Application Note

Biotherapeutics & Biosimilars

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Analysis of Camelid Single-Domain Antibodies Using Agilent AdvanceBio SEC 120 Å 1.9 µm and AdvanceBio HIC Columns

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Abstract

This application note describes the use of size exclusion chromatography (SEC) and hydrophobic interaction chromatography (HIC) for the analysis of camelid single-domain antibodies (nanobodies). Nanobodies are a growing class of single-domain antibody fragments used for therapeutic purposes. The Agilent AdvanceBio SEC 120 Å 1.9 µm column provides a unique advantage over other SEC columns for high-resolution separation of nanobody aggregates and fragments. The Agilent AdvanceBio HIC column enables analysis of nanobody post-translational modifications (PTMs) such as glutamine/pyroglutamate conversion. An SEC-based native LC/MS method is also demonstrated for greater understanding of PTM and impurity characterization.

Introduction

Despite the success of biotherapeutics such as monoclonal antibodies (mAbs), many significant drawbacks still exist for this class of drugs. For example, IgG mAbs, the most widely used biologic drugs, have a complex structure and rather large size (150 kDa). The large size hampers their efficient *in vivo* delivery to diseased cells such as those found in tumors. Alternatively, single-domain antibodies, also known as nanobodies, provide tremendous opportunity in terms of reaching their intended targets.[1]

Nanobodies are small (~15 kDa), natural single-domain proteins derived from the camelid heavy chain antibody (Figure 1). They are recombinantly produced antigen-binding V_{HH} fragments with binding affinity equivalent to that of conventional IgG mAbs. Due to their small size, nanobodies can bind to antigen motifs that are frequently inaccessible to conventional mAbs, providing access to presently "undruggable" targets. In addition, the relatively simple protein conformation offers many advantages to drug developers such as ease of manufacturing and different administrative routes.[1] These promising features make nanobodies and V_{HH} fragment-derived biologics the rising stars in the biopharma research and development pipeline.[2]

This application note presents thorough characterization of two V_{HH} fragments (anti-PD1 and anti-PDL1 single-domain antibodies) using SEC and HIC, both with UV detection. Furthermore, we demonstrate SEC-based native LC/MS analysis of the two samples. The Agilent AdvanceBio SEC 120 Å 1.9 µm column is designed for aqueous SEC separation of small biomolecules such as proteins in the molecular weight range of 1 to 80 kDa. The column is perfectly suitable for nanobody analysis. The proprietary

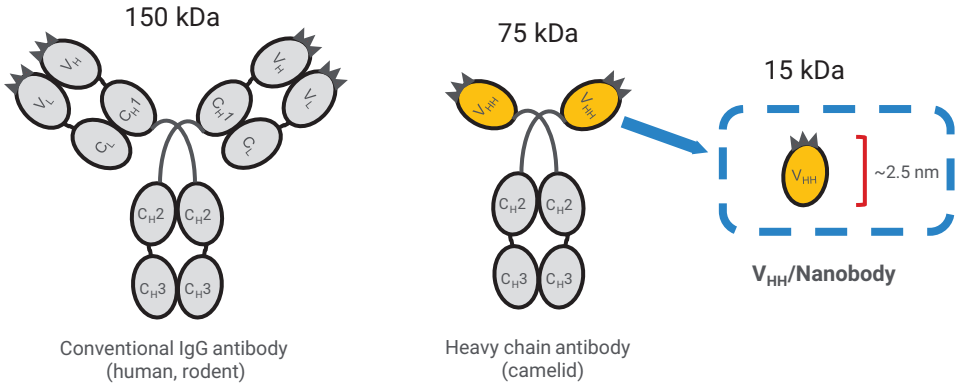

Figure 1. Schematic of a V_{HH} single-domain antibody (nanobody) compared to a conventional mAb.

sub-2 µm hydrophilic polymer-coated silica packing technology enables higher resolving separation of aggregates and fragments compared to SEC columns from other vendors. HIC analysis allows reserved-phase-like separation of protein variants in a native condition. The Agilent AdvanceBio HIC column exhibits optimal hydrophobicity and selectivity for nanobody PTM characterization. Using a generic HIC method (without organic solvent modifier), a common PTM, glutamine/pyroglutamate conversion is revealed. The combination of these approaches offers a complete solution for in-depth analysis of nanobody purity and critical quality attribute assessment.

