Application Note

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Complementary Methodologies for Analysis of NISTmAb Impurities

Authors

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Abstract

Size heterogeneity is a critical quality attribute (CQA) of monoclonal antibodies (mAb) as both aggregation and degradation can impact the safety and efficacy of therapeutic antibodies. Size-based impurities consist of low molecular weight (LMW) degradation products and high molecular weight (HMW) aggregates. To fully characterize mAb size heterogeneity, both Size Exclusion Chromatography (SEC) and Capillary Electrophoresis-sodium dodecyl sulfate (CE-SDS) are used, with each providing key insights into mAb monomeric purity. SEC uses native conditions that allow for characterization of HMW aggregates, while CE-SDS provides high-resolution separations of LMW species. In this application note, the NISTmAb reference standard is used to compare monomeric purity measurements by SEC and CE-SDS while highlighting the complementary nature of these techniques.

Introduction

The presence of both HMW and LMW impurities can alter the effectiveness or safety profile of therapeutic antibodies. As a result, regulatory agencies mandate a thorough characterization of therapeutic antibodies as part of the approval process for new biologics and quality control release criteria[1]. Several CQAs are therefore assessed during the development and production of biotherapeutic monoclonal antibodies. One key CQA used as a measure of sample integrity is size heterogeneity, or the percent monomeric purity. Monoclonal antibodies require precise analytical techniques for size heterogeneity characterization. To aid in this assessment, two techniques, SEC and CE-SDS offer complementary, yet unique insights into the impurity composition of these antibodies[1,2].

SEC separations occur in native environments allowing for the separation of HMW multimeric mAb aggregates from the monomeric mAb and LMW mAb fragments. HMW aggregates elute first and are composed of multimeric species.

Depending on the SEC system used, resolution of the different aggregate species, such as the dimer and trimer, may be possible. The monomeric mAb elutes as the largest main peak, followed by the LMW species. The LMW species generally elute as a single peak and are composed of partial mAb fragments. SEC excels in analyzing soluble noncovalent HMW aggregates of monoclonal antibodies. However, resolution of individual LMW fragment species is typically not possible[1,2].

In contrast, CE-SDS is performed using denaturing conditions which break apart noncovalent aggregates. This allows for high resolution separations of the LMW fragments when analyzed under nonreducing conditions, but prevents its use for HMW aggregate characterization. Nonreducing CE-SDS is used to determine monomeric percent purity and quantitation of each individual LMW fragment. By preparing the mAb sample in the presence of a reducing agent, such as Dithiothreitol (DT T), the same method can be used to assess heavy and light chain relative abundance, heavy chain glycan occupancy, and nonreducible species content of the mAb[1,2,3]. These CQAs are unable to be determined by SEC analysis. The choice between SEC and CE-SDS hinges on the specific requirements of the analysis, with each offering different, yet complementary insights about the integrity of the antibody.

Agilent provides both SEC and CE-SDS solutions for size heterogeneity analysis, including high-quality biocompatible ultrahigh performance liquid chromatography (UHPLC) instrumentation, sub-2 µm column technology, and the ProteoAnalyzer system. The biocompatible Agilent 1290 Infinity II Bio LC system perfectly copes with the high salt concentrations often found in SEC buffers, providing confident results at the lowest maintenance cost. To enable optimal performance, a combination of sub-2 µm columns and a UHPLC instrument with dead volumes as low as possible is preferred. Large dead volumes destroy the resolution obtained by these columns due to dispersion effects[4]. Complementing this is the Agilent ProteoAnalyzer system, which provides high-resolution CE-SDS analysis of 12 samples in parallel. In this application note, the NIST mAb standard is analyzed on the Agilent 1290 Infinity II Bio LC for SEC analysis and Agilent ProteoAnalyzer system for CE-SDS purity analysis, highlighting the orthogonal nature of the two techniques. In particular, the ability of each technology to provide unique data in relation to HMW and LMW impurities is examined.

Experimental

CE-SDS analysis

NISTmAb (Sigma p/n NIST8671, aliquot from Reference Material 8671, Lot 14HB-D-002)[1] was prepared in PBS at a concentration of 2,000 ng/µL under both reducing and nonreducing conditions according to the Agilent Protein Broad Range P240 (p/n 5191-6640) manual[5]. The samples were covalently labeled by incubating with the supplied reagents at 70 °C for 10 minutes. The reduced and nonreduced antibodies were analyzed across multiple capillaries of an Agilent ProteoAnalyzer system[6] with the Agilent Protein Broad Range P240 kit LM Only method. For nonreduced conditions, the sample injection was decreased to 7 kV 6 seconds for optimal results. Analysis of the samples from the ProteoAnalyzer was compared to the known specifications of the NISTmAb from datasheets and published results[1,7].

