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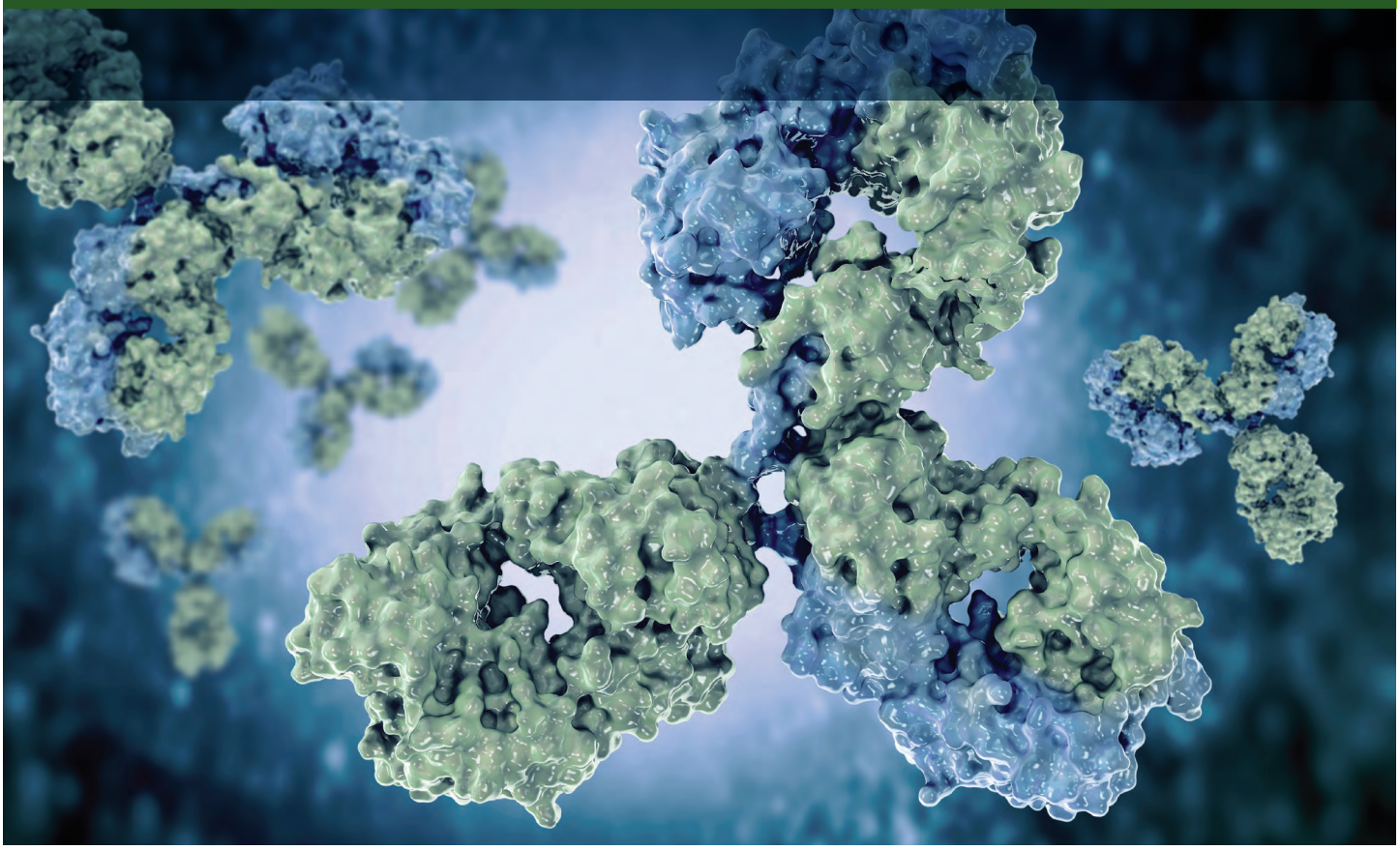
Agilent-NISTmAb

Aggregate and Fragment Analysis

Agilent BioHPLC Columns
Application Compendium



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Agilent-NISTmAb Standard (P/N 5191-5744; 5191-5745) was aliquoted from NISTmAb RM 8671 batch. Quality control (QC) testing is performed using Agilent LC-MS system. QC batch release test includes aggregate profile, charge variants and intact mass information. A certificate of analysis (CoA) can be found in each product shipment with test results.

Please note that authors used various monoclonal antibodies including Agilent-NISTmAb Standard and NISTmAb RM 8671 to demonstrate critical quality attribute workflows.

