Effect of Flow Rate on Reversed Phase Separations of Monoclonal Antibodies

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The analysis of intact monoclonal antibodies (mAbs) by reversed phase HPLC is a common technique for assessing protein variation, such as clipping and heterogeneity due to post-translational modifications. Whether for LC-UV impurity analysis or for confirming primary sequence using LC-MS, the analysis of intact mAbs can provide useful insight on a protein therapeutic.

Because of their slow diffusion rates, peak broadening is commonly observed with reversed phase analysis of proteins and other large molecules. This is also exacerbated by fundamental denaturing conditions of reversed phase LC, which can cause conformational changes to the protein, leading to further peak distortion.

However, analysis using superficially porous (core-shell) particles can minimize the diffusion distance, thus the amount of peak dispersion, that is inherent with large molecule LC analysis. Further, the significantly lower surface area of wide pore core-shell particles lends to an overall less hydrophobic phase and consequently lower organic solvent for elution. Utilizing a bioZen™ 2.6 µm WidePore C4 LC column helps to improve peak shape by reducing band broading and enhancing efficiency but other adjustments can be made to further improvements.

As such, core-shell particles allow for expanding experimental design to include flow-rate, which can have a significant impact on the separation, both selectivity and peak capacity. Here, we investigate the impact of increasing flow-rate for NIST mAb, a common protein standard for evaluation and benchmarking analytical methods for protein characterization.

In assessing chromatographic performance, peak width at half height was used as the target attribute. **Figure 1** shows a comparison of intact NIST mAb, run at varying flow-rates, from 0.5 mL to 0.8 mL. There was a marked improvement in peak widths running at 0.8 mL/min; interestingly though 0.6 mL/min performed slightly worse than 0.5 mL/min, as there is a drop in resolution between the pre-peak and main peak (**Figure 2**).

The effect of flow-rate changes is even more impactful for subunit analysis of mAbs. **Figure 3** shows a series of stacked chromatograms for reduced NIST mAb. Overall, there is a reduction in peak widths for both heavy chain and light chain with an increase in flow-rate. However, with heavy chain, we can observe not only a decrease in peak width at half height, but also an improvement in impurity profile, as another shoulder is observed at 0.8 mL/min when compared to 0.5 mL/min **(Figure 4).**

In summary, increases in flow-rate with core-shell particles, such as the bioZen WidePore C4, can overcome the slow diffusion rates inherent with large molecules. In general, increases in flow-rate should reduce peak widths, thus improving overall chromatographic performance. This can be most impactful with subunit analysis of monoclonal antibodies. However, as observed with intact NIST mAb at 0.6 mL/min, one should experimentally determine the chromatographic impact of running higher flow-rates, as unexpected results may occur.

LC Conditions

Column:	bioZen 2.6 µm WidePore C4
Catalog No.:	00D-4786-AN
Dimensions:	100 x 2.1 mm
Mobile Phase A :	0.1 % TFA in Water
Mobile Phase B:	0.1 % TFA in Acetonitrile
Gradient:	25-45% B in 6 minutes
Flow Rate:	*As indicated*
Temperature:	90°C
Detection:	UV-Vis @ 280 nm (Fig 1,2) UV-Vis @ 214 nm (Fig 3,4)
Sample:	NIST mAb (1 mg/mL)
Injection Volume:	2 µL

Figure 1:

Stacked chromatograms of intact NIST mAb. An improvement in peak width at half height is observed at 0.8 mL/min when compared to 0.5 mL/min (0.030 vs 0.033, respectively)

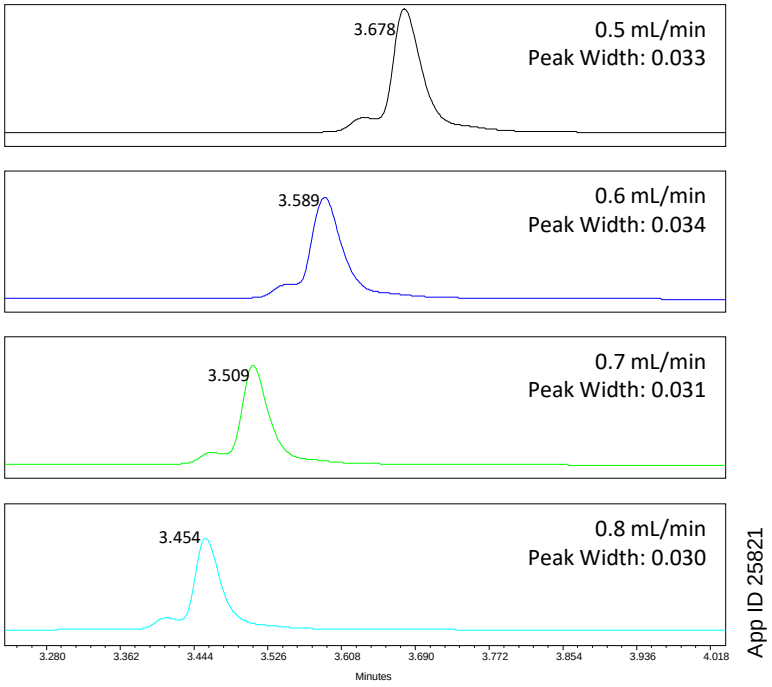


Figure 2:

Overlay of intact NIST mAb; note the loss of resolution with 0.6 mL/min. when compared to 0.5 mL/min. This may be due to linear velocity not being scaled with change in organic solvent per column volume.

