Improve Your Analyses of Large Proteins, Antibodies and Antibody-Drug Conjugates Using Reversed-Phase Liquid Chromatography by Maximizing Pore Access

Introduction

For many years pharmaceutical companies have taken a deliberate approach for creating new small molecule drugs for disease treatment. These new chemical entities and their analogs were often synthesized in large numbers and then tested for efficacy for treatment of various diseases. With the small molecule drug hit rate dropping and with recombinant protein synthesis becoming available and growing explosively, it became possible to manufacture biopharmaceuticals which could be used to treat diseases that could not have been considered previously. Many pharmaceutical companies have focused more and more of their efforts in this area, and new biopharma companies have grown around this important area. A number of large, small-molecule-focused pharmaceutical companies have also purchased or merged with startup biopharmaceutical companies to acquire their pipelines and their expertise.

Currently, there are tens of FDA-registered and commercialized biopharmaceuticals and more than 400 biotherapeutics (including monoclonal antibodies [mAbs], antibody-drug conjugates [ADCs], hybridized antigen binding structures and biosimilars), under development or on the market. Challenging diseases such as various forms of cancer, autoimmune diseases (arthritis, Crohn's disease), neurodegenerative diseases and many others can now, or will soon be, treated using these new biotherapeutics using a more effective targeted approach.

These new protein biopharmaceuticals have high molecular weights (MWs), are very large in solution, and are very complex, because many of them are based on molecules from the human or animal immune systems. In fact, their MWs usually exceed 100 kilodaltons (kDa) and, specifically, the MWs of mAbs range between 140 and 150 kDa (Figure 1) [1]. The volumes of these molecules in solution are very large, compared to small molecule pharmaceuticals, peptides, or even small to medium MW polypeptides and proteins.

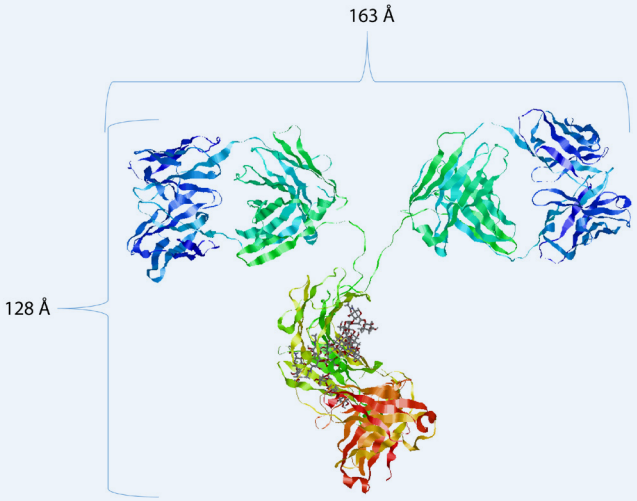

Figure 1. The crystal structure for a canine IgG2A monoclonal antibody. Its molecular dimensions are 128 Å by 163 Å, with a 69 Å Z-dimension (depth).

In addition to their high MW and size, these large biotherapeutics are also very complex, having multiple protein chains held together by disulfide linkages, and having complex, heterogeneous glycan modifications.

Characterization of New Biopharmaceuticals

The complexity of these biopharmaceuticals and the requirements for thorough characterization of such new entities or biosimilars for regulatory purposes and to ensure identity, purity and quality are substantial compared to those of small molecule drugs. It has been stated that "***In terms of size and rough complexity, if an aspirin were a bicycle, a small biologic would be a Toyota Prius, and a large biologic would be a F-16 fighter jet.*** [2]" Among the important analytical techniques that are applied for characterization of biotherapeutics such as mAbs are various modes of liquid chromatography (LC) for separations of intact mAbs, their fragments, their enzymatic peptide digests and their associated glycans. One of the most important modes for protein characterization has, for a long time, been reversed-phase HPLC (RPLC).

Reversed-Phase HPLC as a Preferred Method for Protein Analysis

For several decades, RPLC has been the dominant method for pharmaceutical and small molecule analyses, and it is employed at every stage of the drug discovery and development, manufacturing and quality assurance process. Since the early 1990s, a central role for RPLC for the analysis of proteins has emerged—initially with protein fragments (chemical and enzymatic digests), protein subunits, and, more recently, for intact protein analyses.

