APPLICATION

Mobile Phase Optimization for Aggregate Analysis of Monoclonal Antibodies

Dani Xing and Brian Rivera
Phenomenex, Inc., 411 Madrid Ave., Torrance, CA 90501 USA

Dani Xing
Technical Specialist
Traveling is becoming one of my favorite things to do but I love finding a good place to eat or napping in the afternoon after reading a good book. I hike too and Krav Maga (is that a verb?)! …So I can keep eating :)

Introduction

Size exclusion chromatography (SEC) is a non-adsorptive separation that fractionates analytes based upon hydrodynamic volume. Because it is non-denaturing, it is the most common method for determination of protein aggregate in a sample. For protein therapeutics, quantitation of aggregate is a critical quality attribute (CQA) since aggregate can have lower efficacy and potentially be immunogenic.

Ideally, aggregate analysis by SEC is done in the protein formulation buffer. However, it is critical that an SEC separation be completely "entropic"- that is, reducing or eliminating electrostatic and hydrophobic interactions that might occur between the protein and the functionalized SEC silica column. Buffer formulations for the protein therapeutic, depending on ionic strength and composition, might not have optimal chromatography.

This is especially true with monoclonal antibodies (mAbs), which are the most common protein therapeutic platform, with approximately 70 mAbs predicted to be in the global market by 2020.[1] mAb characterization is complicated by the varied physicochemical properties of these complex, 150 kD proteins- isoelectric points vary, typically 7-9. Relative hydrophobic index also is variable and can be unpredictable based upon many factors like buffer formulation and protein concentration.[2]

For some mAbs, a formulation like 1X Phosphate Buffered Saline might be an acceptable mobile phase. However, recent work by Goyon and colleagues showed the effects of sodium and potassium additives in SEC, and how salt concentration, as well as buffer type and concentration, can affect separation and aggregate recovery significantly.[3]

With advances in chromatography, sub-2 µm are becoming the common not only in small molecule, but large molecule as well. SEC is no exception, as throughput demands and characterization needs increase. mAb aggregate is often at very low levels (<0.1 % by peak area compared to monomer) and fragment separation is also a method requirement. In these instances, the high efficiency, sub-2 µm SEC column can increase sensitivity of the method and improve separation of closely eluting fragments. This is of course, provided optimal mobile phase conditions are used.

In this technical note, two monoclonal antibodies are analyzed using sub-2 µm SEC columns using optimized mobile phase conditions. Modulation of the salt in the mobile phase showed improvements in peak shape and recovery.

Materials and Methods

Reagents and Chemicals

Monoclonal antibodies samples were graciously gifted from a customer. Potassium Chloride and Potassium Phosphate (monobasic and dibasic) were purchased from Sigma Aldrich (St. Louis, MO, USA). Mobile phase was filtered using a 0.2 µm Phenex™ Nylon Filtration Membrane and samples were filtered with a 0.45 µm Phenex Syringe Filter.

Experimental Conditions

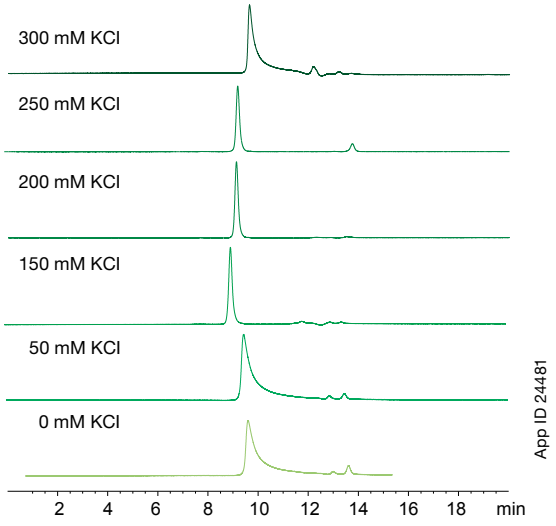
HPLC Conditions

HPLC was performed using Yarra™ 1.8 µm SEC-X300 run on an Agilent® 1100 equipped with a UV-Vis Detector. Because minimizing system dwell volume is critical for high performance with sub-2 µm SEC columns, it is important to note that the system used was equipped with a 2 µL micro flow cell (G1315-60024) and was plumbed with 0.005" ID PEEK tubing.

