Application Note

Biopharma

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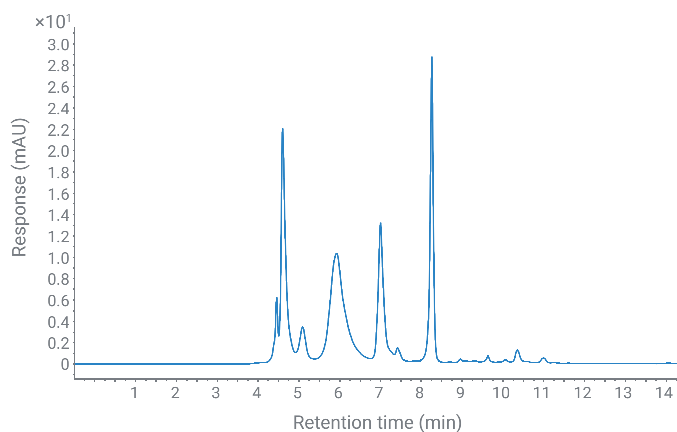
Elevate Your mAb Aggregate Analysis

High-resolution SEC with the Agilent 1290 Infinity II Bio LC System



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Abstract

This application note demonstrates the superior resolution in size exclusion chromatography (SEC) protein separation made possible by the Agilent 1290 Infinity II Bio LC System equipped with the Agilent AdvanceBio SEC column and ultralow dispersion capillaries. The biocompatible UHPLC system enables analysis using corrosive salty buffers and therefore saves maintenance expense. The resolution in SEC analysis was compared for capillaries of different inner diameter (0.17, 0.12, and 0.07 mm). A protein standard mixture and monoclonal antibodies (mAbs), including aggregates, were separated and compared for resolution. In addition, molecular weight was determined by the Agilent OpenLab GPC/SEC add-on software in one software solution, enabling a one-step workflow.

Introduction

Modern biopharmaceuticals, such as mAbs, are highly heterogeneous compounds. Aggregation monitoring, one of the most important critical quality attributes (CQAs), is typically executed by SEC. With this technique, the identity of the compound can be determined by the calculation of the molecular weight after a standard column calibration. In addition, it confirms the purity by showing the presence of unwanted higher molecular weight compounds such as dimeric and higher aggregates. To achieve the necessary resolution, modern SEC columns with sub-2 µm particle material are recommended. 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. In addition, the completely biocompatible 1290 Infinity II Bio LC perfectly copes with the high salt concentrations often found in SEC buffers, providing confident results at the lowest maintenance cost.

This application note demonstrates the use of modern sub-2 µm SEC columns on the 1290 Infinity II Bio LC and illustrates the benefit of using instruments with the lowest possible dead volumes. To demonstrate the effect of dead volume on the separation of proteins and aggregates, capillaries with different inner diameters were used. The well-characterized NISTmAb will be used to generate more aggregates by pH and thermal stress with subsequent separation of dimers, trimers, and higher aggregates.

Experimental

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 Variable Wavelength Detector (G7114B), equipped with a biocompatible micro flow cell, 3 mm, 2 µL

Additional parts

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

Software

Agilent OpenLab Version 2.5 and GPC/SEC add-on software V. 1.2

LC method

Parameter	Value
Solvent	Phosphate-buffered saline (PBS), pH 7.4
Flow Rate	0.35 mL/min
Isocratic Separation	
Column Temperature	30 °C
Sample Temperature	4 °C
Needle Wash	3 s water
Injection Volume	5 µL
Detection (VWD)	280 nm, data rate 20 Hz

Column

Agilent AdvanceBio SEC, 200 Å, 4.6 × 300 mm, 1.9 µm (part number PL1580-5201)

Samples

- Protein mix for calibration (part number 5190-9417): thyroglobulin (670,000 Da), γ-globulin (150,000 Da), ovalbumin (45,000 Da), myoglobin (17,000 Da), angiotensin II (1,000 Da)
- Humanized monoclonal antibody (mAb) trastuzumab, marketed as Herceptin, was obtained from Roche (Basel, Switzerland). The trastuzumab was dissolved in 30 mM phosphate buffer, pH 6.8.
- Agilent NISTmAb, humanized IgG1κ mAb (part number 5191-5745)

Protocol for pH/temperature-stressed NISTmAb

The mAb was diluted in the mobile phase to a final concentration of 2 mg/mL. pH stress was carried out as described elsewhere with slight modification:[1] 1 M HCl was slowly added dropwise to the sample solutions to change the pH from 6.0 to 1.0. Then, 1 M NaOH was added to adjust the pH to 10.0. Finally, 1 M HCl was added again to adjust the pH back to 6.0. There was approximately 1 minute waiting time between the pH shifts, with constant, slight stirring. The resulting solution was incubated at 60 °C for 60 minutes.