Aggregate and Fragment Analysis- Size Exclusion Chromatography

Introduction

Proteins are often susceptible to aggregation, forming dimers and higher order aggregates because of exposure to stress conditions. This is particularly problematical in biotherapeutic protein manufacture since the target protein will be subjected to a wide variety of conditions that may induce aggregation. Changes in temperature and concentration during fermentation, changes in pH and concentration during downstream processing. Even shear forces (from impeller blades, stirrers and other engineering plant equipment) can result in stress related aggregation. The presence of aggregates, particularly very large multimers up to sub-visible particles, are potentially harmful to health. It is therefore a prerequisite that the level of aggregation is quantified and determined, and limits are put into place. Size exclusion chromatography is one of the popular techniques that is particularly suited to the separation of monomer peaks from higher order aggregates and lower molecular fragments, in combination with a suitable concentration detector such as UV or DAD, quantification as relatively straightforward. More complex macromolecules such as monoclonal antibodies (mAbs) may be more challenging due to their larger structure (150kDa) and hydrophobicity that can result in unwanted interactions with many size exclusion chromatography columns. To address this issue, Agilent has developed a new stationary phase that demonstrates greatly reduced risk of secondary interactions. Agilent AdvanceBio SEC columns are packed with highly uniform particles with newly featured 1.9 µm, containing a low-binding, polymer-coated, silica stationary phase that provides efficient separations and minimal non-specific interactions. The AdvanceBio SEC columns are therefore ideally suited for rapid separation and quantification of aggregates and low molecular fragments.

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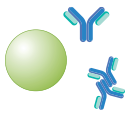

Aggregate/ Fragment Analysis

Size exclusion chromatography

Accurate, precise quantitation for a broad range of biomolecule separations

AdvanceBio SEC

Versatile performance for routine and challenging applications

Attribute	Advantage
Hydrophilic polymer coating	Avoid secondary interactions
Increased analytical speed	Meet vital deadlines
Higher reproducibility	Reduce rework
Greater sensitivity	Quantitate aggregates, even at low levels

Bio SEC-3 and Bio SEC-5

Extra wide pore and scale-up options

Attribute	Advantage
Compatibility with most aqueous buffers	Method flexibility
Wide range of pore size options, including 1000 Å and 2000 Å	Options for everything from peptides to VLPs
Analytical and semi-prep dimensions	Easy scale up or down

Size exclusion chromatography (SEC) is a chromatographic method where molecules in solution are separated by size or, more specifically, by hydrodynamic radius. When applied to large molecules such as mAbs, the larger components of the sample mixture, like aggregates, are excluded from the pores in the chromatographic media and elute first, whereas the smaller components (e.g., heavy chain, fragments, free light chain, clips) diffuse further into the pores and elute later in subsequent peaks according to their size.

Currently, SEC separations are performed in HPLC or UHPLC mode (i.e., SEC-HPLC or SEC-UHPLC) under native or denaturing conditions using specialized, high-performance systems. SEC-UHPLC mode, which operates under the same principles as HPLC, is a step improvement in liquid chromatography based on advances in column particle chemistry performance, system optimization, detector design, and data processing. For mAbs, an SEC chromatogram with good separation will feature an antibody monomer elution peak with a symmetrical shape and allow optimal resolution of higher molecular weight forms (i.e., aggregates) to resolve as distinct earlier peaks relative to the main monomer peak.

Since protein aggregation can occur as a result of external factors, one of the most important steps in aggregate analysis is sample preparation. It is necessary to ensure the proteins are fully dissolved in the mobile phase but not subjected to factors that may alter the level of aggregation, such as sonication, temperature, pH and excessive concentration.

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Size exclusion chromatography is a relatively straightforward technique relying on isocratic elution. The mobile phase conditions should ensure there is no secondary interactions between the protein and the column stationary phase. Typically, aqueous buffers such as sodium phosphate (100-200 mM) or PBS (phosphate buffered saline) at neutral pH are used. It is important to use freshly prepared buffer solutions and to filter it through 0.2 µm filter. Under no circumstance should unused buffer be left on the instrument for a prolonged period since these eluents provide ideal conditions for bacterial growth. SEC separations are generally monitored by UV detection at 280 nm, low wavelengths (210 – 220 nm) can greatly improve the sensitivity of the technique.

The resolved molecular species can also be characterized by on-line light scattering techniques such as MALS or may be collected for off-line analysis. With use of volatile buffers such as ammonium acetate it is possible to perform native SEC-MS and this technique is being widely explored for online SEC monitoring with direct mass spec characterization. Preservatives, such as 20% ethanol, are recommended for long term column storage, however care must be taken since the viscosity of the mobile phase containing organic modifiers is often significantly higher and column damage could ensue when performed at higher operating flowrates.

The featured Application note (5994-0876EN) demonstrates method development with an Agilent AdvanceBio SEC 200 Å 1.9 µm column for SEC analysis of the NISTmAb.

