Doing HPLC Method Development for Monoclonal Antibody Aggregates? Wait a “SEC”!

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Contrary to popular belief, **size exclusion chromatography** (SEC) isn’t straightforward. However, it should be—it’s a non-absorptive separation, right? Just look up an SEC column’s calibration curve, use a standard mobile phase like 1X PBS, and *voila*, done and done.

Of course, that isn’t the case. For a true, “ideal SEC” separation, the separation must be completely “entropic.” ***What does that mean?*** To separate analytes by their hydrodynamic volume, electrostatic and hydrophobic interactions must be minimized. These secondary interactions are a source of band broadening and poor peak shape that can drastically effect an already inherently low-resolution technique. These secondary interactions can also be strong enough to effect protein recovery. And most SEC methods, especially for proteins and aggregate analysis, are quantitative. So, recovery of aggregate in an SEC method might be a bit of a concern.

Monoclonal antibodies are a special situation, as reducing both secondary **ion exchange** and hydrophobic interactions is a tall order. mAbs are relatively large (~150kD), so their aggregates are ginormous (yes, that is a word). So, you can imagine, mAb aggregate; i.e. agglomerated mAb monomers, can get very hydrophobic. Further, depending on the sequence, mAbs can be heavily charged—especially under physiological pH. Panitumumab

and Palivizumab, for example, both have isoelectric points greater than 9, so secondary ion exchange interactions are to be expected with pH’s that high.

As such, method development should be considered to develop a robust, reproducible SEC method. So much for SEC being plug and play.

There are plenty of SEC method development parameters to adjust for a mAb SEC profile. Certainly, pore size/exclusion range of a column is a consideration, though, for the most part, the standard 290-300Å pore size has shown time and time again that this is the best column for mAb aggregate analysis. Flow rate can also be used to improve separation of higher molecular weight species; lower the flow rate, instant increase in resolution.

But the single most impactful thing that one can do for improved SEC separation of mAbs is optimization of mobile phase.

Now, 1X PBS or the standard 0.2 M Potassium Phosphate, 250 mM Potassium Chloride, pH 6.2 might work for your method. The mAb you’re working with could be relatively well behaved under those conditions. However, physicochemical properties—namely hydrophobicity—of mAbs can be nearly impossible to predict. And each mAb behaves differently. Cetuximab will not behave like trastuzumab, which won’t behave like rituximab. And these are IgG1’s—we haven’t even talked about isotypes like IgG4’s, which present a whole host of other challenges.

Further complicating this is the fact that, admittedly, every **HPLC column** manufacturer uses different surface chemistry to create an inert surface for ideal SEC separations. To assume that every SEC column behaves the same would be similar to thinking every C18 is the same, when we know, of course they are not.

Recall that silica is inherently acidic—simply using unmodified silica media for SEC of proteins would lead to strong electrostatic interactions that likely would cause adsorption. So HPLC media is functionalized with a hydrophilic silane, typically a “diol”. But even the so called “hydrophilic silane” will have some carbon and this can cause unwanted hydrophobic interactions.

Worst case scenario is, as mentioned before, poor recovery of very hydrophobic high molecular weight aggregate.

Thus, since every SEC chemistry is different, mobile phase can have a huge implication in method performance. Bonded density of the silane can affect the overall hydrophobicity of the SEC media, in which case, a kosmotropic buffer like phosphate might “salt out” the protein too much, thus causing hydrophobic interactions that lead to band broadening. Conversely, a lower bonded density could lead to higher silanol activity, thus electrostatic interactions might be too high. In this case, more salt, sodium chloride for instance, might be best.

So, I know you’re thinking to yourself, get to the point Brian, I’ve got to develop a method for a new lead mAb, where do I start?

Our best chance of success on our column chemistry: 50 mM Potassium Phosphate, 250 mM Potassium Chloride, pH 6.8. This should be the starting point for mAb analysis on any of our SEC columns.

Need to see more? Check out our latest technical note,

Mobile Phase Optimization for Aggregate Analysis of Monoclonal Antibodies

And the most enlightening insight about this study—potassium salts and buffers. Never again with the 1X PBS.

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Summary

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