Preliminary mobile phase conditions started with the standard United States Pharmacopeia method of 0.2 M KH_2PO_4 250 mM KCl, pH 6.2. However, peak shape was considerably better using 50 mM KH_2PO_4 at pH 6.8 (data not shown). From there, KCl amounts were varied as indicated in **Table 1**.

Column:	Yarra 1.8 µm SEC-X300
Dimensions:	300 x 4.6 mm
Part No.:	00H-4745-E0-SS
Mobile Phase:	50 mM KH_2PO_4, pH 6.8 KCl (as indicated)
Flow Rate:	0.3 mL/min
Detection:	UV-Vis @ 280 nm
Temperature:	Ambient
LC System:	Agilent 1100

Figure 1:
mAb 1, SEC Profiles



APPLICATIONS

Table 1.
mAb 1, Results

Salt Concentration (mM KCl)	Retention Time (t)	Peak Width	Peak Symmetry
0	9.46	0.445	0.215
50	9.47	0.4374	0.223
150	9.02	0.1665	0.764
200	9.10	0.1557	0.788
250	9.15	0.1594	0.785
300	9.52	0.3069	0.193

Figure 2.
mAb 2, SEC Profiles

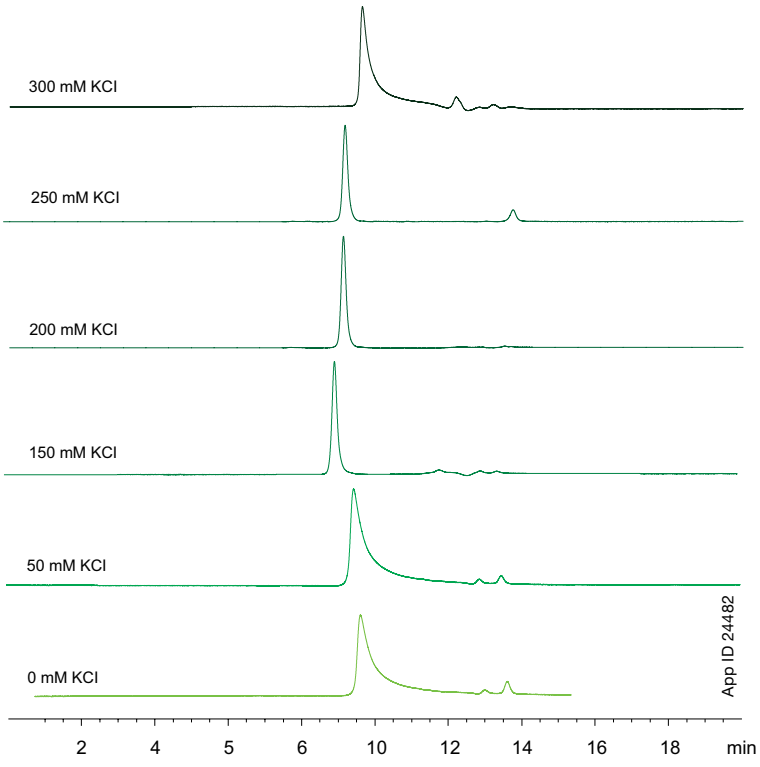


Figure 3.
mAb 2, SEC Profile

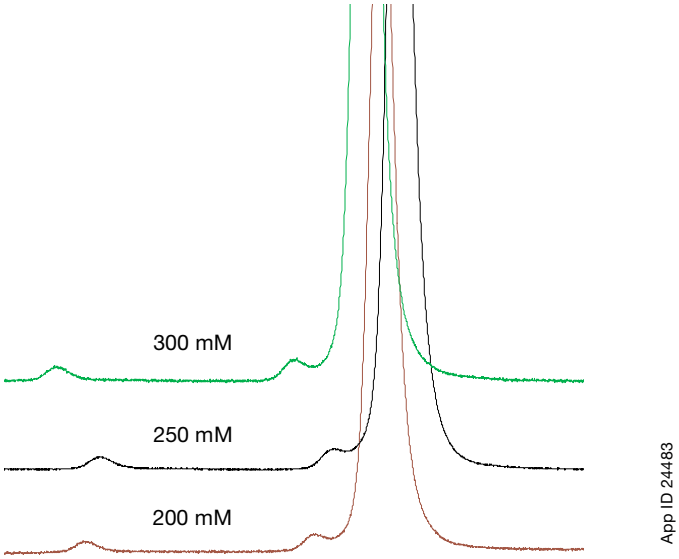


Table 2.
mAb 2, Results

Salt Concentration (mM KCl)	Retention Time (t)	Peak Width	Peak Symmetry
0	9.63	0.1472	0.661
50	9.60	0.1718	0.78
150	9.77	0.1753	0.785
200	9.82	0.1767	0.788
250	9.91	0.1822	0.778
300	9.96	0.1745	0.784

Results and Discussion

The results from mAb 1 clearly show how modulation of salt can affect peak shape. In **Figure 1**, we can see increases in salt concentration affect peak shape and chromatography. With no potassium chloride, peak shape is unacceptable. It isn't until 150 mM potassium chloride are peak symmetries within a reasonable value of 0.764. However, with the addition of more potassium chloride past 200 mM, there is a decrease in performance, with 300 mM showing even worse peak shape than no salt. This can be attributed to the addition of more chloride causing a "salting out" effect. That is, the mAb is now more hydrophobic, leading to secondary interactions with the stationary phase, causing the peak tailing. For mAb 1,200 mM gives the superior peak shape.

mAb 2 is an example of a more well-behaved mAb. That is, even with no potassium chloride in the mobile phase, peak shapes look reasonably good. The addition of even 50 mM potassium chloride gives peak symmetry of 0.78, with incremental improvements with each subsequent increase in potassium chloride concentration. In **Figure 2**, we do a see a small increase in retention time from 50 mM to 300 mM, indicating slight hydrophobic retention, but not to the determinant of overall peak shape.