Size Exclusion Chromatography (SEC)

Application	Agilent Columns	Notes	USP Designation
Peptides, proteins, aggregate analysis	AdvanceBio SEC	Robust hydrophilic polymer coating yielding minimal secondary interactions; 2.7 µm particles with 130 Å or 300 Å, or 1.9 µm particles with 120 Å or 200 Å pore size for highest resolution.	L59
	Bio SEC-3	Higher resolution and faster separation from 3 µm particles, with 100 Å, 150 Å, and 300 Å pore sizes	L59
Large biomolecules and samples with multiple molecular weight components	Bio SEC-5	More pore size options (100 Å, 150 Å, 300 Å, 500 Å, 1000 Å, and 2000 Å) to cover a wider range of analytes.	L59
Globular proteins, antibodies	ProSEC 300S	Single column option for protein analysis in high salt conditions	L33
Proteins, globular proteins	ZORBAX GF-250/450	Legacy products that should be employed where protocols still require use of USP designation L35.	L35

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General recommendations to consider in SEC separation

Column selection

- To ensure sample integrity, SEC is carried out slowly on long columns
- Column lengths are typically 250 or 300 mm
- Normal flow rate is 1.0 mL/min on a 7.5 or 7.8 mm id column and 0.35 mL/min on a 4.6 mm id column
- Columns are often run in series to increase resolution in biopolymer applications
- Smaller particle sizes are used to increase resolution in protein applications
- Separations done on 150 mm columns with smaller particle sizes can reduce analytical time

Column media choice

- There should be no non specific interactions between analytes and column media
- Silica-based sorbents are used for analyzing peptides and proteins
- Polymer-based sorbents are for analyzing biopolymers

Column parameters

- **Pore size**–depends on the molecular weight range of the sample to avoid exclusion of sample components and maximize volume in the required separation region
- **Particle size**–use smaller particles for higher resolution (but higher backpressure)
- **Column length**–compromise between resolution and analysis time
- **Column id**–use smaller columns for reduced solvent consumption and smaller injection volume

Sample preparation

- Ideally, samples should be dissolved in the mobile phase
- If the sample is cloudy, it may be necessary to change the mobile phase conditions
- Filtration or centrifugation can be used to clarify samples, but these processes could alter the molecular weight composition of the sample
- To dissolve a sample, gentle heating, vortexing, or sonication is sometimes used, but should be applied with caution because this can alter the molecular weight composition
- Care should also be taken to ensure the sample does not change during storage
- Samples should be made up fresh and analyzed as soon as possible
- Bacterial growth can develop quickly in buffer solutions
- Samples made up at high concentration can also change over time, leading to aggregation or even precipitation

Mobile phase

- Mobile phase should contain buffer/salt to overcome ionic interactions, but too much may cause hydrophobic interactions
- Do not alter the analyte to avoid degradation/ aggregation, etc
- Make up fresh mobile phase and use promptly, as bacterial growth is rapid in dilute buffer stored at room temperature
- Buffer shelf life is less than 7 days unless refrigerated
- Filter before use to remove particulates in water (less likely) or in buffer salts (more likely)
- High pH phosphate buffers (particularly at elevated temperature) can significantly reduce column lifetime when using silica columns

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Quick Reference Guide

Size Exclusion Chromatography UV/DAD Workflow

Agilent AdvanceBio SEC 2.7 µm Columns

In this document Agilent applications chemists share their recommendations for an optimum LC system and its confi guration for characterizing biomolecules. They also offer guidance on a generic method to get you started, and how this method can be further optimized to meet your specifi c separation goals. Additional application information is available at **www.agilent.com/chem/advancebio**.

Guidelines

- Protein aggregation is impacted by various environmental factors, including pH, ionic strength, and temperature. To quantify levels of aggregation use a mobile phase that does not affect the sample. Typically, start with 150 mM phosphate buffer, pH 7.0.
- For routine SEC use a 300 mm column.
- To increase sample throughput use a 150 mm column and increase the flow rate.
- To increase resolution use two columns in series.
- SEC is a non-interactive LC technique and so small injection volumes must be used to achieve efficient separations. Sample size should be ≤5% of total column volume.
- AdvanceBio 2.7 µm SEC columns are recommended for SEC/DAD, SEC/UV, and SEC/LS applications. For SEC/MS methods Agilent recommends Bio SEC-3 columns.
- To maximize column lifetime Agilent recommends using a guard column and not to exceed 200 bar operating pressure when using a single column or 400 bar for two columns in series.

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Agilent 1260 Infinity Bio-Inert LC System

Mobile phases

Isocratic elution with freshly prepared aqueous or aqueous/ organic buffers. Ensure all components of the mobile phase are soluble and filter, 0.2 µm, before use.

Pump (G5611A)

0.1 to 2.0 mL/min for 7.8 mm id
0.1 to 0.7 mL/min for 4.6 mm id.

Sample injection (G5667A)

5-10 µL injection for samples contain 1-5 mg/mL of protein.

Column compartment (G1316C)

20-30 °C is typical temperature used for SEC of biologically active proteins.

Detection (G1315D)

DAD with a bio-inert standard flow cell, 10 mm.