UHPLC SEC analysis

Instrument

Agilent 1290 Infinity II Bio High-Speed Pump (G7132A)

Agilent 1290 Infinity II Bio Multisampler (G7137A) including integrated Sample Thermostat (#101)

Agilent 1290 Infinity II Multicolumn Thermostat (G7116B) with biocompatible Heat Exchanger

Agilent 1290 Infinity II Diode Array Detector (G7117B), equipped with a biocompatible light sensitive sample flow cell, 10 mm, 1 µL

Additional parts

Agilent 1290 Infinity II Bio Ultra Low Dispersion kit (G7132A#006)

Software

Agilent OpenLab, version 2.5

LC method

- **Solvent:** 150 mM sodium phosphate pH 7.0
- **Flow rate:** 0.1 mL/min isocratic separation
- **Column temperature:** 30 °C
- **Sample temperature:** 4 °C
- **Injection volume:** 1 µL
- **Detection:** 280 nm – 20 Hz

Column

Agilent AdvanceBio SEC 1.9 µm column, PEEK Lined 2.1 x 150 mm – 620 bar.

Solvents and chemicals

A phosphate buffer mobile phase was prepared by dissolving sodium phosphate dibasic and sodium phosphate monobasic in fresh ultrapure water obtained from a Milli-Q integral system equipped with LC-Pak polisher and a 0.22 µm membrane point-of-use cartridge. The pH was adjusted to 7.0 with phosphoric acid at 25 °C. All UHPLC chemicals were purchased from Sigma, USA. The NISTmAb was prepared in the mobile phase at a concentration of 2 mg/mL.

Results and discussion

Analysis of the NISTmAb by SEC clearly separated the HMW, monomer, and LMW species. The HMW species split into two populations, the dimer and trimer (Figure 1). As expected, the LMW species all co-eluted as a single peak. The monomeric purity of the NISTmAb by SEC was found to be 97.83%, compared to the NISTmAb reference material sheet value of 96.63%. The HMW species were found to compose a total of 2.05%, with the LMW species representing 0.12% (Table 1). These values were comparable to the NISTmAb Reference Material Sheet[7].

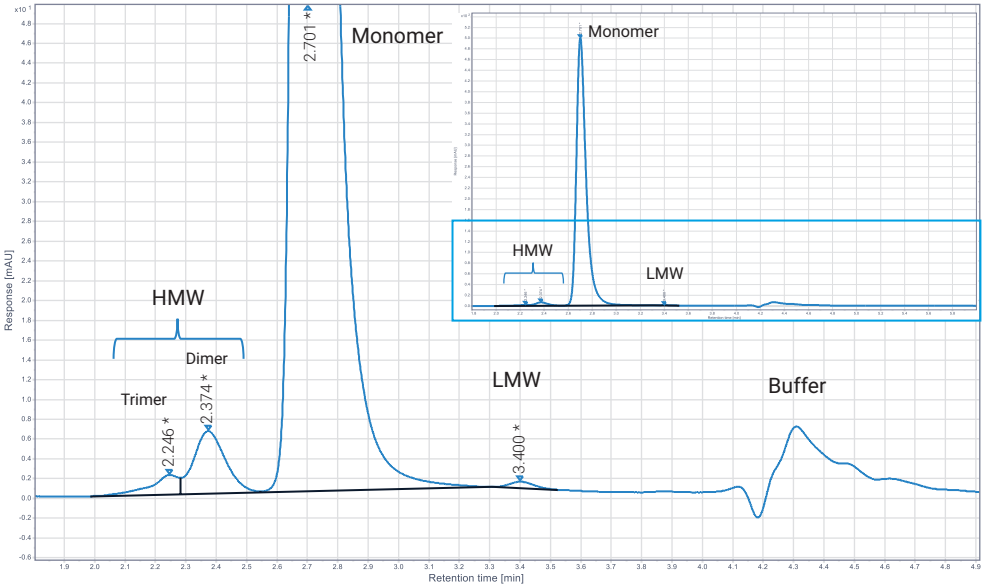

Figure 1. Results of SEC NISTmAb separation using the Agilent 1290 Infinity II Bio LC system.