Solvents and chemicals

- **PBS:** One tablet dissolved in 200 mL of deionized water yields 0.01 M phosphate buffer, 0.0027 M potassium chloride, and 0.137 M sodium chloride, pH 7.4, at 25 °C.
- Chemicals were purchased from VWR, Germany.
- Fresh ultrapure water was obtained from a Milli-Q integral system equipped with LC-Pak polisher and a 0.22 µm membrane point-of-use cartridge (Millipak).

Results and discussion

Modern columns for SEC separation of proteins comprise material with sub-2 µm particles for optimum resolution. However, this requires instruments that have optimized low dead volume, because especially capillaries of larger inner diameters can destroy the achieved resolution. The separation of a mixture of five proteins, including three dimers, with the 1290 Infinity II Bio LC is shown in Figure 1A. To minimize dead volume and dispersion effects, capillaries with an inner diameter of 0.07 mm were used for the separation. Even the early-eluting dimer of thyroglobulin (4.947 min) was partially separated. To set up a calibration for molecular weight determination, all the proteins in this mixture were used to generate the calibration curve (Figure 1B). The best curve fit was obtained for a fourth order.

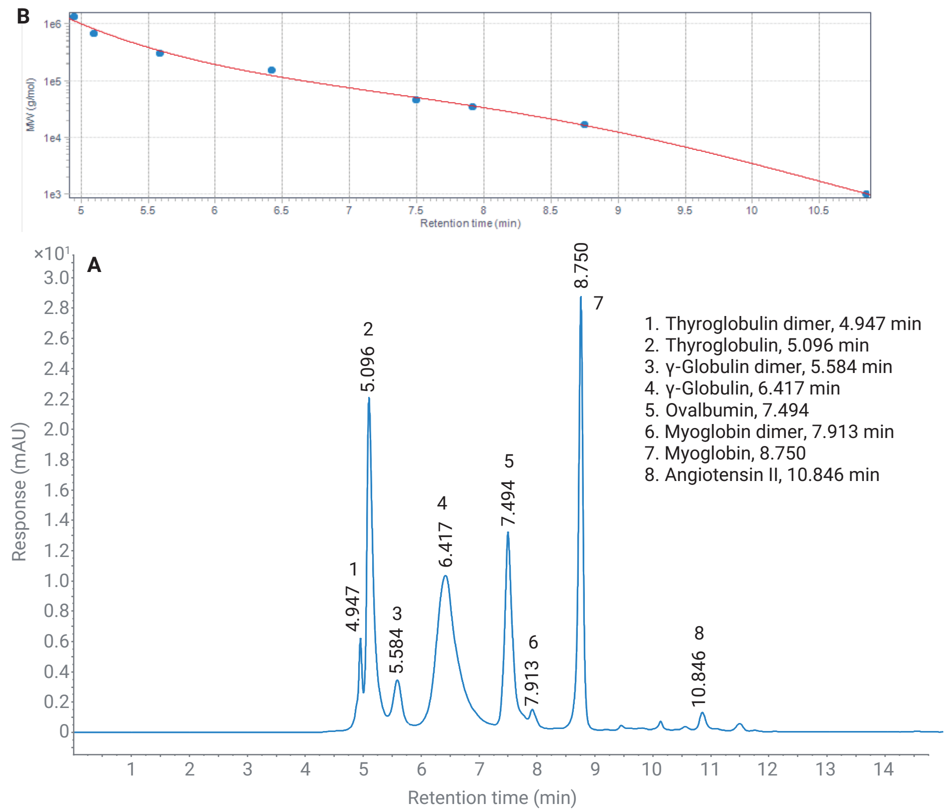
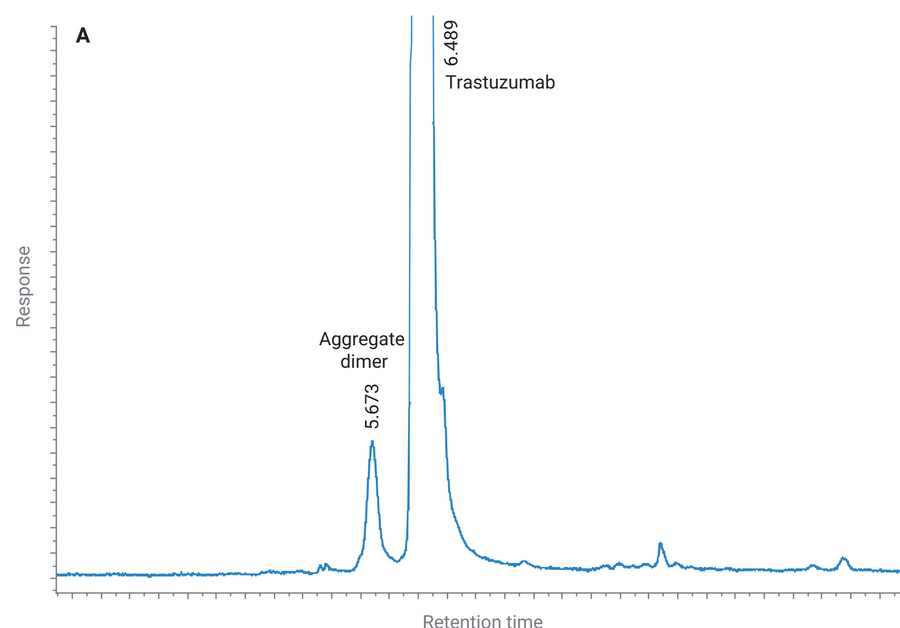
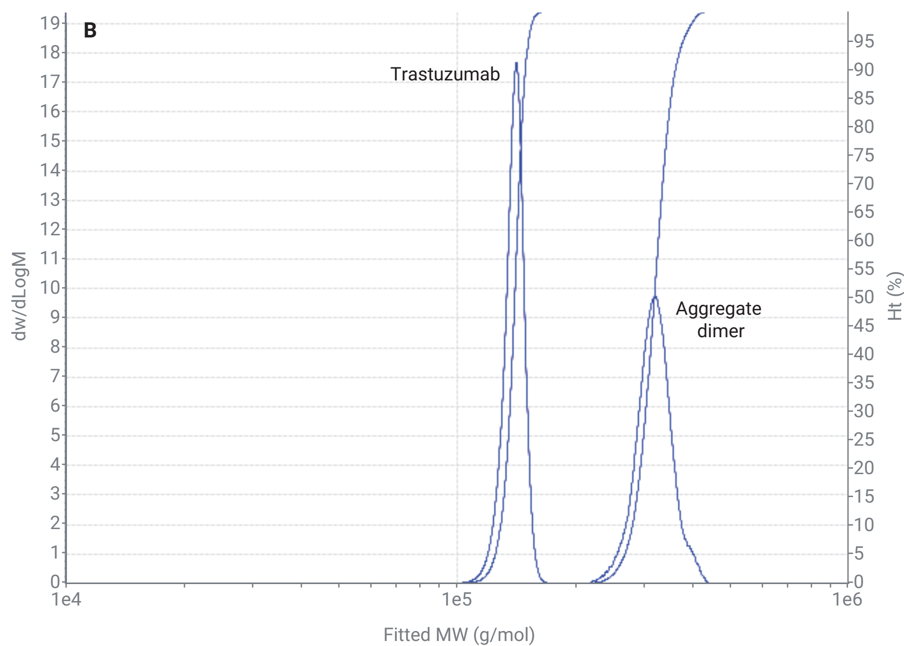


Figure 1. (A) Separation of a five-protein mixture including three dimers on the Agilent AdvanceBio SEC, 200 Å, 4.6 × 300 mm, 1.9 µm in combination with the Agilent 1290 Infinity II Bio Ultra Low Dispersion Kit comprising 0.07 mm capillaries. (B) Calibration curve of SEC for molecular weight determination with a protein mixture featuring some dimers.