Analytical columns

Description	130Å	300Å
4.6 x 300 mm, 2.7 µm	PL1580-5350	PL1580-5301
4.6 x 150 mm, 2.7 µm	PL1580-3350	PL1580-3301
7.8 x 300 mm, 2.7 µm	PL1180-5350	PL1180-5301
7.8 x 150 mm, 2.7 µm	PL1180-3350	PL1180-3301

Analytical guards

Description	130Å	300Å
4.6 x 50 mm, 2.7 µm	PL1580-1350	PL1580-1301
7.8 x 50 mm, 2.7 µm	PL1180-1350	PL1180-1301

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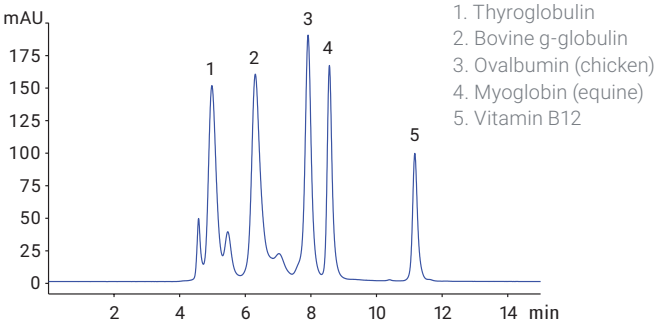
AdvanceBio SEC column choice

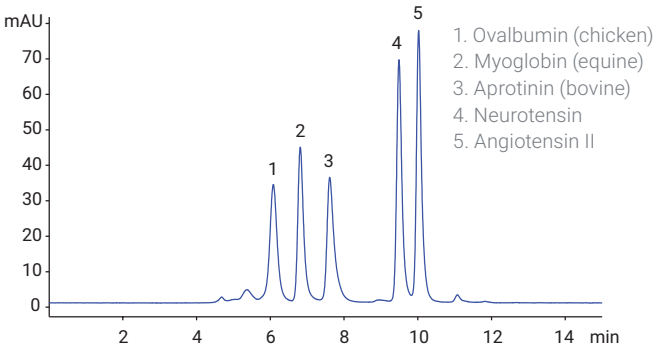
AdvanceBio SEC 300Å, protein resolving range 5 – 1,200 KD



Conditions

Parameter	Value
Column:	AdvanceBio SEC 300Å, 7.8 x 300 mm
	BioRad Gel Filtration Standard #1511901
Mobile phase:	150 mM sodium phosphate, pH 7.0

AdvanceBio SEC 130Å, protein resolving range 0.1 – 100 KD

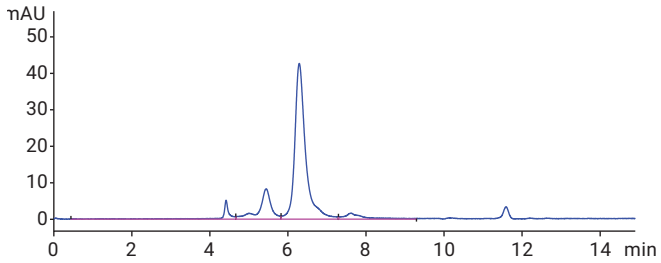


Conditions

Parameter	Value
Column:	AdvanceBio SEC 130Å, 7.8 x 300 mm
Mobile phase:	150 mM sodium phosphate, pH 7.0

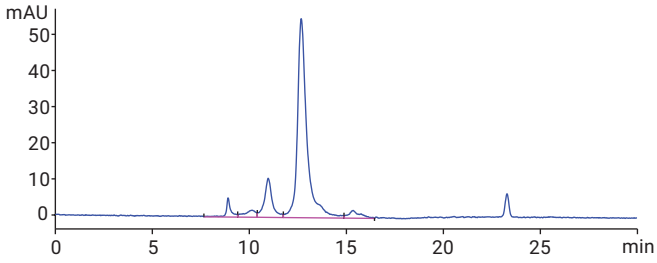
Increasing resolution

Two columns can be run in series to increase resolution.



Conditions

Parameter	Value
Column:	1 x AdvanceBio SEC 300Å, 7.8 x 300 mm)
Run time:	15 min Rs (monomer/dimer) = 2.06
Mobile phase:	150 mM sodium phosphate, pH 7.0
Sample:	IgG



Conditions

Parameter	Value
Column:	2 x AdvanceBio SEC 300Å, 7.8 x 300 mm)
Run time:	30 min Rs (monomer/dimer) = 2.59
Mobile phase:	150 mM sodium phosphate, pH 7.0
Sample:	IgG

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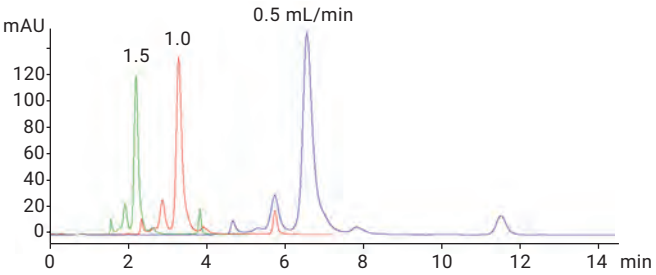
Reducing analysis time

Use a shorter column, 150 mm, and for further savings run at higher flow rate.



