Analyses of Large Proteins, Antibodies, and Modified Proteins Using A New Series of Advanced Large Pore HPLC Materials

Introduction

The list of FDA-registered and commercialized therapeutic proteins continues to expand rapidly, including more than 400 biotherapeutics, such as enzymes, monoclonal antibodies (mAbs), antibody-drug conjugates (ADCs), and hybrid antigen binding structures. Various human and veterinary diseases, such as cancers, autoimmune diseases, neurodegenerative diseases, and many others can be treated using these new molecules, employing a more effective targeted approach [1].

Protein biopharmaceuticals have higher molecular weights (MWs), are large in aqueous solution, with shapes that can be very complex having tertiary and quaternary structural elements. The polypeptide chains fold in specific ways, with highly hydrated charged amino acid side chains oriented towards the surface containing domain structures that are stabilized by specific and cumulative ion pairs and hydrophobic interactions. Many of these proteins are composed of more than one polypeptide chain, associated by both covalent and non-covalent bonds. In many cases, the proteins are based on molecules from the human or animal immune systems, and often their MWs usually exceed 100 kilodaltons (kDa). For example, the MWs of antibodies, including therapeutic mAbs, range between 140 and 150 kDa (Figure 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.

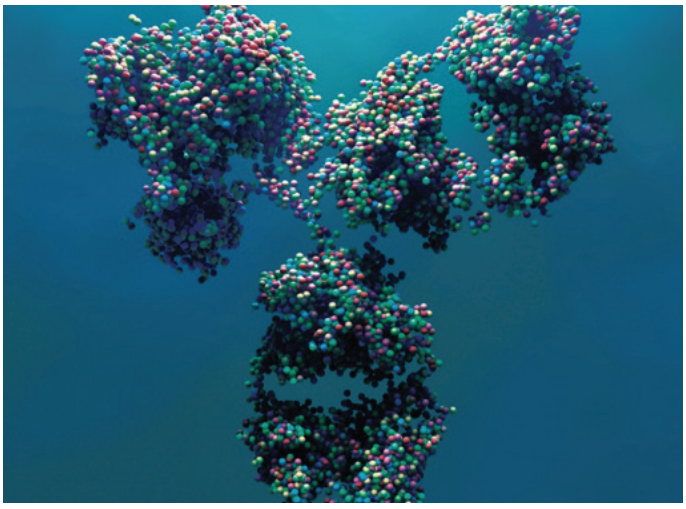

Figure 1. Representative Therapeutic Monoclonal Antibody Structure

In addition to their high MW and hydrated volumes, large biotherapeutic proteins have additional structural complexities due to inherent or induced heterogeneity. For example, many biotherapeutics are glycoproteins, often with a mixture of neutral and acidic glycan structures at one or more specific sites of the protein structures. As mentioned above, proteins may be composed of multiple protein chains, and some are covalently linked by specific interchain or intrachain disulfide linkages, or may have a mixture of fully bridged disulfide and free sulfhydryl groups. Moreover, the polypeptide chains themselves may have heterogeneous lengths, with terminus variations of one or two amino acids (ragged ends). In many therapeutic proteins these various sources of heterogeneity may coexist to a varying degree, as the biological and production process variables that lead to these heterogeneities are not all under complete control during the preparation of a useful therapeutic biopharmaceutical.

Characterization of Biopharmaceutical Proteins

The complexity of these biopharmaceuticals, and the regulatory requirements for thorough characterization of novel entities, or of biosimilars, to ensure identity, purity and quality, are substantial compared to those of small molecule drugs. 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 digests to yield smaller peptide fragments, and their associated glycans. A key analytical approach for protein characterization remains reversed-phase HPLC (RPLC), due to the unique and informative capabilities of this method.

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 drug discovery and development, manufacturing and quality assurance processes. 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 coincides with the development of increasingly useful and appropriately designed chromatographic materials. Methods for their use include the analysis of protein enzymatic digests, intact polypeptides and intact proteins, and the various derivatives of all of the above.

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 detectors, most notably, high speed and high resolution mass spectrometers (MS). Most advanced MS platforms are capable of being efficiently interfaced with HPLC and UHPLC instruments via electrospray ionization (ESI). Unlike some HPLC modes, RPLC is easily accommodated by MS detection, 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 [2], 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) [3]. 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.