The calibration was used to determine the molecular weight of the mAb trastuzumab and a comprised dimeric aggregate (Figure 2). The antibody elutes at 6.489 minutes and the corresponding dimeric aggregate elutes at 5.673 minutes (Figure 2A). The determined molecular weight at the peak maximum of trastuzumab and the dimer were Mp 141,566 Da and Mp 321,609 Da, respectively. The molecular weight distribution is shown in Figure 2B and the calculated molecular weights are outlined in the included table (2C).





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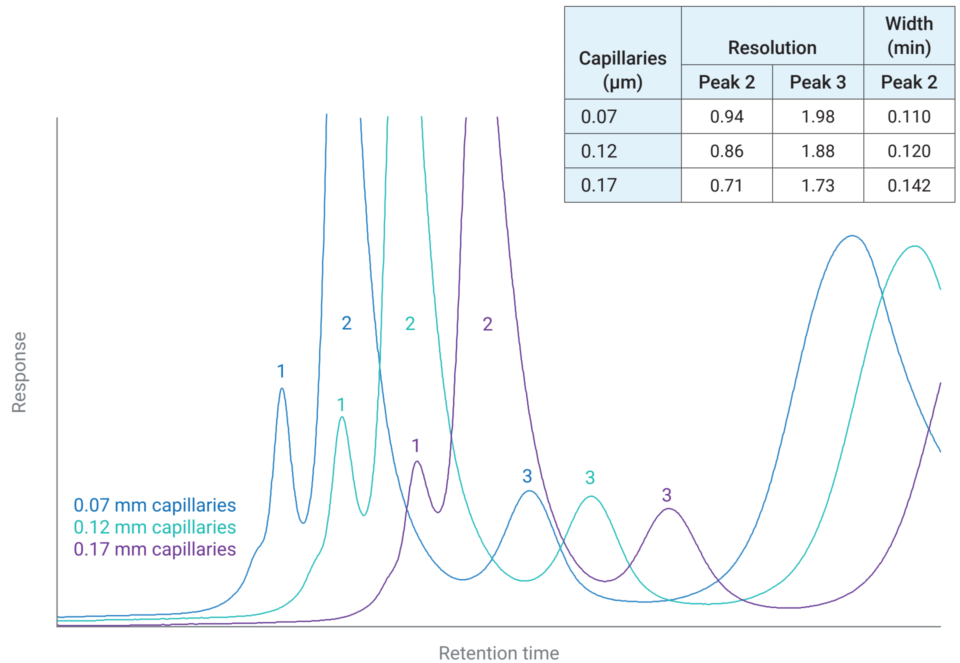
Compound	RT (min)	Mp (g/mol)
Trastuzumab Aggregate	5.673	321609
Trastuzumab	6.489	141566

Figure 2. Trastuzumab and dimeric aggregate, determination of molecular weights. (A) SEC separation of the monomer of trastuzumab from a dimeric aggregate. (B) The molecular weight of trastuzumab and the dimeric aggregate. (C) Table with molecular weights of trastuzumab and its aggregate. Mp: molecular weight at peak maximum.

The influence of the inner diameter of the used capillaries could be shown in a comparison of capillaries with an inner diameter of 0.07 (ULD), 0.12 and 0.17 mm. To demonstrate the effect, the resolution of the second and third peak of the protein mixture (shown in Figure 1) was determined (Figure 3). The best values of resolution for peak 2 and peak 3 could be obtained by means of capillaries with 0.07 mm inner diameter (table in Figure 3). For the determination of the peak width at half-height, peak 2 of the protein mixture was used. From the measured values, it could be seen that the peak width increases when using capillaries of larger inner diameters.

The influence of the capillaries on the separation of trastuzumab and its aggregate is shown in Figure 4. Here, it can be seen that an additional lower molecular weight compound was hidden under the main peak, which is only separated as a slight shoulder with the 0.17 mm capillaries and is more clearly visible using the 0.07 mm capillaries.



Capillaries (µm)	Resolution		Width (min)
	Peak 2	Peak 3	Peak 2
0.07	0.94	1.98	0.110
0.12	0.86	1.88	0.120
0.17	0.71	1.73	0.142

Figure 3. Comparison of capillaries with increasing inner diameters and their influence on resolution and peak width.