Experimental

Samples and chemicals

- Llama anti-PD1 single-domain antibody [F12A8]; purchased from ProSci Inc (Poway, CA)
- Llama anti-PDL1 single-domain antibody [F2G2]; purchased from ProSci Inc (Poway, CA)
- Glutaminyl-peptide cyclotransferase; purchased from R&D Systems (Minneapolis, MN)
- Human anti-IL8 IgG monoclonal antibody; produced in house from CHO cells

All chemicals and solvents used were HPLC grade or higher. Sodium phosphate monobasic and dibasic, sodium chloride, and ammonium sulfate were from Sigma-Aldrich. Water was purified using a Milli-Q Integral system equipped with LC-Pak Polisher and a 0.22-µm membrane point-of-use cartridge (Millipore).

Columns

- Agilent AdvanceBio SEC 1.9 µm 120 Å, 4.6 × 300 mm (p/n PL1580-5250)
- Agilent AdvanceBio SEC 1.9 µm 120 Å, 4.6 × 150 mm (p/n PL1580-3250)
- Agilent AdvanceBio HIC, 4.6 × 100 mm (p/n 685975-908)

Instrumentation

For HPLC experiments, an Agilent 1260 Infinity II Bio-inert LC system was used comprising:

- Agilent 1260 Infinity II Bio-inert Pump (G5654A)
- Agilent 1260 Infinity II Bio-inert Multisampler (G5668A) with sample cooler (option 100)
- Agilent 1260 Infinity II Multicolumn Thermostat (G7116A) with bio-inert heat exchanger (option 019)
- Agilent 1260 Infinity II Variable Wavelength Detector (G7114A)

For LC/MS experiments, an Agilent 6224 accurate-mass time-of-flight (TOF) LC/MS and 1290 Infinity II LC were used comprising:

- Agilent 1290 Infinity II High Speed Pump (G7120A)
- Agilent 1290 Infinity II Multisampler (G7167B)
- Agilent 1290 Infinity II Thermostatted Column Compartment (G7116B)
- Dual ESI Agilent 6224 accurate-mass time-of-flight (TOF) LC/MS

Results and discussion

SEC is a gold standard technique for characterizing aggregation of biologics. The chromatographic separation mechanism is unique in that analytes are not retained by the stationary phase; instead, they are separated based on accessibility to available particle pore volume. Therefore, careful selection of column pore size based on the protein analyte's size in solution (hydrodynamic radius) is important. Nanobodies are small proteins with molecular weight of approximately 15 kDa, translating to a hydrodynamic radius of approximately 25 Å. SEC columns packed with narrow pore (120 to 130 Å) particles offer linear separation in the range suitable for such an application.[3,4] Figure 2 shows an SEC separation of anti-PD1 single-domain antibody (sdAb) using a standard method with sodium phosphate pH 7 as the mobile phase (to maintain the native state of the protein). Excellent separation of sdAb monomer from impurities, i.e., high-molecular weight (HMW) and low molecular weight (LMW) species, can be

HPLC Conditions (SEC Analysis)	
Column	Agilent AdvanceBio SEC 1.9 µm 120 Å, 4.6 × 300 mm (p/n PL1580-5250)
Mobile Phase	150 mM sodium phosphate, pH 7.0
Flow Rate	0.35 mL/min
Column Temperature	25 °C
Injection Volume	5 µL, 1 mg/mL
Total Run Time	15 min
Detection	UV at 214 nm