This growing importance and application of RPLC for protein analyses has coincided with the development of increasingly useful and appropriately designed chromatographic materials, and methods for their use, for the analysis of protein enzymatic digests, and of intact polypeptides and proteins.

The increased role of RPLC has also been accelerated by the development of improved LC instrumentation (hardware and software), and the advancements in high speed and high resolution mass spectrometers (MS) capable of being interfaced with HPLC and UHPLC instruments. Unlike some HPLC modes, RPLC is readily interfaced with MS instrumentation, and RPLC-MS can be used at scaled flow rates ranging from nanoliter/min to mL/min accommodating nano (< 300 µm ID), capillary (300 µm–1 mm ID) and analytical (1–4.6 mm ID) columns.

The relative simplicity of RPLC and the widespread availability of automated, computer-controlled instrumentation and qualified software have made the technique indispensable for protein identification and quantitation. It was shown in the 1980s that RPLC of even moderate size proteins (ca. 15–20 kDa), because of their molecular size, required larger-pore-size column packing materials [3], and careful selection of mobile phases and analysis conditions that maintain protein solubility and enhance recovery. Most analytical RPLC protein separations use acidic mobile phase additives (e.g., formic or trifluoroacetic acid), elevated column temperatures (40–90 °C), and an organic modifier-water gradient using acetonitrile or acetonitrile mixed with a short chain aliphatic alcohol (propanol, butanol or isopropanol). An example RPLC separation of a mixture of intact proteins, separated using a typical acetonitrile/water gradient, with UV absorbance detection at 215 nm, is shown in Figure 2.

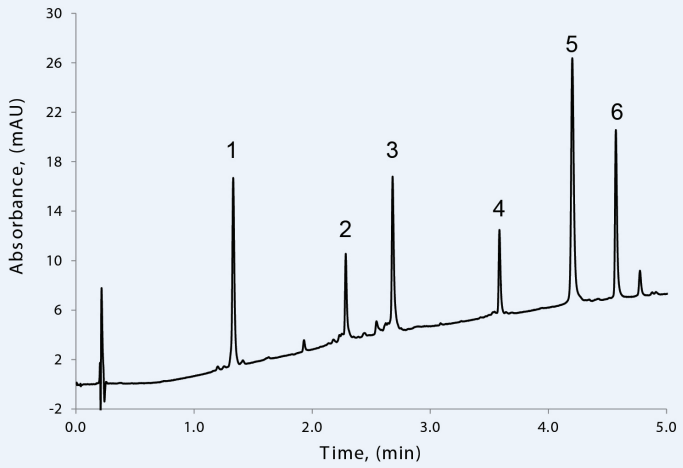

Figure 2. Gradient RPLC separation of a mixture of intact proteins using a 400 Ångstrom HALO Protein C4 SPP column. Conditions: Column: 2.1 x 100 mm, 3.4 µm; Mobile phase A: water (0.1% TFA); Mobile phase B: ACN (0.1% TFA); Gradient: 23–52% B in 5 min; Flow rate: 1.1 mL/min; Temperature: 60 °C; Injection volume: 1 µL; Instrument: Agilent 1200 SL; Detection: UV at 215 nm. Sample: (1) ribonuclease A; (2) cytochrome c; (3) lysozyme; (4) α-lactalbumin; (5) catalase; (6) enolase.

Until very recently, researchers have used existing column technology for RPLC of intact mAbs, ADCs and their resulting fragments. Typically, such packing materials have consisted of 200–300 Ångstrom pore-size, silica-based, fully porous particle (FPP) bonded phases. However, a new, very wide, 1000 Ångstrom pore size superficially porous packing for RPLC of very large molecules has been developed to improve the resolution for, and the information gained from, chromatography of these important biotherapeutics.

HALO® 1000 Ångstrom Protein C4 Column

The HALO 1000 Ångstrom Protein C4 column is an innovative product that is built upon the success and performance of the family of Fused-Core® superficially porous particle (SPP) HALO columns (Table 1). The objective in the design of the various HALO columns has always been to develop particles whose pore size had been chosen carefully to balance retention and pore access for the targeted analyte sizes.

Table 1. HALO Fused-Core Column Family: Pore Size Designed for Target Analytes.