Capillaries (µm)	Resolution	Width (min) Herceptin	Width (min) Aggregate
0.07	3.34	0.096	0.189
0.12	3.05	0.107	0.205
0.17	2.83	0.121	0.216

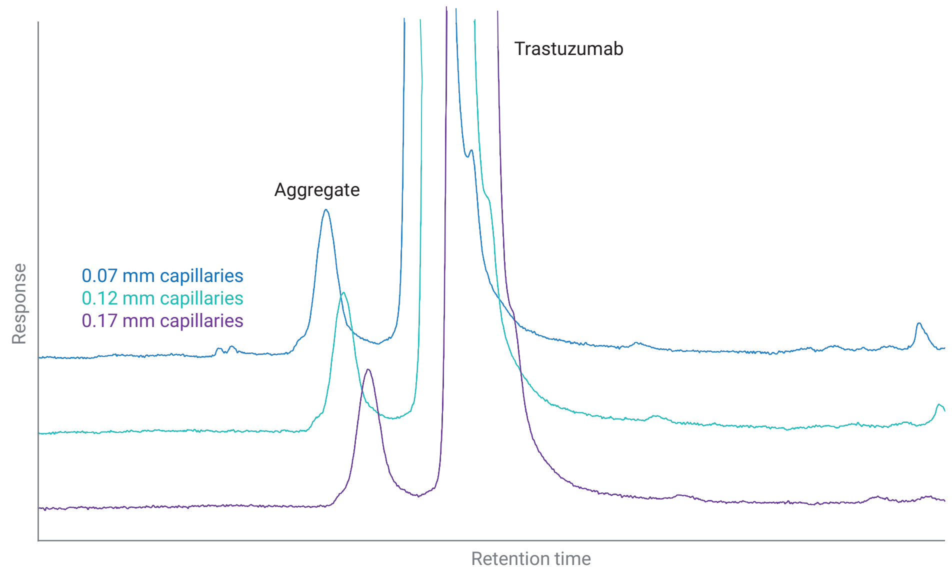


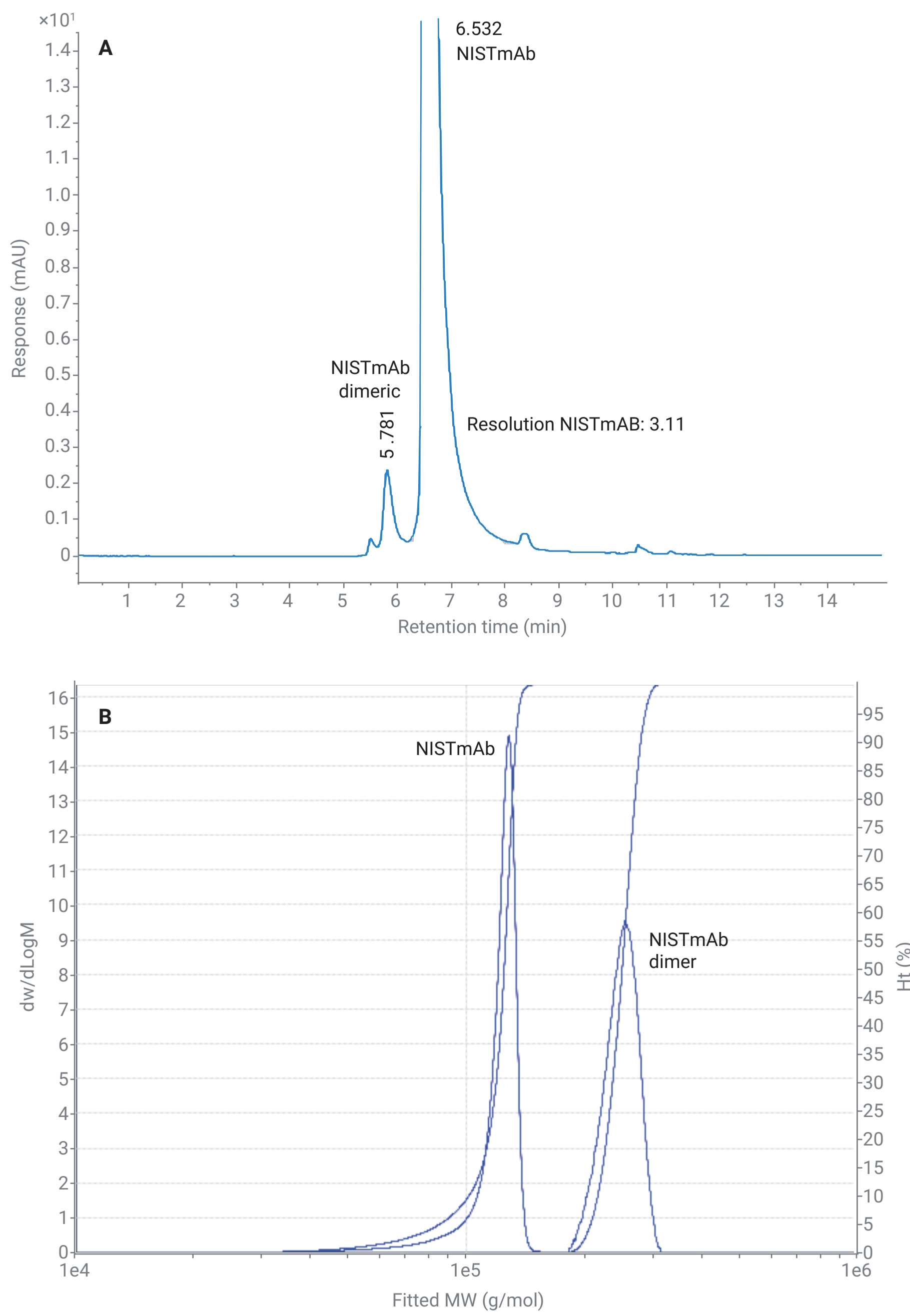
Figure 4. Resolution of trastuzumab from its dimeric aggregate and peak width depending on the inner diameter of the used capillaries.