Conditions

Parameter	Value
Column:	1 x AdvanceBio SEC 300Å, 7.8 x 150 mm
Mobile phase:	150 mM sodium phosphate, pH 7.0
Sample:	IgG

Conditions

Flow rate	Rs (monomer/dimer)	Run time
0.5 mL/min	1.94	15 min
1.0 mL/min	1.63	8 min
1.5 mL/min	1.46	4 min

Application Note

Biologics

Size Exclusion Chromatography Method Development of NISTmAb Using an Agilent AdvanceBio SEC 200 Å 1.9 µm Column

Author

Veronica Qin
Agilent Technologies, Inc.

Abstract

This Application Note demonstrates method development with an Agilent AdvanceBio SEC 200 Å 1.9 µm column for size exclusion chromatography (SEC) analysis of the NIST monoclonal antibody (mAb). A wide range of mobile phase combinations can easily be screened with the bio-inert quaternary pump of the Agilent 1260 Infinity II Bio-inert LC system and Agilent Buffer Advisor software.

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Introduction

SEC is a commonly used technique to characterize and quantify size variants from biotherapeutic proteins. A variety of different mobile phase conditions often need to be evaluated to improve peak shape and resolution for a protein of interest. The AdvanceBio SEC 200 Å 1.9 µm column with its unique bonding chemistry offers reduced secondary interactions under different buffer conditions. SEC method development can be time-consuming, with the requirement of screening a number of different buffer compositions and pH combinations. However, Buffer Advisor software, combined with a bio-inert quaternary LC pump provides a simple way of online mobile phase optimization for SEC analysis. This Application Note presents SEC method development for characterizing the NISTmAb.

Experimental

Materials

NISTmAb (RM 8671) (10 mg/mL) was purchased from NIST SRM standards. Monobasic and dibasic sodium hydrogen phosphate (NaH_2PO_4/ Na_2HPO_4) and sodium chloride (NaCl) were purchased from MilliporeSigma. All chemicals used were ≥99.5 % pure. Water was purified from a Milli-Q A10 water purification system (Millipore). Mobile phases were prepared fresh daily and filtered through a 0.22 µm membrane filter prior to use.

Instrumentation

An Agilent 1260 Infinity II Bio-inert LC system with the following configuration was used:

- Agilent 1260 Infinity II Bio-inert quaternary pump (G5654A)
- Agilent 1260 Infinity II Bio-inert multisampler (G5668A) with sample cooler (option #100)
- Agilent 1260 Infinity II multicolumn thermostat (G7116A) with bio-inert heat exchanger (option #019)
- Agilent 1260 Infinity II variable wavelength detector (G7114A)

Column

Agilent AdvanceBio SEC 200 Å 1.9 µm, 4.6 × 300 mm (p/n PL1580-5201)

Software

- Agilent OpenLab CDS 2.2 software
- Agilent Buffer Advisor software

Results and discussion

Conditions

Parameter	Settings
Column Temperature:	25 °C
Mobile phase:	A) Water B) 1 M NaCl C) 245 mM NaH_2PO_4 D) 420 mM Na_2HPO_4
Flow Rate:	0.35 mL/min
Injection Volume:	3 µL
Detection:	UV at 280 nm

To screen a variety of mobile phase conditions for NISTmAb SEC analysis, the following stock solutions were prepared:

- 1 M NaCl
- 245 mM NaH_2PO_4
- 420 mM Na_2HPO_4

Using these stock solutions, it was possible to create sodium phosphate buffer concentrations from 150 to 350 mM without the addition of NaCl, using Buffer Advisor software. In addition, combinations of sodium phosphate buffer with NaCl present at varying concentration were also evaluated. Mobile phase pH was tested at four different values: pH 6.6, 6.8, 7.0, and 7.4. In Buffer Advisor software, we can enter different method development screening conditions including buffer concentration, salt concentration, and pH. The software can then automatically calculate the correct percentage of each stock solution needed to achieve the desired mobile phase conditions. Table 1 shows several selected screening conditions and mobile phase compositions calculated by Buffer Advisor software.

Table 1. Selected screening conditions and mobile phase compositions calculated by Buffer Advisor software.