Column Name	Pore Size (Å)	Particle Size(s) (µm)	Surface Area (m^2/g)	Stationary Phases	Target Analytes
HALO	90	2, 2.7, 5	120, 135, 90	C18, C8, RP-Amide, Phenyl-Hexyl, PFP, ES-CN, Penta-HILIC, HILIC (silica), Glycan*	Small molecules and peptides < 5 kDa
HALO Peptide	160	2, 2.7, 5	65, 90, 60	ES-C18, ES-CN	Peptides and polypeptides < 20 kDa
HALO Protein 400	400	3.4	15	C4, ES-C18	Peptides, polypeptides and proteins < 500 kDa
HALO Protein 1000	1000	2.7	20	C4	Large proteins, mAbs, mAb fragments, and ADCs < 500 kDa

**Note: HALO Glycan columns can be used for analytes up to 15–20 kDa, and are only available in 2.7 µm particle size.*

The performance improvement afforded by HALO 1000 Å, 2.7 µm particles (Figure 3) for RPLC analyses of large biotherapeutics, compared to commercially available, smaller pore size columns, is attributable to these main advantages:

- Larger pore size allows completely unrestricted access to, and movement via diffusion of the biomolecules to, the particle surface and bonded phase
- Superficially porous layer provides a shorter diffusion path (even vs. fully porous sub-2-µm particles) for larger biomolecules having much lower diffusion coefficients, while maintaining sufficient surface area for necessary loading capacity, resulting in reduced peak broadening and high resolution of minor components

Unrestricted Pore Access

Unrestricted pore access by large biomolecules to the large 1000 Å pores produces narrow peaks, which allows high resolution separations of protein and mAb variants, in addition to improved sample loading. For existing columns with smaller pore sizes, molecular exclusion and restricted diffusion of large molecules can occur, which produces broader peaks, poorer loading behavior, and less resolution. While the minimum pore size required to fully accommodate very large biomolecules has not yet been determined, consideration of the effects of diffusion into an open cylindrical channel suggests [4, 5] that particle pore size should be on the order of 10 times the effective hydrodynamic diameter of an analyte for optimal chromatographic performance.

The pore size distribution of HALO 1000 Å particles, as measured by nitrogen adsorption is shown in Figure 4A. The center of the distribution is at 1000 Å for these SPPs. For comparative purposes, the pore size distribution of 1.7 µm, 300 Å fully porous particles (FPPs) is also shown in Figure 4B. The difference in the pore size distributions is apparent, with the 1000 Å HALO material having a significantly larger population of pore sizes greater than 400 Å. Note that the HALO SPP pore size distribution extends well above its mode of 1000 Å, permitting free access for mAbs and larger proteins to the stationary phase available within the porous shell structure.

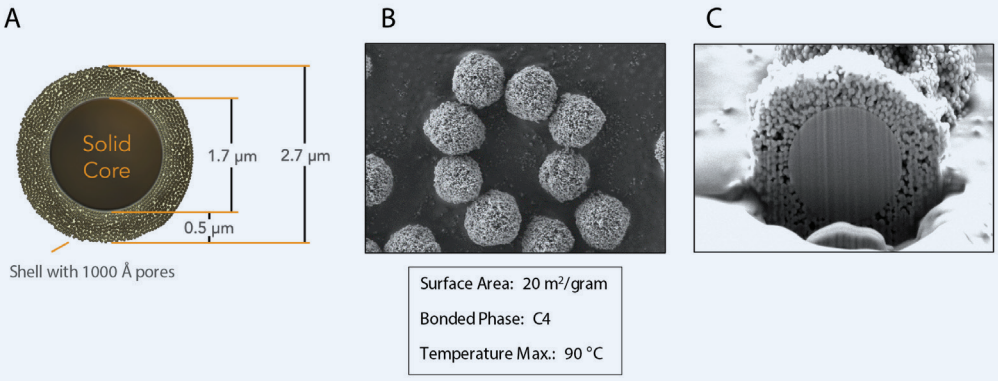

Figure 3. (A) Schematic drawing of a 1000 Å superficially porous, 2.7 µm particle; (B) Scanning electron microscope (SEM) image of several 2.7 µm superficially porous 1000 Å particles; (C) Focused-ion-beam-sliced SEM image of a 1000 Å SPP showing the 1.7 µm core with 0.5 µm porous shell.