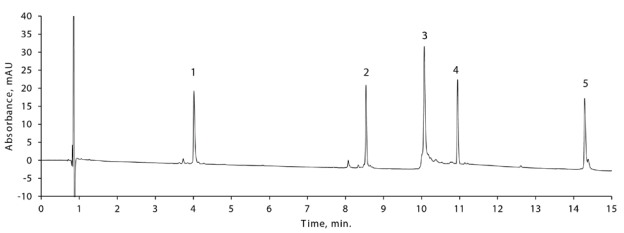

Figure 2. Gradient RPLC Separation of a Mixture of Intact Proteins

TESTING CONDITIONS:

Column: HALO 1000 Å ES-C18, 2.7 µm, 2.1 x 150 mm
Mobile phase A: Water (0.1% TFA)
Mobile phase B: 80/20 ACN/Water/0.085% TFA
Gradient: 27–60 %B in 15 min
Flow rate: 0.4 mL/min
Temperature: 60 °C
Injection volume: 2 µL
Instrument: Shimadzu Nexera XR
Detection: UV at 280 nm
Sample: (1) ribonuclease A; (2) lysozyme; (3) SigmaMAb; (4) α-lactalbumin; (5) enolase

Until very recently, researchers have used column technology for RPLC of intact mAbs, ADCs and their resulting fragments which has been fundamentally unchanged since the mid-1990s. Typically, such packing materials have consisted of 200 – 300 Å pore size, silica-based, fully porous particle (FPP) bonded phases. However, new, very wide, 1000 Å pore size superficially porous packings for RPLC of very large molecules have been developed to improve the resolution for, and the information gained from, higher resolution chromatographic separations of protein mixtures.

HALO® 1000 Å Protein Column Series

The HALO 1000 Å Protein columns are innovative products that are built upon the success and performance of the family of small particle diameter

COLUMN NAME	PORE SIZE (Å)	PARTICLE SIZE(S) (um)	SURFACE AREA (m^2/g)	STATIONARY PHASES	TARGET ANALYTES
HALO Glycan	90	2.7	135	Proprietary	Glycans, glycopeptides, glycoproteins < 20 kDa
HALO Peptide	160	2,2.7,5	65, 90, 60	ES-C18, ES-CN, Phenyl-Hexyl	Peptides and polypeptides 100 Da < MW < 15 kDa
HALO Protein	400	3.4	15	C4, ES-C18	Peptides, polypeptides, and proteins 2 kDa < MW < 500 kDa
HALO Protein	1000	2.7	22	C4, ES-C18, Diphenyl	Large proteins, mAbs, mAb fragments, and ADCs > 50 kDa

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

Fused-Core® superficially porous particle (SPP) HALO columns (Table 1), a particle type pioneered by Advanced Materials Technology. 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.

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 of biomolecules to the interior domain of particles, and movement within the volume element adjacent to the particle surface and associated 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 (impurities and structural variants).

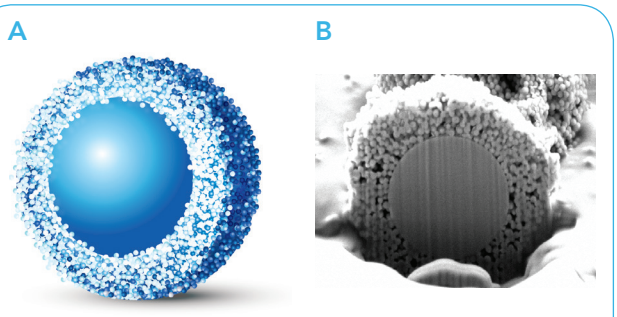

Figure 3. (A) Schematic drawing of a 1000 Å superficially porous 2.7 µm particle; (B) Focused-ion-beam-sliced SEM image of a 1000 Å SPP showing the 1.7 µm core with 0.5µm shell

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 load tolerance before band broadening. For existing columns with smaller pore sizes, molecular exclusion and restricted diffusion of large molecules occurs and produces broader peaks, poorer loading behavior, and less resolution. While the minimum pore size required to fully accommodate very large biomolecules is determined by many factors, 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 HALO 1000 Å pore size distribution permits free access for mAbs and larger proteins to the stationary phase available within the porous shell structure.