In this case, it is more prudent to then look visually at chromatography. **Figure 3** shows an overlay with a zoomed in view of monomer and separation of pre-peak and high molecular weight aggregate. In this instance, there is an improvement between separation of pre-peak and monomer.

Conclusion

When developing a method for aggregate analysis of mAb's by SEC, it is critical to optimize mobile phase conditions to prevent non-specific secondary interactions. Here, the effect of salt is investigated for two different biosimilar mAb's. The first mAb required a moderate amount of salt for acceptable peak shape. The second mAb performed well even with no salt. However, increases in salt showed incremental improvements in peak shape.

Ideally, buffer and salt concentration are optimized, based upon the requirements for the method. However, when there is a need for a platform method- a general method used to evaluate several different mAb's- a good starting point for method development would be 50 mM KH_2PO_4 250 mM KCl, pH 6.8.

References:

1. Ecker DM, Jones SD, Levine HL. The therapeutic monoclonal antibody market. mAbs. 2015;7(1):9-14. doi:10.4161/19420862.2015.989042.
2. Haverick M, Mengisen S, Shameem M, Ambrogelly A. Separation of mAbs molecular variants by analytical hydrophobic interaction chromatography HPLC: overview and applications. mAbs. 2014;6(4):852-858. doi:10.4161/mabs.28693.
3. Goyon A, Beck A, Veuthey JL, Guillarme D, Fekete S. Comprehensive study on the effects of sodium and potassium additives in size exclusion chromatographic separations of protein biopharmaceuticals. J Pharm Biomed Anal. 2016. doi: 10.1016/j.jpba.2016.09.031

Ordering Information

Yarra™ 1.8 µm SEC Stainless Steel Columns (mm)

	Analytical	Analytical	SecurityGuard™ ULTRA Cartridges***
Phases	**150 x 4.6**	**300 x 4.6**	**3/pk**
Yarra 1.8 µm SEC-X150	00F-4631-E0-SS	00H-4631-E0-SS	AJ0-9512
Yarra 1.8 µm SEC-X300	00F-4743-E0-SS	00H-4743-E0-SS	AJ0-9513
***SecurityGuard ULTRA cartridges require holder, Part No.: AJ0-9000			For Stainless Steel Only