Buffer (mM)	NaCl (mM)	pH	Software-Calculated Mobile Phase Compositions			
			A%	B%	C%	D%
150	0	6.6	49.9	0.0	34.5	15.6
150	0	6.8	52.6	0.0	27.9	19.5
150	0	7	55.3	0.0	21.5	23.2
150	0	7.4	59.6	0.0	11.3	29.1
50	250	6.6	58.8	25.0	10.2	6.0
50	250	6.8	59.8	25.0	7.9	7.3
50	200	7.4	66.8	20.0	3.1	10.1
25	250	7.0	67.8	25.0	3.0	4.2

A: Water
B: 1 M NaCl
C: 245 mM NaH_2PO_4
D: 420 mM Na_2HPO_4

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Figure 1 shows SEC chromatograms of NISTmAb under 150 mM sodium phosphate without the addition of any NaCl at pH ranging from 6.6 to 7.4. As shown in Table 2, at this buffer concentration, a higher pH at 7.4 results in better peak shape and dimer/monomer resolution. In addition, the peak area percentage of high molecular weight species (HMW%) increases gradually from pH 6.6 to 7.4 (Figure 2), and at pH 7.4 the value is close to 3 % as reported by NIST's evaluation of RM 8671[1].If we set pH at 7.4, and increase the buffer concentration from 150 mM up to 350 mM, peaks have less tailing at 300 and 350 mM, but dimer/monomer resolution continues to drop with increased concentration (Table 3). Under mobile phase compositions without NaCl, 150 mM of sodium phosphate at pH 7.4 gives the best result, with a balance of peak shape and resolution and more accurate HMW%.

In addition, mobile phases consisting of different concentrations (25 to 100 mM) of sodium phosphate and 250 mM NaCl at the four pH values were evaluated by comparing peak symmetry and dimer/monomer resolution (results shown in Table 4). It was shown that 50 mM sodium phosphate and 250 mM NaCl at pH 6.8 gave the best result, with a balance of peak shape and resolution. Using 25 mM phosphate, and 250 mM NaCl, pH at 6.8 or above, gave more accurate HMW% results (Figure 3).

Table 2. Effect of pH on peak symmetry and dimer/monomer resolution.

Buffer (mM)	NaCl (mM)	pH	Asymmetry (As)	Resolution (Dimer/Monomer)
150	0	6.6	1.49	2.33
150	0	6.8	1.43	2.35
150	0	7	1.42	2.67
150	0	7.4	1.41	2.78

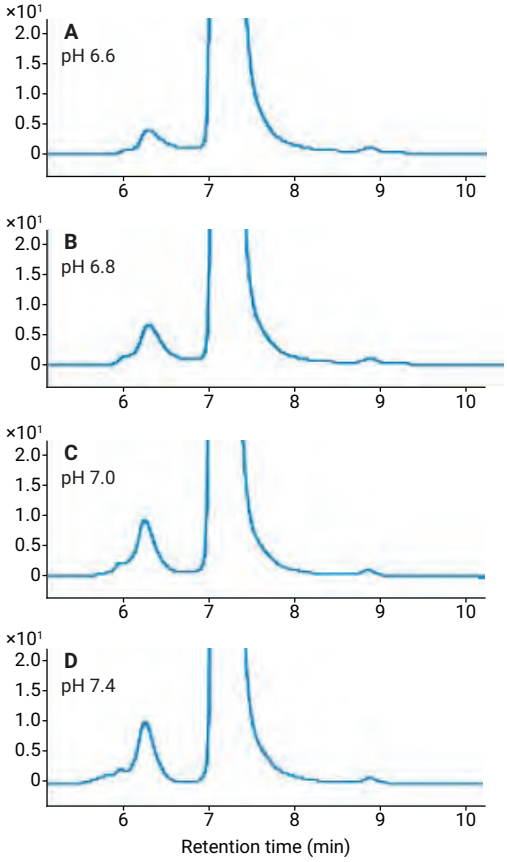

Figure 1. Size exclusion chromatograms of NISTmAb using 4.6 × 300 mm SEC columns running at 0.35 mL/min under 150 mM sodium phosphate at A) pH 6.6, B) pH 6.8, C) pH 7.0, and D) pH 7.4.

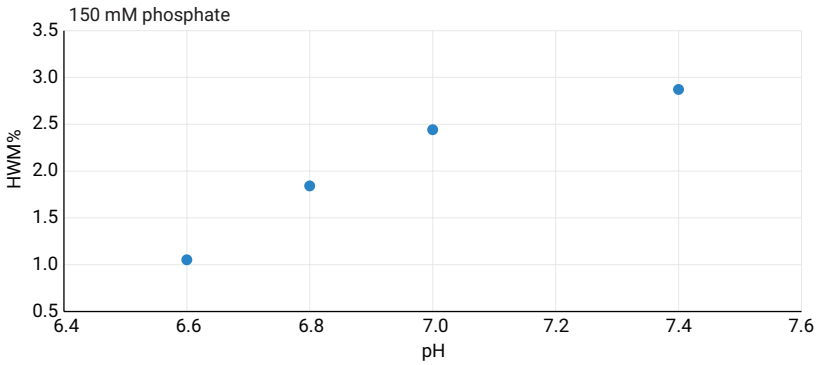

Figure 2. Peak area percentage of HMW of NISTmAb under 150 mM phosphate at different pH values.

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Table 3. Effect of buffer concentration (without NaCl) on peak symmetry and dimer/monomer resolution.