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 the other Fused-Core® product series (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 Protein SPP columns to tolerate increasing load for protein separations have been investigated using various preparations. The effects of sample load for a highly purified IgG1 mAb peak width are shown in Figure 4 both for a HALO 1000 Å C4 column and a 300 Å FPP C4 column (2.1 x 150 mm sizes) using trastuzumab as a model. These results show that, for all load levels, the HALO 1000 Å C4 column afforded smaller peak widths than those for the 300 Å FPP C4 column.

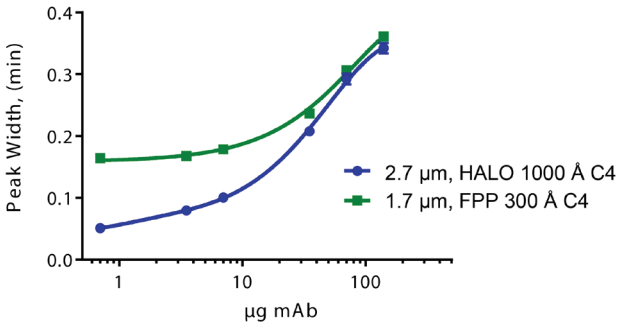

Figure 4. Effect of Sample Mass on Peak Width

TESTING CONDITIONS:

Column: 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 m2/g compared to about 20 m2/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 in the example of trastuzumab, it 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.

The HALO® 1000 Å Protein Column Bonded Phase Series

The traditional bonded phase for RPLC separations of proteins has been the short chain alkyl bonded phase, typically based on a form of butyl-silane (C4). This material works well, and has repeatedly shown high performance separations of proteins using default gradient RP conditions. To enhance the utility of 1000 Å wide pore bonded phases for protein development, Advanced Materials Technology has recently introduced specially-selected versions of the octyldecyl-silane (ES-C18) and diphenyl-silane (Diphenyl) surface modified bonded phases (surface structures shown in Figure 5).

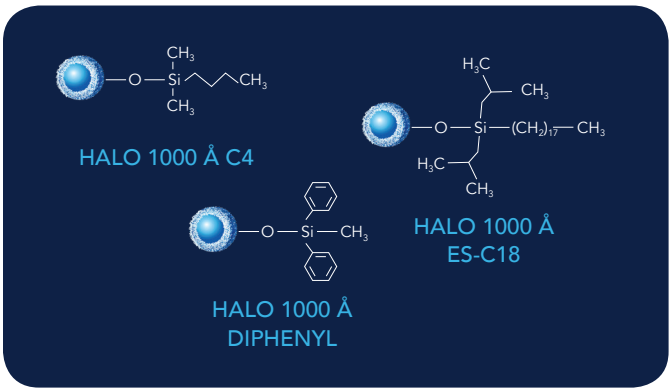

Figure 5. HALO 1000 Å Bonded Phase Structures

The three bonded phase RPLC materials selected for the HALO 1000 Å Protein series was derived from the comparison of many materials, with specific reference to stability for applications of low pH and elevated temperature that are often required to obtain high recovery of proteins in RPLC separations. All three materials exhibit acceptable column lifetimes when operated under such conditions, and an example of the HALO 1000 Å Diphenyl stability, when challenged with highly aggressive low pH (<2), elevated temperature conditions (90°C) is shown in Figure 6.

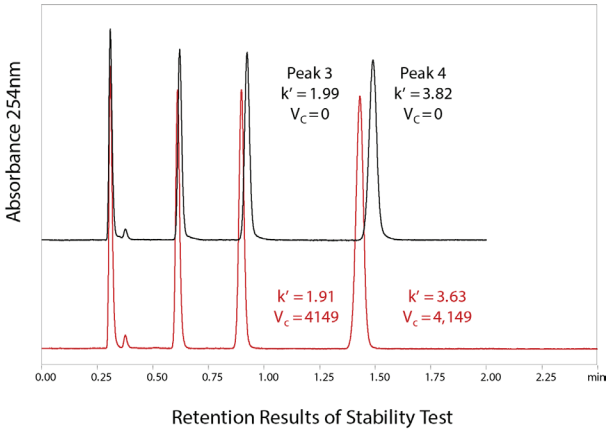

Figure 6. High Temperature and Low pH Stability

TESTING CONDITIONS:

Column: HALO 1000 Å Diphenyl, 2.7 µm, 2.1 x 50 mm
Mobile phase A: Water
Mobile phase B: ACN
Isocratic: 55/45 A/B
Flow rate: 0.4 mL/min
Temperature: 25 °C
Injection volume: 0.2 µL
Instrument: Shimadzu Nexera
Detection: UV at 254 nm
Sample: (1) uracil; (2) hexanophenone; (3) octanophenone; (4) decanophenone

Black trace shows the initial results and red trace shows the column performance after 4000 column volumes.