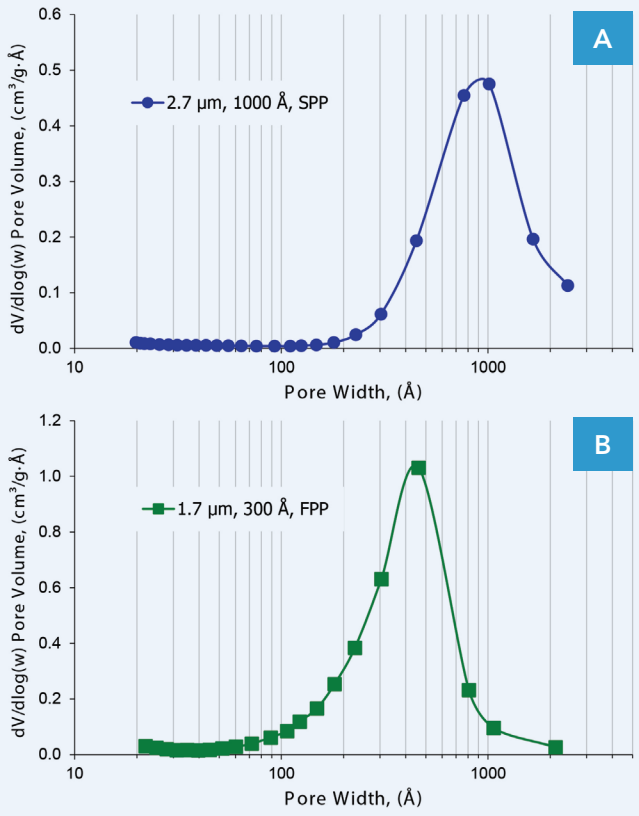

Figure 4. (A) Pore size distribution for 2.7 µm, 1000 Å SPPs; (B) Pore size distribution for 1.7 µm, 300 Å FPPs.

Short Diffusion Path

Another contributing factor to the superior performance of the HALO 1000 Å particle is its porous shell morphology, which it shares with other members of the Fused-Core® family (see Table 1 and Brief History Section). The unique particle design of SPPs includes a solid silica core surrounded by a porous shell of sub-micron thickness. This thin shell does not require that the slowly-diffusing biomolecules traverse the entire radius of the particle as it does for FPPs. This reduced diffusion path confers improved mass transfer and sharper peaks, and permits faster separations. For a review on SPPs, see Hayes, et al. [6], and for descriptions of superficially porous particles and their advantages for larger protein separations, see Kirkland, et al. [7].

Sample Loading

The very wide pores and short diffusion distances not only provide narrower peaks and improved resolution for large biotherapeutic molecules, but they also enable greater sample load capacity and tolerance. What this means is that a larger amount (mass) of sample can be injected, with less peak broadening, so that minor impurities such as subtle variants of mAbs and ADCs can be detected and quantified.

The ability of the HALO 1000 Å C4 Protein SPP column (hereafter HALO 1000 Å C4) to tolerate increasing load for a mAb separation has been investigated using trastuzumab as a model analyte. The effects of sample load on peak width are shown in Figure 5 both for a HALO 1000 Å C4 column and a 300 Å FPP C4 column (2.1 x 150 mm sizes). These results show that, for *all load levels*, the HALO 1000 Å C4 column afforded smaller peak widths than those for the 300 Å C4 column.

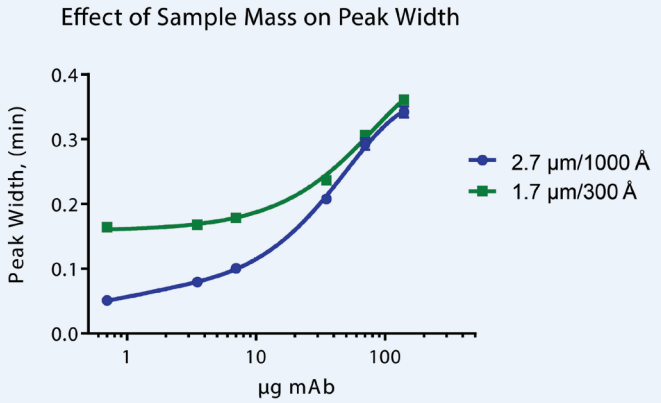

Figure 5. Sample loading using trastuzumab on both 1000 Å, 2.7 µm HALO C4 column and 300 Å, 1.7 µm C4 columns. Conditions: Columns: 2.1 x 150 mm; Mobile phase A: water (0.1% DFA); Mobile phase B: ACN (0.1% DFA); Gradient: 27–37% B in 10 min; Flow rate: 0.5 mL/min; Temperature: 80 °C; Sample: trastuzumab; Injection volume: 0.1, 0.5, 1, 5, 10, and 20 µL of 7 mg/mL mAb in water; Instrument: Shimadzu Nexera; Detection: UV at 280 nm with 350 nm reference wavelength. Note: Peak widths measured at 50% height.

