Selecting the Correct Pore Size of Your Analytical Columns for Better RPLC Separations of Biomolecules

Molecular Weights of Biomolecules and Particle Pore Sizes

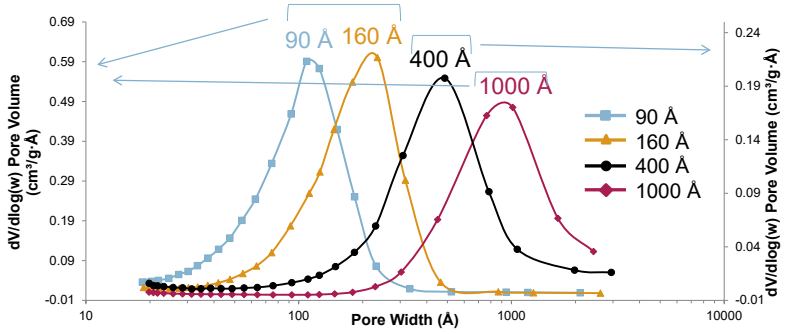
The physical size of an analyte in solution, and that size relative to the aperture of the particles' pores is what matters most for obtaining unrestricted diffusion into and out of the pores, where most of the bonded phase and surface area resides. The relatively thin porous layer, which superficially porous particles possess, also improves stationary phase access and makes diffusion less hindered compared to totally porous particles—even those that have diameters less than 2 microns.

Some Key Points to Remember

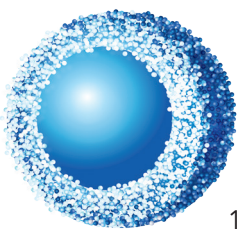
- Biomolecules can have different shapes and sizes in solution for the same or similar MWs.
- Column particle pore sizes are typically characterized by column manufacturers using nitrogen adsorption, mercury porosimetry or other techniques, and are usually expressed in Ångstrom units (Å).
- Particles for HPLC and UHPLC columns usually have a broad pore size distribution range as shown in Figure 1 for HALO® particles having different nominal average pore sizes.
- Consensus has not yet been reached on how large pore size should be relative to molecular size to avoid unnecessary loss of LC performance (broader and shorter peaks with less retention).
- Rule of thumb: Particle pore size should be a minimum of 8–10 fold larger than the molecular diameter for minimal size exclusion effects and for better access to stationary phase and less restricted diffusion for large analytes (Figure 1).

Figure 1 Pore Size Selection Based on Analyte Type and Size

Pore Sizes of HALO® Silica for Different Molecules



Molecule	Molecule Diameter	Recommended Minimum HALO® Pore Size
Small drugs	5-20 Å	90 Å
Small peptides	20-30 Å	160 Å
Large peptides	30-50 Å	160 Å
Small proteins	50-100 Å	400 Å
Large proteins	100-200 Å	1000 Å