Examples of HALO® 1000 Å Protein Column RPLC Performance

HALO® 1000 Å SPP C4 vs. 300 Å FPP C4

RPLC separations of intact denosumab (IgG2 type) are compared for HALO 1000 Å SPP and 300 Å FPP columns in Figure 7. For the HALO 1000 Å C4 column separation, the denosumab peak widths are not only narrower compared to that for the 300 Å C4 column, but there is also much better resolution of the IgG2 disulfide bridge isoform variants. The narrower peak widths using HALO 1000 Å have also been observed and previously described using IgG1 type mAbs [8].

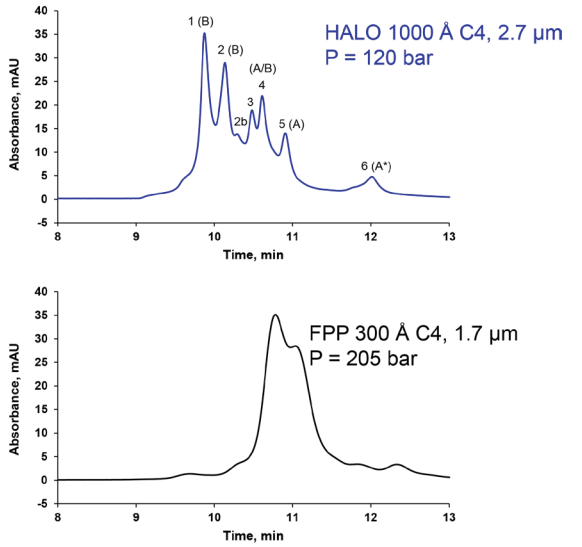

Figure 7. Intact Denosumab Separation Using 1000 Å SPPs and 300 Å FPPs

TESTING CONDITIONS:

Columns: 2.1 x 150 mm
Flow rate: 0.2 mL/min
Mobile Phase A: 88/10/2 H2O/ACN/n-Propanol + 0.1% DFA
Mobile Phase B: 70/20/10 n-Propanol/ACN/H2O + 0.1% DFA
Gradient: 14–24% B in 20 min
Injection Volume: 2 µL of 2 mg/mL denosumab in Water + 0.1% DFA
Temperature: 80 °C
Detection: PDA at 280 nm

These observations, along with the sample loading results shown in Figure 4 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, 69% narrower using the HALO 1000 Å C4 column compared to those obtained using the 300 Å FPP C4 column (Figure 8).

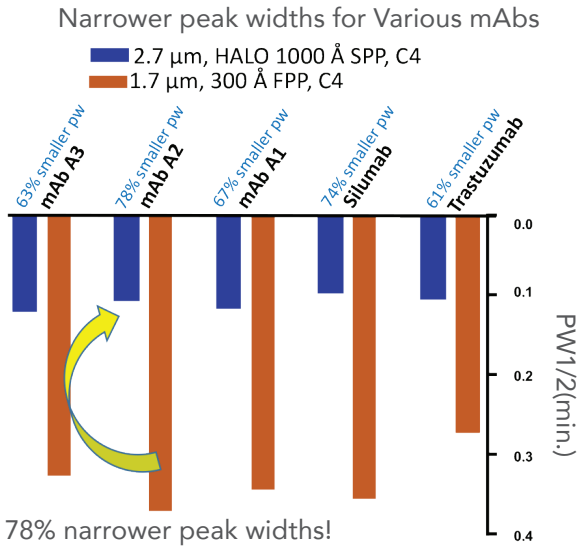

Figure 8. Narrower Peak Widths for Various mAbs. This bar graph compares the average peak widths for five different mAbs using a HALO 1000 Å C4 column and a 300 Å FPP C4 column.