The RSD values of retention time and peak area are excellent for all capillaries (Table 1).

As another example, the well-characterized NISTmAb (humanized IgG1κ mAb) was used for separation from aggregates and determination of molecular weights (Figure 5).

Table 1. Retention time and peak area RSDs of trastuzumab for all used capillaries. The increase in retention times is due to the increasing volumes of the different sets of capillaries.

	0.07 mm Capillaries		0.12 mm Capillaries		0.17 mm Capillaries	
	RT	Area	RT	Area	RT	Area
Average	6.464	1736.13	6.500	1727.05	6.554	1717.29
RSD (%)	0.02	0.10	0.01	0.28	0.01	0.25



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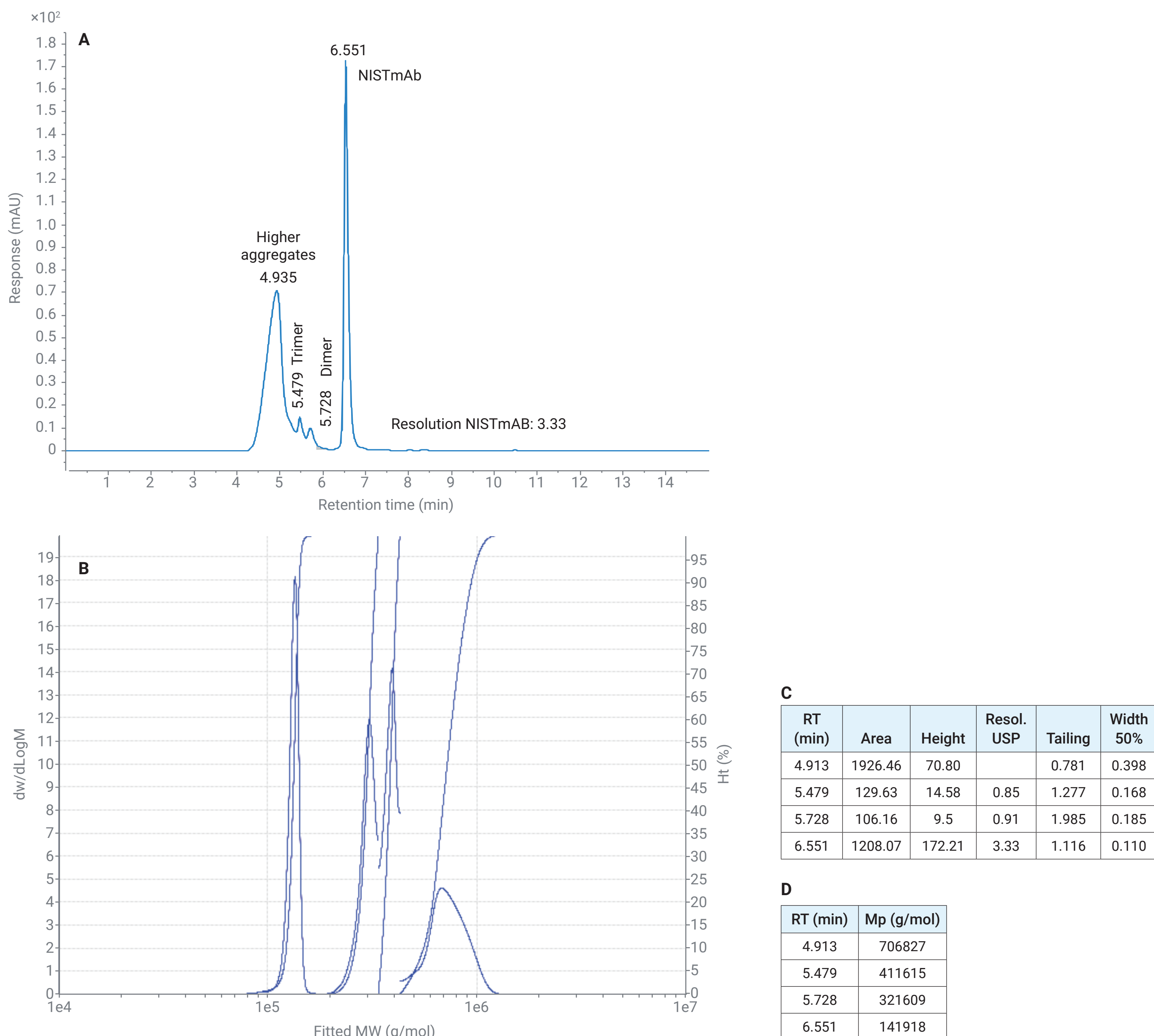
RT (min)	Area	Height	Resol. USP	Tailing	Width 50%
5.781	23.80	2.08		1.940	0.175
6.532	3006.00	409.40	3.11	1.569	0.100

D

RT (min)	Mp (g/mol)
5.781	305626
6.532	144767

Figure 5. (A) Separation of NISTmAb from its main dimeric aggregate with resolution 3.11. (B) Molecular weights in the NISTmAb and its aggregate. (C) Table showing the values of major peak characterization from NISTmAb and its aggregate. (D) Tables showing the molecular mass of NISTmAb and its aggregate.

Under pH- and temperature-stress conditions (see Experimental section), this mAb can form higher aggregates (Figure 6). With the ultralow dispersion capillaries, the higher aggregates could be separated (Figure 6A). Their molecular weight distribution and values for peak characterization are outlined in Figure 6B and the associated tables.