Buffer (mM)	NaCl (mM)	pH	Asymmetry (As)	Resolution (Dimer/Monomer)
150	0	7.4	1.41	2.78
200	0	7.4	1.45	2.60
250	0	7.4	1.42	2.57
300	0	7.4	1.40	2.45
350	0	7.4	1.38	2.33

Table 4. Effect of buffer concentration (with NaCl) and pH on peak symmetry and dimer/monomer resolution.

Buffer (mM)	NaCl (mM)	pH	As	Rs (Dimer/Monomer)
25	250	6.6	1.36	2.73
25	250	6.8	1.36	2.86
25	250	7	1.35	2.83
25	250	7.4	1.37	2.86
50	250	6.6	1.35	2.87
50	250	6.8	1.33	2.86
50	250	7	1.36	2.85
50	250	7.4	1.36	2.84
100	250	6.6	1.36	2.87
100	250	6.8	1.36	2.89
100	250	7	1.35	2.83
100	250	7.4	1.37	2.80

Using 50 mM phosphate and 250 mM NaCl, or 100 mM phosphate and 250 mM NaCl, we obtained accurate HMW% at the four pH values were evaluated by comparing peak symmetry and dimer/monomer resolution (results shown in Table 4). It was shown that 50 mM sodium phosphate and 250 mM NaCl at pH 6.8 gave the best result, with a balance of peak shape and resolution. Using 25 mM phosphate, and 250 mM NaCl, pH at 6.8 or above, gave more accurate HMW% results (Figure 3).

Using 50 mM phosphate and 250 mM NaCl, or 100 mM phosphate and 250 mM NaCl, we obtained accurate HMW% at any of the four pH values. Therefore, with these two mobile phase compositions, pH had negligible effect on HMW% results (Figure 4). Table 5 compares the effect of salt concentration and pH on peak symmetry and dimer/monomer resolution when setting buffer concentration at 50 mM. Among these conditions, 50 mM sodium phosphate with 250 mM NaCl at pH 6.8 gave the best result, with a balance of peak shape and resolution. Using 50 mM phosphate, and 200 mM NaCl, similar to 25 mM phosphate with 250 mM NaCl, pH at 6.8 or above, gave more accurate HMW% results (Figure 5). Overall, considering peak symmetry, dimer/monomer resolution, and HMW% accuracy, the optimum mobile phase composition is 50 mM sodium phosphate, 250 mM NaCl at pH 6.8 (chromatogram shown in Figure 6).

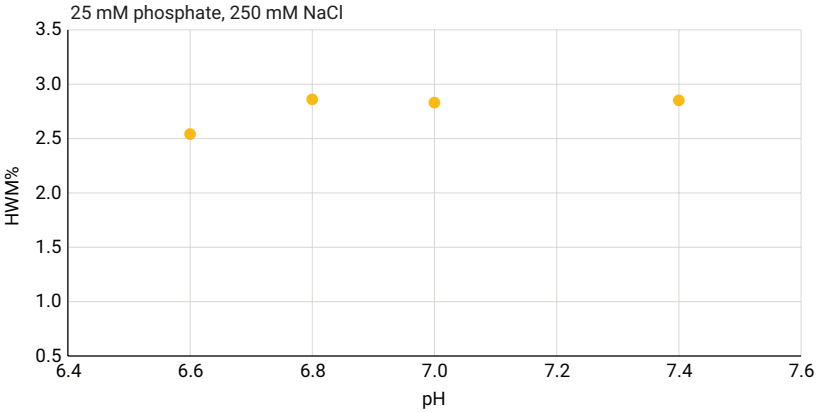

Figure 3. Peak area percentage of HMW of NISTmAb under 25 mM phosphate, 250 mM NaCl at different pH values.

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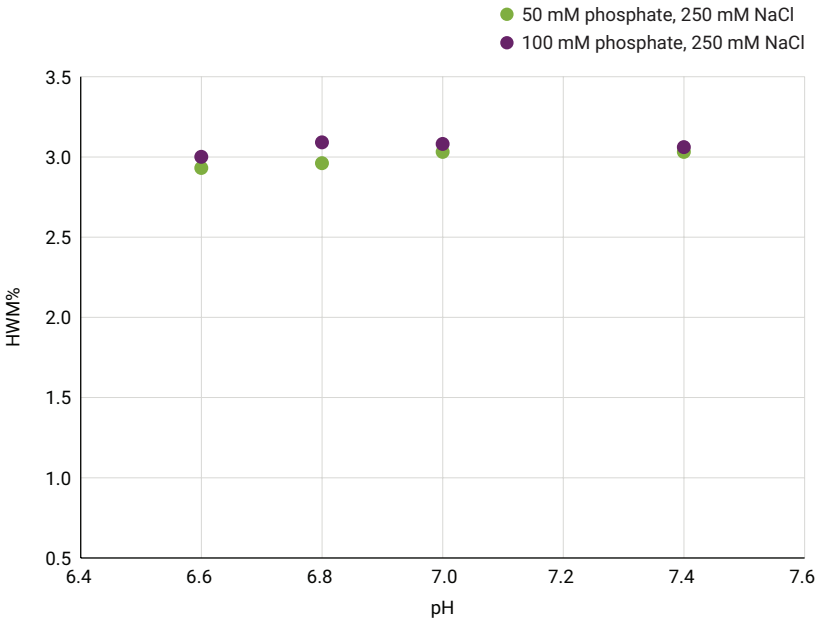

Figure 4. Peak area percentage of HMW of NISTmAb under 50 mM phosphate, 250 mM NaCl or 100 mM phosphate, 250 mM NaCl at different pH values.