These results are counterintuitive, when one considers the actual surface areas of these two silica column packing materials. The surface area (nitrogen adsorption, surface BET analysis) of the 300 Å FPPs is about 90 m^2/g compared to about 20 m^2/g for the 1000 Å SPPs. The surface area of the latter is about 4.5-fold smaller, yet the sample loading capacity (inversely correlated with peak width) is much better for the 1000 Å SPPs at lower sample loads, and comparable to the FPPs at high loads. These findings suggest that trastuzumab has much greater access to the bonded phase surface of the 1000 Å SPPs, compared to the 300 Å FPPs, which have higher absolute surface area.

Examples of HALO 1000 Å Protein Column RPLC Performance

HALO 1000 Å SPP C4 vs. 300 Å FPP C4

RPLC separations of intact trastuzumab (IgG1 type) are compared for HALO 1000 Å SPP and 300 Å FPP columns in Figure 6. For the HALO 1000 Å C4 column separation, the trastuzumab peak width is not only 43% narrower compared to that for the 300 Å C4 column, but there is also much better resolution of the minor variant peaks, which elute after the main trastuzumab peak. In addition, there is increased retention of the trastuzumab on the 1000 Å SPP column compared to the 300 Å FPP column, even though the actual surface area of the 300 Å FPPs is much greater.

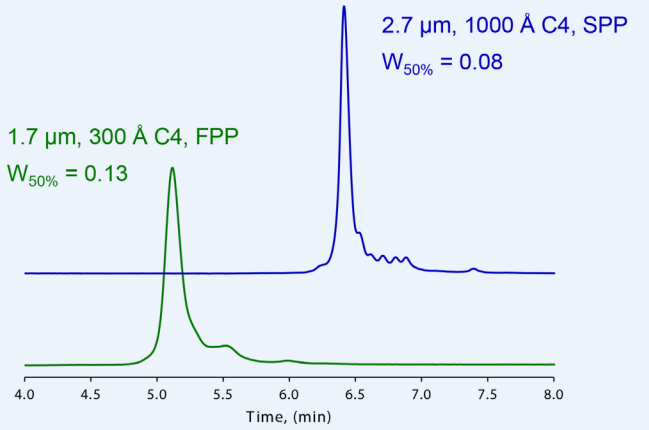

Figure 6. Intact trastuzumab separation using 1000 Å SPPs and 300 Å FPPs. Conditions: Columns: 2.1 x 150 mm; Mobile phase A: water (0.1% TFA); Mobile phase B: ACN (0.1% TFA); Gradient: 32–38% B in 12 min; Flow rate: 0.4 mL/min; Temperature: 80 °C; Sample: trastuzumab; Injection volume: 2 µL of 0.5 mg/mL in water; Instrument: Shimadzu Nexera; Detection: UV at 280 nm with 350 nm reference wavelength.

When a set of small neutral molecule probes was analyzed isocratically using both the HALO 1000 Å C4 column and the 300 Å C4 column, the latest-eluting analyte (1-chloro-4-nitrobenzene) was retained ~50% longer using the 300 Å C4 column, consistent with its much higher surface area (90 vs. 20 m^2/g) [8].

These observations, along with the sample loading results shown in Figure 5 demonstrate that large molecules have much greater pore access for bonded phase interactions using the HALO 1000 Å SPP column, compared to the 300 Å FPP column.

RPLC analyses of four additional mAbs were carried out using both columns. The peak widths of these four mAbs (in addition to trastuzumab) were, on average, 68% narrower using the HALO 1000 Å C4 column compared to those obtained using the 300 Å FPP C4 column (Figure 7).