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RT (min)	Area	Height	Resol. USP	Tailing	Width 50%
4.913	1926.46	70.80		0.781	0.398
5.479	129.63	14.58	0.85	1.277	0.168
5.728	106.16	9.5	0.91	1.985	0.185
6.551	1208.07	172.21	3.33	1.116	0.110

D

RT (min)	Mp (g/mol)
4.913	706827
5.479	411615
5.728	321609
6.551	141918

Figure 6. (A) Separation of aggregates from a pH-stressed NISTmAb. (B) Distribution of molecular weight of aggregates occurring under stress conditions from NISTmAb. (C) Table of values of major peak characterization of NISTmAb and its aggregates. (D) Tables showing the molecular mass of NISTmAb and its aggregates.

Conclusion

This application note demonstrates the capability of the 1290 Infinity II Bio LC together with the AdvanceBio SEC column to separate proteins and their aggregates with the highest resolution due to minimized system dead volume and ultralow dispersion capillaries. The 1290 Infinity II Bio LC is a completely biocompatible system capable of operating with highly salted buffers. This offers the lowest maintenance costs at the highest resolution performance.

Reference

1. Quantitation of mAb and ADC Aggregation Using SEC and an Aqueous Mobile Phase. *Agilent Technologies application note*, publication number 5991-6303EN, **2016**.

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