Table 1. Critical quality attributes of purity for the NISTmAb were determined by SEC using the Agilent 1290 Infinity II Bio LC system and compared to the NIST datasheet[7]. 1290 Infinity II Bio LC: N = 10.

	NIST Datasheet SEC		Agilent SEC	
	Size heterogeneity (%)	Combined standard uncertainty (%)	Size heterogeneity (%)	%CV
Monomeric Purity (nonreduced)	96.63	0.15	97.83	0.04
High Molecular Weight	3.17	0.15	2.05	1.99
Low Molecular Weight	0.20	0.008	0.12	3.00

When the NISTmAb was analyzed by nonreduced CE-SDS, the LMW fragments were clearly resolved into the individual constituents, followed by a large peak representing the mAb monomer (Figure 2). To the right of the mAb monomer is a small peak, representative of the HMW species that were not denatured by SDS and heat. The monomeric purity was calculated as previously described[1,8] and found to be 98.18% with CE-SDS by the ProteoAnalyzer, compared to 98.47% as reported in the NISTmAb Reference Material Sheet. The remaining 1.82% of the sample was composed of the LMW fragments, including the Light Chain (LC), Heavy Chain (HC), fragment antigen binding (Fab) region, HC:LC, HC:HC, and HC:HC:LC impurities (Table 2). The ProteoAnalyzer values were consistent with those reported in the NIST Material Reference Sheet[7].

The monomeric purity observed by SEC and CE-SDS were found to be only 0.86% different, while the LMW fragment percentage values differed significantly. SEC found the LMW impurities to be 0.15%, while CE-SDS found these species to compose 1.82% of the total sample. The under-quantification of LMW species by SEC compared to CE-SDS was consistent with values previously reported by NIST, and may be the result of SDS releasing fragments held together by noncovalent interactions[1].

While some HMW material was detected by CE-SDS, the presence of SDS breaks up aggregates, preventing its use for HMW material quantification[1,2]. The HMW species was not included in the CE-SDS monomeric purity calculation in accordance with the measured and recorded analytes reported by NIST[1]. Analysis of reduced samples by CE-SDS can also be used to determine the percent glycosylation and thioether content. The ProteoAnalyzer system has previously been shown to provide values for these CQAs that are consistent with those reported in the NIST Material Reference Sheet[7].