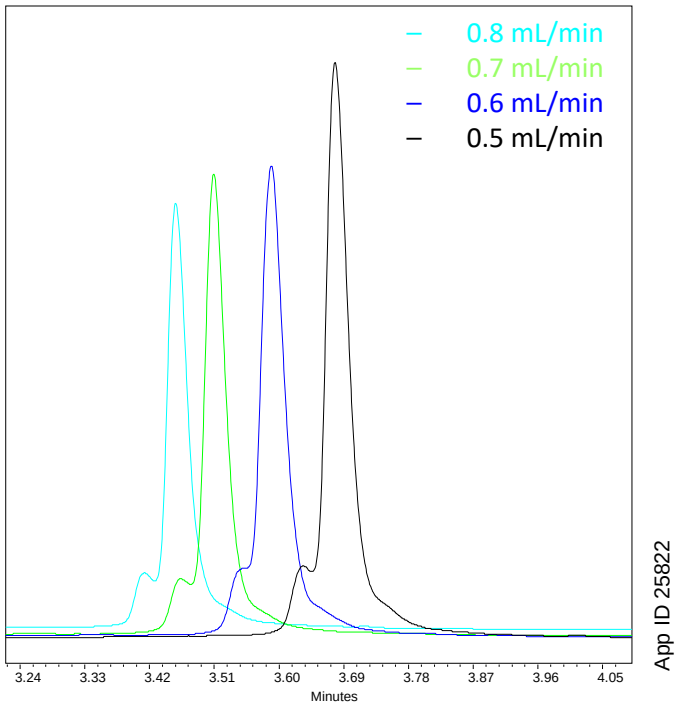


Figure 3:

Light Chain and Heavy Chain for NIST mAb. Peak widths at half height improved for the earlier eluting light chain with increasing linear velocity; with peak widths improving when flow-rate is increased to 0.8 mL/min compared to 0.5 mL/min (0.025 vs 0.029, respectively).

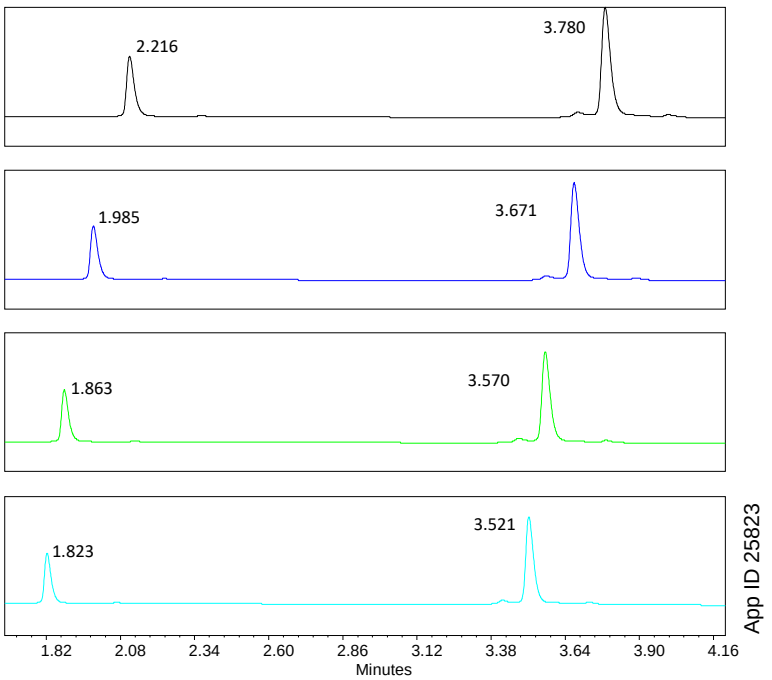
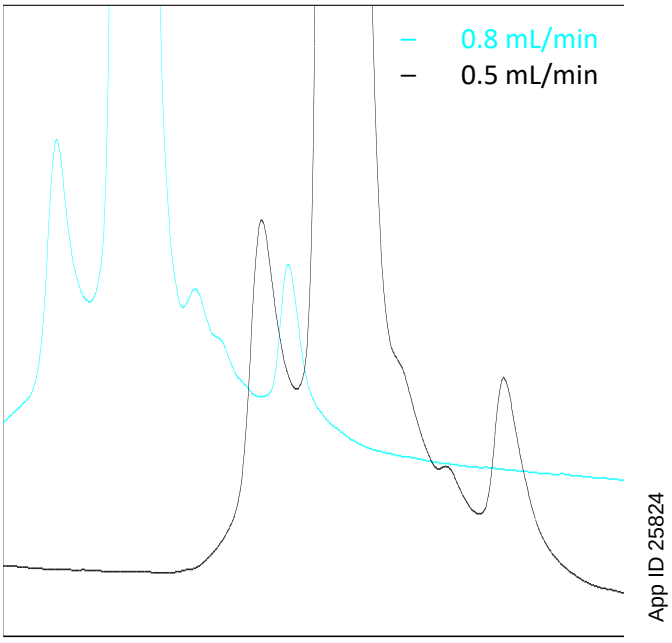


Figure 4:

Heavy Chain Overlay comparing 0.5 mL/min and 0.8 mL/min. Note the improvement in impurity profile with 0.8 mL/min, showing a shouldering of another late eluting variant.



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