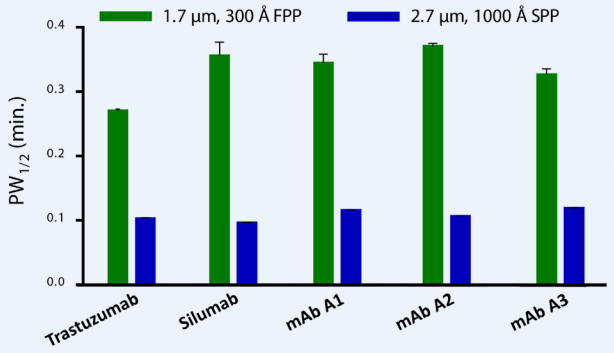

Figure 7. Bar graph comparing the average peak widths for five different mAbs using 1000 Å SPP C4 column and a 300 Å FPP C4 column. Conditions: Columns: 2.1 x 150 mm; Mobile phase A: water (0.1% DFA); Mobile phase B: ACN (0.1% DFA); Gradient: 27–37% B in 20 min; Flow rate: 0.4 mL/min; Temperature: 80 °C; Samples: as indicated; Injection volume: 2 µL (1 µg each in 0.1% TFA); Instrument: Shimadzu Nexera; Detection: UV at 280 nm with 350 nm reference wavelength. Note that peak widths measured at 50% height.

RPLC separations of an IgG2 mAb sample were also compared using the HALO 1000 Å C4 column and the 300 Å C4 column (Figure 8). The IgG2 mAb was resolved into several distinct bands using the 1000 Å C4 column unlike for the separation obtained using the 300 Å C4 column. The retention of the IgG2 was comparable using both columns, but the separation of the IgG2 mAb into multiple peaks using the 1000 Å C4 column shows enhanced resolution of the IgG2 variants.

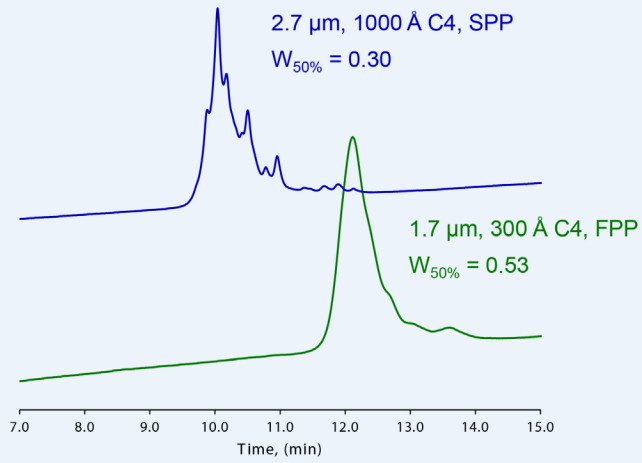

Figure 8. Intact IgG2 separation using 1000 Å SPP and 300 Å FPP columns. Conditions: Columns: 2.1 x 150 mm; Mobile phase A: 5/95 n-propanol/water (0.1% DFA); Mobile phase B: 70/20/10 n-propanol/ACN/water (0.1% DFA); Gradient: 14–24% B in 20 min; Flow rate: 0.4 mL/min; Temperature: 80 °C; Sample: IgG2; Injection volume: 4 µL of 0.5 mg/mL in water; Instrument: Shimadzu Nexera; Detection: UV at 280 nm with 350 nm reference wavelength.

For all other mAbs that have been analyzed so far (data not shown), narrower peak widths have been observed

consistently using HALO 1000 Å C4 compared to 300 Å C4 columns tested. All results to-date indicate that both pore size and pore-size distribution play very significant roles in RPLC analyses of very large proteins such as mAbs.

HALO 1000 Å C4 SPP vs. 1500 Å Polymeric RP FPP

RPLC separations of intact trastuzumab were also compared using the HALO 1000 Å, 2.7 µm C4 column and a 1500 Å, 4 µm, polymeric FPP column (Figure 9). The trastuzumab peak was about 24% narrower using the 1000 Ångstrom C4 column, compared to the larger pore size polymeric column. The trastuzumab peak may be sharper due to the combination of smaller particle size and the thin shell of the 1000 Å SPP column (2.7 µm with 0.5 µm shell) compared to the 4 µm particle size of the 1500 Å fully porous polymeric particles. Resolution of the minor trastuzumab variants was much better using the 1000 Å C4 SPP column.