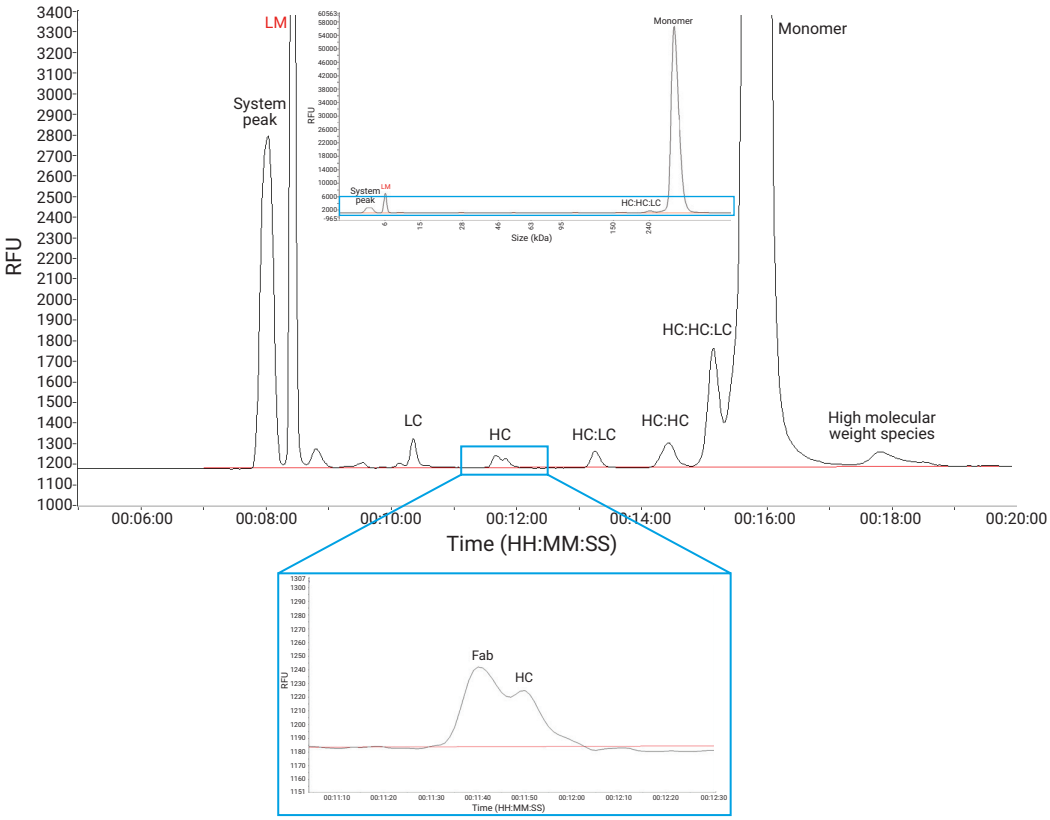

Figure 2. Results of the nonreduced NISTmAb using the Agilent ProteoAnalyzer system.

Table 2. Critical quality attributes of purity for the NISTmAb were determined by the Agilent ProteoAnalyzer system and compared to the NIST datasheet[7]. ProteoAnalyzer: N = 11.

	NIST Datasheet CE-SDS		ProteoAnalyzer CE-SDS	
	Size heterogeneity (%)	Combined standard uncertainty (%)	Size heterogeneity (%)	%CV
Monomeric Purity (nonreduced)	98.47	1.0	98.18	0.09
High Molecular Weight	Not calculated	Not calculated	Not calculated	Not calculated
Low Molecular Weight	Not calculated	Not calculated	1.82	5%

Conclusion

The data presented here demonstrates the orthogonality of SEC and CE-SDS when performing mAb monomeric purity assessments. Monomeric purity assessments by SEC provide accurate quantification of HMW aggregates, but can under-quantify low molecular weight species as shown in the NIST mAb Reference Material Sheet and confirmed here. This is because SEC is more robust for measuring monomer and HMW species, but the quantitation of LMW species can vary depending on the mAb studied[2]. On the other hand, CE-SDS provides improved resolution of closely related size-variants and is better suited for accurately quantifying LMW impurities. For precise quantification of low molecular weight antibody fragments, CE-SDS is the preferred method, while SEC is the preferred method for quantification of HMW aggregates. Combining both techniques provides the best overall characterization of mAbs, as described by organizations such as the USP[2]. Agilent offers reliable solutions for both of these technologies with the Agilent 1290 Infinity II Bio LC and Agilent ProteoAnalyzer systems.

References

1. Turner, A.; Yandrofski, K.; Telikepalli, S.; King, J.; Heckert, A.; Filliben, J.; Ripple, D.; Schiel, J. E. Development of Orthogonal NISTmAb Size Heterogeneity Control Methods. *Anal. Bioanal. Chem*. **2018**, 410 (8), 2095–2110. https://doi.org/10.1007/s00216-017-0819-3.
2. United States Pharmacopeia (2024). General Chapter, *(129) Analytical Procedures for Recombinant Therapeutic Monoclonal Antibodies*. USP-NF. Rockville, MD: United States Pharmacopeia. https://doi.org/10.31003/USPNF_M6297_02_01
3. Michels, D.; Parker, M.; Salas-Solano, O. Quantitative impurity analysis of monoclonal antibody size heterogeneity by CE-LIF: example of development and validation through a quality-by-design framework. *Electrophoresis* **2012**, 33, 815-826.
4. Elevate Your mAb Aggregate Analysis. *Agilent Technologies application note*, publication number 5994-2709EN, **2020**.
5. Agilent Protein Broad Range P240 Kit Quick Guide for the ProteoAnalyzer System. *Agilent Technologies kit quick guide*, publication number D0031125, **2024**.
6. Dependable Denaturing Protein Electrophoresis; Agilent ProteoAnalyzer system. *Agilent Technologies brochure*, publication number 5994-6716EN, **2023**.
7. National Institute of Standards and Technology; U.S. Department of Commerce. *Reference Material 8671: NISTmAb, Humanized IgG1κ Monoclonal Antibody, Lot 14HB-D-002. Reference Material Information Sheet*. Nist.gov. https://tsapps.nist.gov/srmext/certificates/8671.pdf (accessed 2023-10-24).
8. Analysis of NIST Antibody on the Agilent ProteoAnalyzer System. *Agilent Technologies Technical Overview*, publication number 5994-6960EN, **2023**.

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