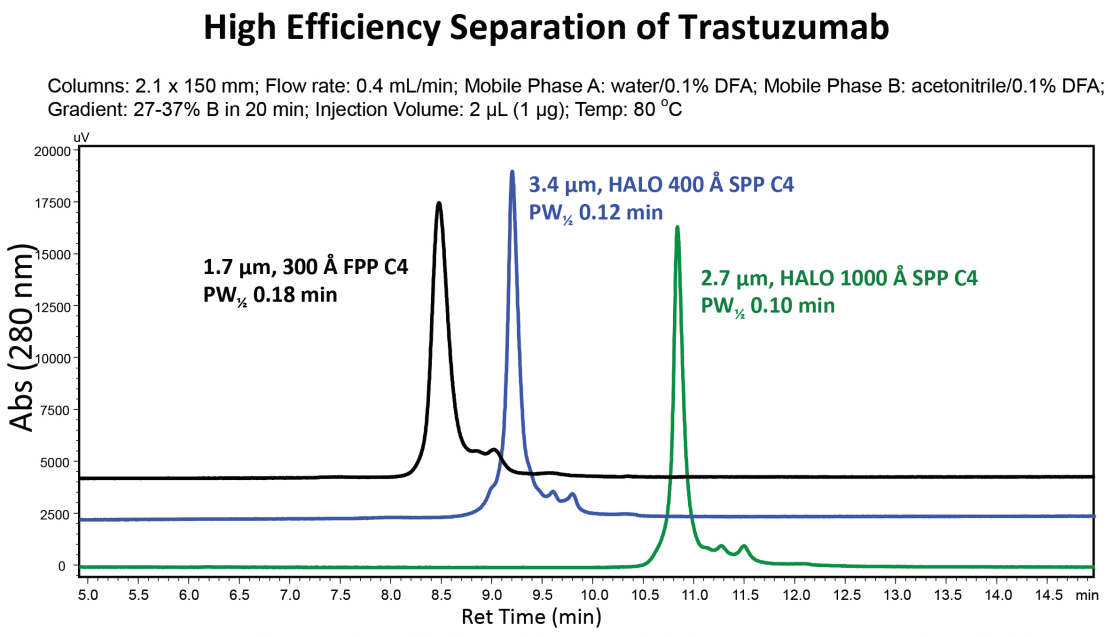


Because 300 Å pore size columns have been the "go-to" size for peptides and proteins for many years, chromatographers assume that if they choose a 300 Å pore size column, they will not observe any problems due to restricted pore access, slow diffusion, and size exclusion effects. However, for many proteins, especially those that are greater than ~50 kDa in size, narrower and taller peaks can be obtained using both 400 Å and 1000 Å pore size superficially porous columns.

The improvement in performance for the separation of a monoclonal antibody, with a MW of about 150 kDa is demonstrated in Figure 2. In this case, the larger pore size of a HALO 1000 Å C4 column provided improved retention, sharper peaks and improved resolution of minor components compared to the 300 Å, 1.7 µm FPP column. In Figure 3, the improved resolving power of the HALO 1000 Å C4, 2.7 µm SPP column is compared to that of a HALO 400 Å C4, 3.4 µm SPP column and a 300 Å, 1.7 µm FPP column for a mixture of IgG2 antibody structural variants. Clearly, more variants can be resolved using the HALO 1000 Å C4 column because there is unrestricted access to the particle surface.

Note: FPP = fully porous particle and SPP = superficially porous particle

Figure 2 Comparison of 300 Å, 400 Å and 1000 Å Columns for RPLC of 148 kDa Monoclonal Antibody



Large reduction in Peak Width and *increased* Retention with Larger Pore SPP, moderate additional improvement in Peak Width with Larger Pores

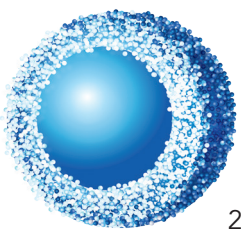
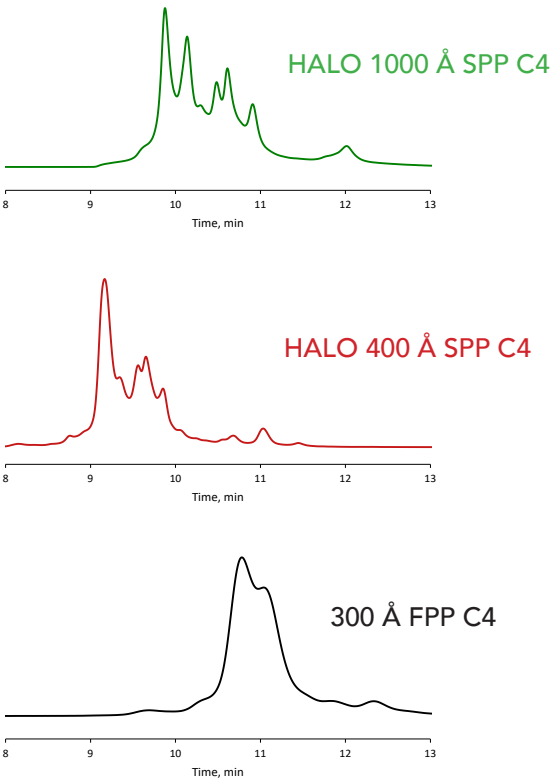


Figure 3 Optimum Resolution of IgG2 Variants shown on a SPP HALO 1000 Å C4, 2.7 µm Column



TEST CONDITIONS
Columns: 2.1 x 150 mm
Flow rate: 0.2 mL/min
Mobile Phase A: 88/10/2 H_2O/ACN/ n-Propanol + 0.1% DFA
Mobile Phase B: 70/20/10 n-Propanol/ACN/ H_2O + 0.1% DFA
Gradient: 14-24% B in 20 min
Injection Volume: 2 µL of 2 mg/mL denosumab in water+0.1% DFA
Temp: 80 °C
Detection: PDA at 280 nm

CONCLUSIONS

Larger pore size superficially porous particles provide improved chromatographic performance for larger biomolecules such as monoclonal antibodies. Increased retention, sharper peaks, and improved resolution of structural variants are some of the benefits provided by larger pores due to unrestricted access to the internal surface area of the particles and fast diffusion into and out of the thin porous shells.

For more information on the HALO 400 Å and 1000 Å columns and their usefulness for proteins, monoclonal antibodies and antibody fragments, please visit our website: www.fused-core.com