Table 5. Effect of salt concentration and pH on peak symmetry and dimer/monomer resolution.

Buffer (mM)	NaCl (mM)	pH	As	Rs (Dimer/Monomer)
50	250	6.6	1.35	2.87
50	250	6.8	1.33	2.86
50	250	7	1.36	2.85
50	250	7.4	1.36	2.84
50	200	6.6	1.36	2.68
50	200	6.8	1.35	2.83
50	200	7	1.36	2.87
50	200	7.4	1.36	2.88

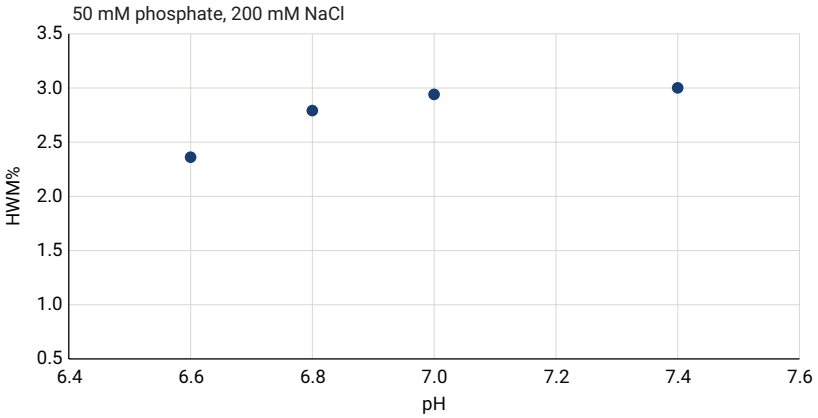

Figure 5. Peak area percentage of HMW of NISTmAb under 50 mM phosphate, 200 mM NaCl at different pH values.

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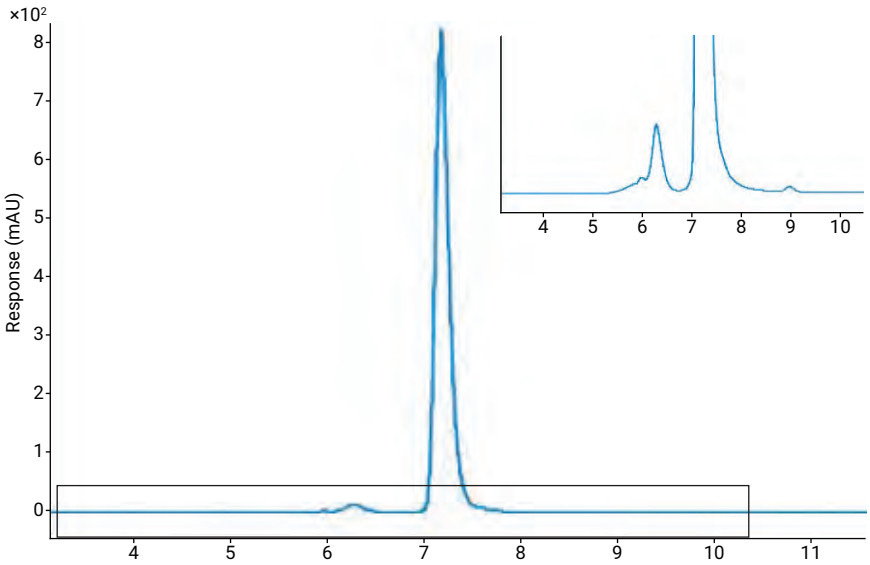

Figure 6. Size exclusion chromatogram of NISTmAb using 4.6 × 300 mm SEC columns running at 0.35 mL/min under 50 mM sodium phosphate, 250 mM NaCl, pH 6.8.

Conclusion

This study demonstrates a simplified approach to mobile phase optimization for SEC analysis of NIST mAb (RM 8671) with the use of an AdvanceBio SEC 200 Å 1.9 µm column together with the bio-inert quaternary pump of the 1260 Infinity II Bio-inert LC system, and Buffer Advisor software. Optimized mobile phase combination and pH is selected considering peak symmetry, dimer/monomer resolution, and quantitation accuracy.

Reference

1. Schiel, J. E.; *et al.* The NISTmAb Reference Material 8671 value assignment, homogeneity, and stability, *Anal. Bioanal. Chem.* **2018**, *410*, 2127–2139.

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