Yarra 1.8 µm SEC Bio-Inert Columns (mm)

	Analytical	Analytical
Phases	**150 x 4.6**	**300 x 4.6**
Yarra 1.8 µm SEC-X150	00F-4631-E0	00H-4631-E0
Yarra 1.8 µm SEC-X300	00F-4743-E0	00H-4743-E0

Phenex™ Syringe Filters		4 mm Diameter for ≤ 2 mL sample volumes		15 mm Diameter for 2 – 10 mL sample volumes		25 - 28 mm Diameter for 10 – 100 mL sample volumes	
	Membrane Type/Size	Part No.	Unit	Part No.	Unit	Part No.	Unit
0.20 µm	NY (Nylon)	AF3-3207-12	100/pk	AF0-2207-12	100/pk	AF0-1207-12	100/pk
		AF3-3207-52	500/pk	AF0-2207-52	500/pk	AF0-1207-52	500/pk
0.45 µm	NY (Nylon)	AF3-3107-12	100/pk	AF0-2107-12	100/pk	AF0-1107-12	100/pk
		AF3-3107-52	500/pk	AF0-2107-52	500/pk	AF0-1107-52	500/pk

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Australia
t: +61 (0)2-9428-6444
f: +61 (0)2-9428-6445
auinfo@phenomenex.com

Austria
t: +43 (0)1-319-1301
f: +43 (0)1-319-1300
anfrage@phenomenex.com

Belgium
t: +32 (0)2 503 4015 (French)
t: +32 (0)2 511 8666 (Dutch)
f: +31 (0)30-2383749
beinfo@phenomenex.com

Canada
t: +1 (800) 543-3681
f: +1 (310) 328-7768
info@phenomenex.com

China
t: +86 400-606-8099
f: +86 (0)22 2532-1033
phen@agela.com

Denmark
t: +45 4824 8048
f: +45 4810 6265
nordicinfo@phenomenex.com

Finland
t: +358 (0)9 4789 0063
f: +45 4810 6265
nordicinfo@phenomenex.com

France
t: +33 (0)1 30 09 21 10
f: +33 (0)1 30 09 21 11
franceinfo@phenomenex.com

Germany
t: +49 (0)6021-58830-0
f: +49 (0)6021-58830-11
anfrage@phenomenex.com

India
t: +91 (0)40-3012 2400
f: +91 (0)40-3012 2411
indiainfo@phenomenex.com

Ireland
t: +353 (0)1 247 5405
f: +44 1625-501796
eireinfo@phenomenex.com

Italy
t: +39 051 6327511
f: +39 051 6327555
italiainfo@phenomenex.com

Luxembourg
t: +31 (0)30-2418700
f: +31 (0)30-2383749
nlinfo@phenomenex.com

Mexico
t: 01-800-844-5226
f: 001-310-328-7768
tecnicomx@phenomenex.com

The Netherlands
t: +31 (0)30-2418700
f: +31 (0)30-2383749
nlinfo@phenomenex.com

New Zealand
t: +64 (0)9-4780951
f: +64 (0)9-4780952
nzinfo@phenomenex.com

Norway
t: +47 810 02 005
f: +45 4810 6265
nordicinfo@phenomenex.com

Puerto Rico
t: +1 (800) 541-HPLC
f: +1 (310) 328-7768
info@phenomenex.com

Spain
t: +34 91-413-8613
f: +34 91-413-2290
espinfo@phenomenex.com

Sweden
t: +46 (0)8 611 6950
f: +45 4810 6265
nordicinfo@phenomenex.com

United Kingdom
t: +44 (0)1625-501367
f: +44 (0)1625-501796
ukinfo@phenomenex.com

USA
t: +1 (310) 212-0555
f: +1 (310) 328-7768
info@phenomenex.com

All other countries
Corporate Office USA
t: +1 (310) 212-0555
f: +1 (310) 328-7768
info@phenomenex.com

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