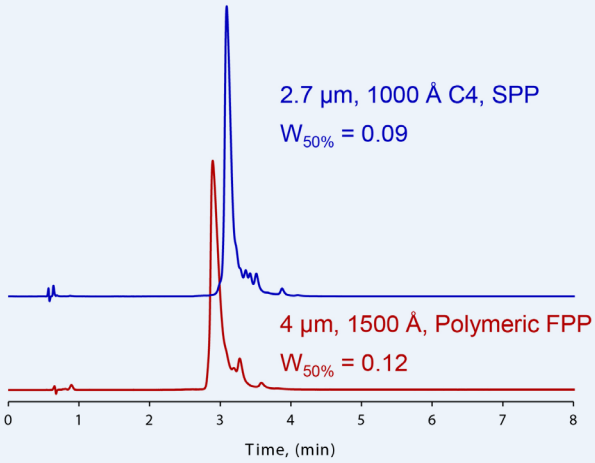

Figure 9. Intact trastuzumab RPLC separations using 1000 Å C4 SPP and 1500 Å FPP columns. Conditions: Columns: 2.1 x 100 mm; Mobile phase A: water (0.1% TFA); Mobile phase B: 80/20 ACN/water (0.085% TFA); Gradient: 40–47.5% B in 8 min; Flow rate: 0.4 mL/min; Temperature: 80 °C; Sample: trastuzumab; Injection volume: 2 µL of 2 mg/mL in water; Instrument: Shimadzu Nexera; Detection: UV at 280 nm with 350 nm reference wavelength

A Brief History of Superficially Porous Particles for HPLC and UHPLC

- Superficially porous particles (SPPs) were originally developed during the 1960s, but enjoyed a new renaissance in the mid-2000s with the commercialization of HALO® Fused-Core® technology by Advanced Materials Technology, Inc (AMT). This revolutionary HALO 90 Ångstrom, 2.7 µm SPP column, first introduced in 2006, rivals the speed, resolution and performance of sub-2-µm columns, which had been introduced in 2003-2004, for small molecule analyses. This new SPP technology has rapidly changed the HPLC materials landscape, and has become very popular in the last ten years.

- These particles consist of a non-porous silica core, surrounded by a porous shell (Figure 3A). The interest in and popularity of these particles is due to the very high column efficiencies at modest back pressures [9]. This superficially porous particle morphology has been so successful that it has been adopted and commercialized by a number of other column manufacturers.

- It is well known that selection of the correct pore size is important to allow unhindered analyte access to the silica surface. Pore access is not a consideration for all but the largest pharmaceuticals (e.g., macrocyclic antibiotics such as tylosin, etc.). However, for peptides, proteins and larger biopharmaceuticals, larger pore size is a critical factor in achieving high efficiencies and narrow peak widths.

- Building upon the success of the 90 Ångstrom HALO particles, 160 Ångstrom pore size HALO Peptide particles, designed for fast and high resolution separations of peptides and small polypeptides, were commercialized in 2011 [10]. HALO Peptide particles are bonded with sterically-protected silanes to promote excellent stability at the low pH, high temperature conditions often used for peptide mapping and other peptide analyses.

- Subsequently, in 2013 a new 400 Ångstrom, 3.4 µm HALO particle was introduced with C4 and sterically-protected C18 bonded phases to provide high efficiency gradient separations of larger polypeptides and proteins [11].

- The HALO 1000 Ångstrom, 2.7 µm particle is the newest addition to the HALO family of columns, and was designed to deliver superior performance for monoclonal antibodies, their fragments, and antibody-drug conjugates.

- AMT is the innovator in this area of superficially porous packing materials, being first to offer very wide pore SPP materials, and is the only company that offers the choice of 400 Å and 1000 Å pore particle materials.

Conclusions

As pharmaceutical companies have shifted their development focus to large-molecule biotherapeutics, the ability to separate intact monoclonal antibodies and antibody-drug conjugates for characterization purposes has become extremely important. To enable this characterization work, new HPLC particle technology has been required. The large 1000 Å pore size of the superficially porous particles used for RPLC described herein enables full access to the bonded phase surface for these larger biomolecules. This improved access to the bonded surface produces narrower peak widths and enhanced resolution of minor mAb variants, and can lead to increased retention under most analysis conditions. Together with new mass spectrometric instrumentation and software, wide-pore superficially porous particle HPLC columns will greatly aid in the advancement of large-molecule biopharmaceutical characterization and development.

List of Abbreviations:

ADC: antibody-drug conjugate
BET: Brunauer, Emmett and Teller (method for calculating surface area)
DFA: difluoroacetic acid
FPP: fully porous particle
IgG: immunoglobulin
kDa: kilodaltons
mAb: monoclonal antibody
SEM: scanning electron microscope
SPP: superficially porous particle
TFA: trifluoroacetic acid

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