TESTING 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
Injection volume: 2 µL (1 µg each in 0.1% TFA)
Instrument: Shimadzu Nexera
Detection: UV at 280 nm with 350 nm reference wavelength
Samples: As Indicated

Note: Peak widths measured at 50% height

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 Å 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, which is expected, as generally the case with silica particles, to exhibit high pressure and mechanical shock resilience, in comparison to polymeric particles.

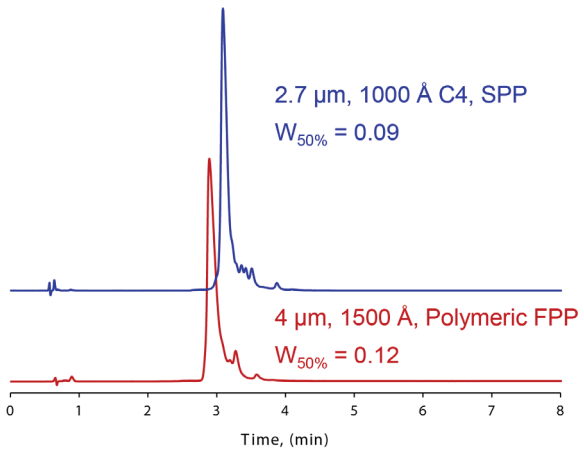

Figure 9. Intact Trastuzumab RPLC Separations Using 1000 Å C4 SPP and 1500 Å FPP Columns

TESTING CONDITIONS:

Columns: 1000 Å SPP and 1500 Å FPP columns 2.1 x 100mm
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

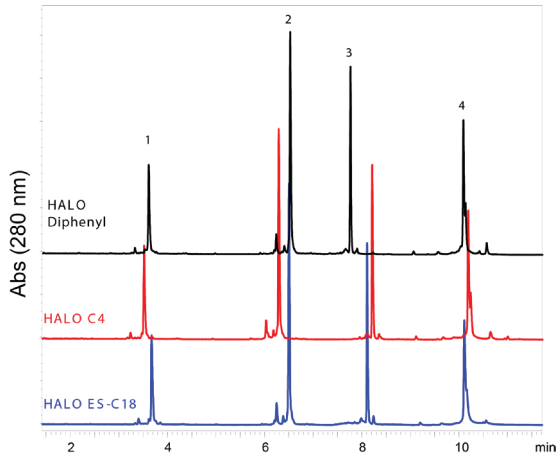

Figure 10. Comparison of Three HALO 1000 Å Bonded Phases Using a Protein Mix

TESTING CONDITIONS:

Columns: HALO 1000 Å, phase as indicated, 2.1 x 150 mm, 2.7 µm
Mobile phase A: Water/0.1% TFA
Mobile phase B: ACN/0.1% TFA
Gradient: 20–60 %B in 15 min
Flow rate: 0.4 mL/min
Temperature: 80 °C
Injection volume: 2 µL
Instrument: Shimadzu Nexera
Detection: UV at 280 nm
Sample: (1) Ribonuclease A; (2) lysozyme; (3) α-lactalbumin; (4) enolase

Use of HALO® 1000 Å Protein Bonded Phase Options for Protein Separations

The HALO 1000 Å Protein series is the only commercially available RPLC material that is a very wide pore SPP particle, and has available three highly stable and reproducible bonded-phases specific for resolving protein mixtures. The separation of four standard proteins is shown in Figure 10, using typical gradient RPLC conditions. Although the Diphenyl bonded phase is a weak RPLC retention material for small molecules, these results show that this bonded phase is comparable in retention to C4 and C18 for protein mixtures (for example comparing retention of peaks 1 and 4 for each column). In addition, this figure also shows that the three bonded phases exhibit subtle selectivity differences, appreciated when examining the relative elution positions for peaks 2 and 3, across the different bonded phase surfaces. Each bonded phase exhibits a unique pattern of separation for this mixture. Such selectivity differences, although appearing modest, become highly relevant when employing gradient elution conditions that are less aggressive, for example as is employed for protein variant analysis for a highly purified protein.

In Figure 11, a high resolution separation of the biotherapeutic mAb trastuzumab is shown. In this example, the materials of interest are the small peaks that elute after the main, correctly formed and fully disulfide bridged intact mAb structure. Following this main peak, a collection of smaller peaks is variably resolved by each of the specific bonded phases. The selectivity differences between these columns show up as a complex collection peaks for the many small protein variant structures. Subsequent studies have revealed that these later eluting peaks are subtle structural variants of trastuzumab, predominantly variants with a single reduced disulfide bridge, leading to pairs of free thiol variants. Similar disulfide bridge variants have been shown in previous studies of other IgG1 and IgG2 mAbs [10,11].

