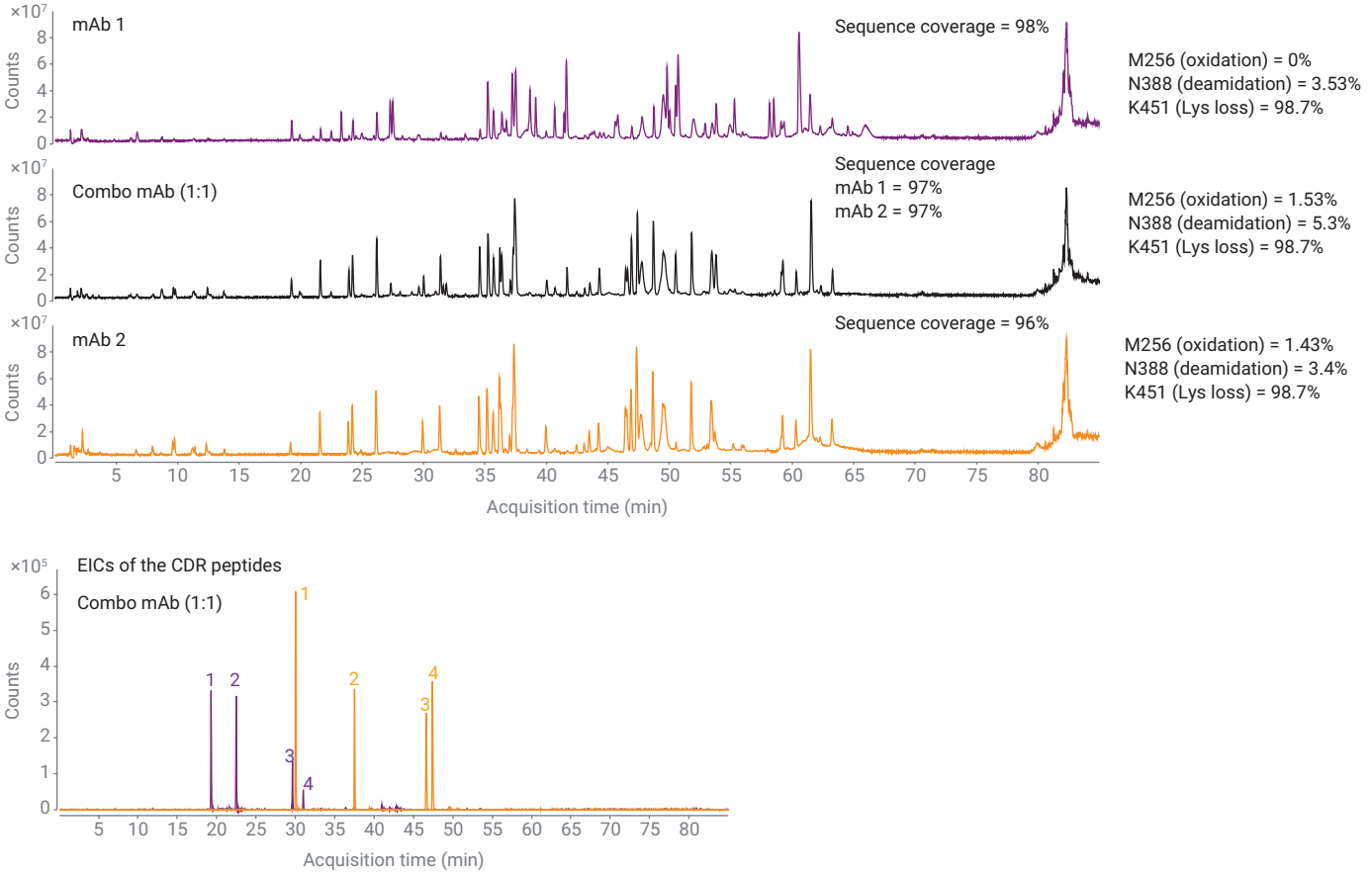


Figure 3. LC/MS separation of tryptic digest with an Agilent AdvanceBio peptide mapping column. Total ion chromatogram (TIC) of mAb 1, mAb 2, and the 1:1 coformulated mAb mixture.

Conclusion

This application note demonstrates the suitability of Agilent LC and LC/MS techniques for different aspects of coformulated mAbs CQA monitoring. The analytical characterization approaches described in this study, including charge variants, aggregation, and PTMs, can be used for coformulated mAbs stability studies.

References

1. Larbouret, C. *et al.* Improving Biologics' Effectiveness in Clinical Oncology: From the Combination of Two Monoclonal Antibodies to Oligoclonal Antibody Mixtures. *Cancers (Basel)* **2021**, 13*(18)*, 4620.
2. Mouchahoir, T.; Schiel, J. E. Development of an LC-MS/MS Peptide Mapping Protocol for the NISTmAb. *Analytical and Bioanalytical Chemistry* **2018**, *410*, 2111–2126.

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