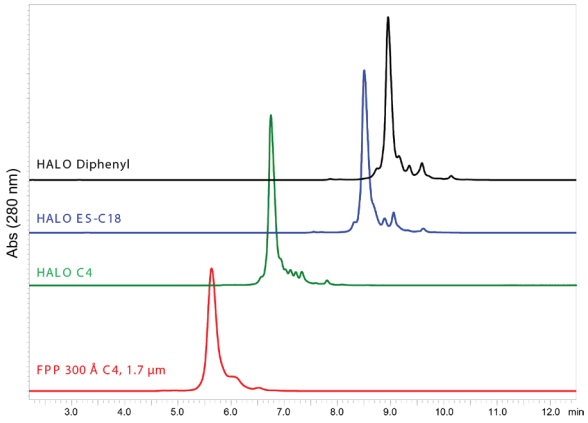

Figure 11. Comparison of Three HALO 1000 Å 2.7 µm Bonded Phases to a 300 Å 1.7 µm FPP C4 Column Using Trastuzumab

TESTING CONDITIONS:

Columns: as indicated, 2.1 x 150 mm
Mobile phase A: Water/0.1% TFA
Mobile phase B: ACN/0.1% TFA
Gradient: 32–40 %B in 16 min
Flow rate: 0.4 mL/min
Temperature: 80 °C
Injection volume: 2 µL
Instrument: Shimadzu Nexera
Detection: UV at 280 nm

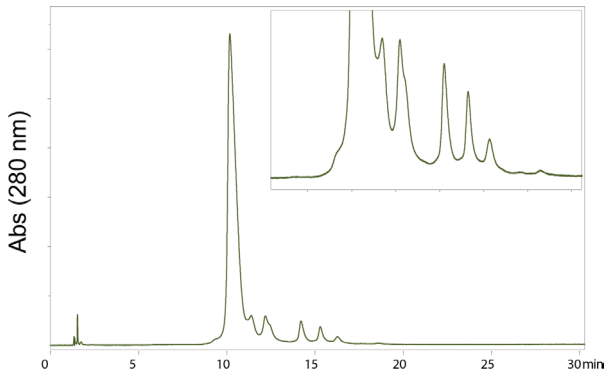

Figure 12. Example of Optimized Trastuzumab Method Development Using a HALO 1000 Å Diphenyl Column

TESTING CONDITIONS:

Mobile phase A: Water (0.1% DFA)
Mobile phase B: 50/50 ACN/n-propanol/0.1% DFA
Gradient: 29–33 %B in 29 min
Flow rate: 0.25 mL/min
Temperature: 60 °C
Injection volume: 2 µL of 2mg/mL trastuzumab in water/0.1% TFA
Instrument: Shimadzu Nexera
Detection: UV at 280 nm

The subtle selectivity difference in the HALO 1000 Å Protein bonded phases can be employed with gradient selectivity optimization and mobile phase modification as independent variables. As an example, Figure 12 shows the high resolution results that can be obtained with bonded phase, temperature and mobile phase optimization to yield a highly informative separation of the main peak and at least 7 variant protein structures present in this biotherapeutic mAb. The separation using this highly efficient HALO 1000 Å Protein Diphenyl column is completed in less than 30 minutes, while being compatible with both UV detection, as well as online high resolution MS detection.

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

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. The recent expansion of the very large pore superficially porous particle family to include several bonded phases (C4, C18, Diphenyl) permits very high resolution separations of lower abundance protein variants, permitting structure analysis and quantification of these variants.

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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. This revolutionary HALO 90 Å, 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 Å HALO particles, 160 Å pore size HALO Peptide particles, designed for fast and high resolution separations of peptides and small polypeptides, were commercialized in 2011 [12]. 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 Å, 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.

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

- Advanced Materials Technology 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.

- The HALO 1000 Å Protein series has been extended to include the C4, ES-C18 and Diphenyl bonded phases, allowing manipulation of separation selectivity, while assuring highly stable and reproducible columns for high resolution separations.

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