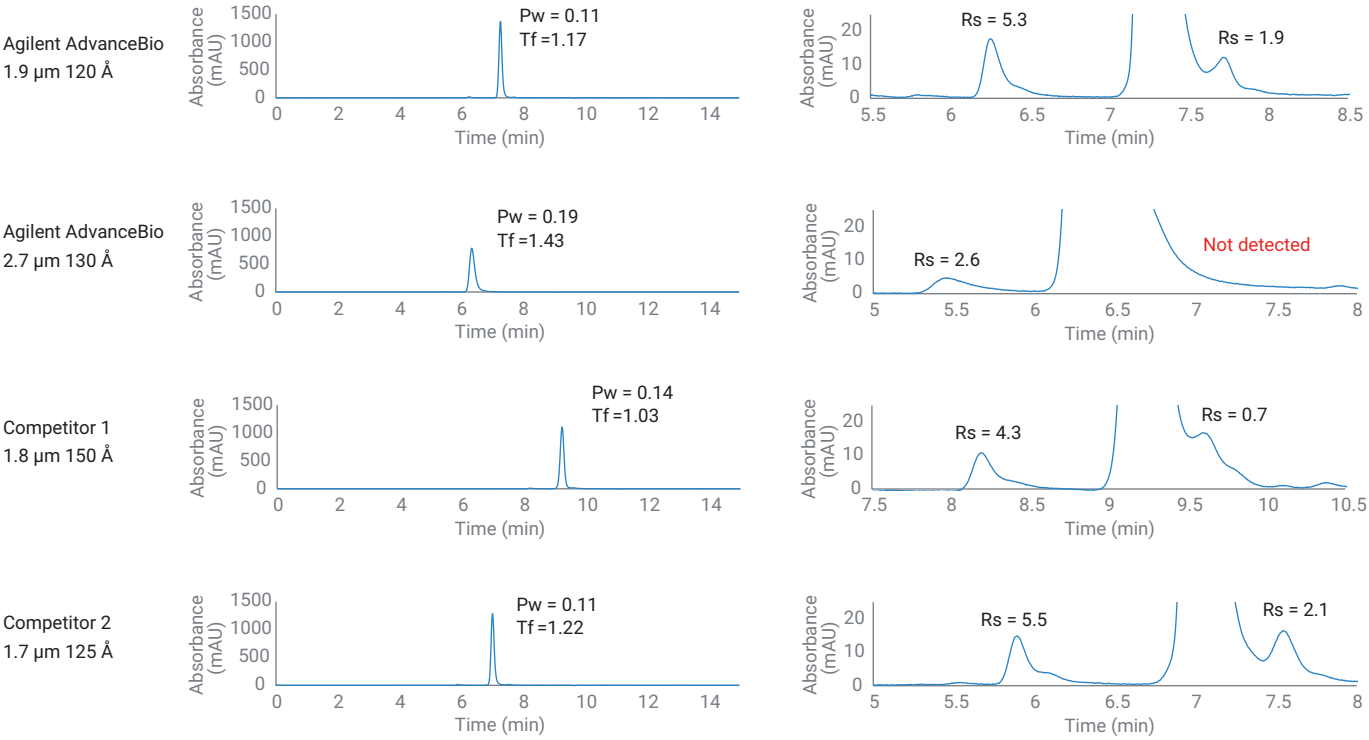

Figure 2. SEC of anti-PD1 single-domain antibody. Right panels show the magnified baseline of the same run on the left. Resolution of the HMW aggregate peak (eluted earlier) or the LMW fragment peak (eluted later) compared to the monomer peak are shown.

seen with the AdvanceBio SEC 1.9 µm 120 Å column. The resolution of HMW and LMW species as well as the peak width and tailing factor of the monomer peak are significantly better compared to columns packed with larger (2.7 µm) particles. Compared to other vendors' offering of sub-2 µm SEC technology, the AdvanceBio SEC 1.9 µm 120 Å column exhibits best-in-class performance.

This is due to careful design of particle surface bonding coverage to eliminate undesirable secondary interactions.[3,4]

SEC is a relatively straightforward chromatographic method where the column is run in isocratic mode. Method development and optimization involve the selection of mobile phase parameters (pH, salt concentration, etc.) to minimize potential secondary interactions such as ionic or hydrophobic interaction. Figure 3 shows SEC salt plot studies for method optimization of anti-PD1 and anti-PDL1 sdAb analysis. A shorter column of 15 cm length was selected for this experiment to achieve higher throughput and speed up the method optimization process. Results showed that anti-PD1 sdAb had minimal or no secondary interaction with the column at the range of mobile phase

HPLC Conditions (SEC Salt Plot Study)	
Column	Agilent AdvanceBio SEC 1.9 µm 120 Å, 4.6 × 150 mm (p/n PL1580-3250)
Mobile Phase	20 mM sodium phosphate, pH 7.0 with concentration of sodium chloride indicated in Figure 3
Flow Rate	0.35 mL/min
Column Temperature	25 °C
Injection Volume	2 µL, 1 mg/mL
Total Run Time	7 min
Detection	UV at 214 nm

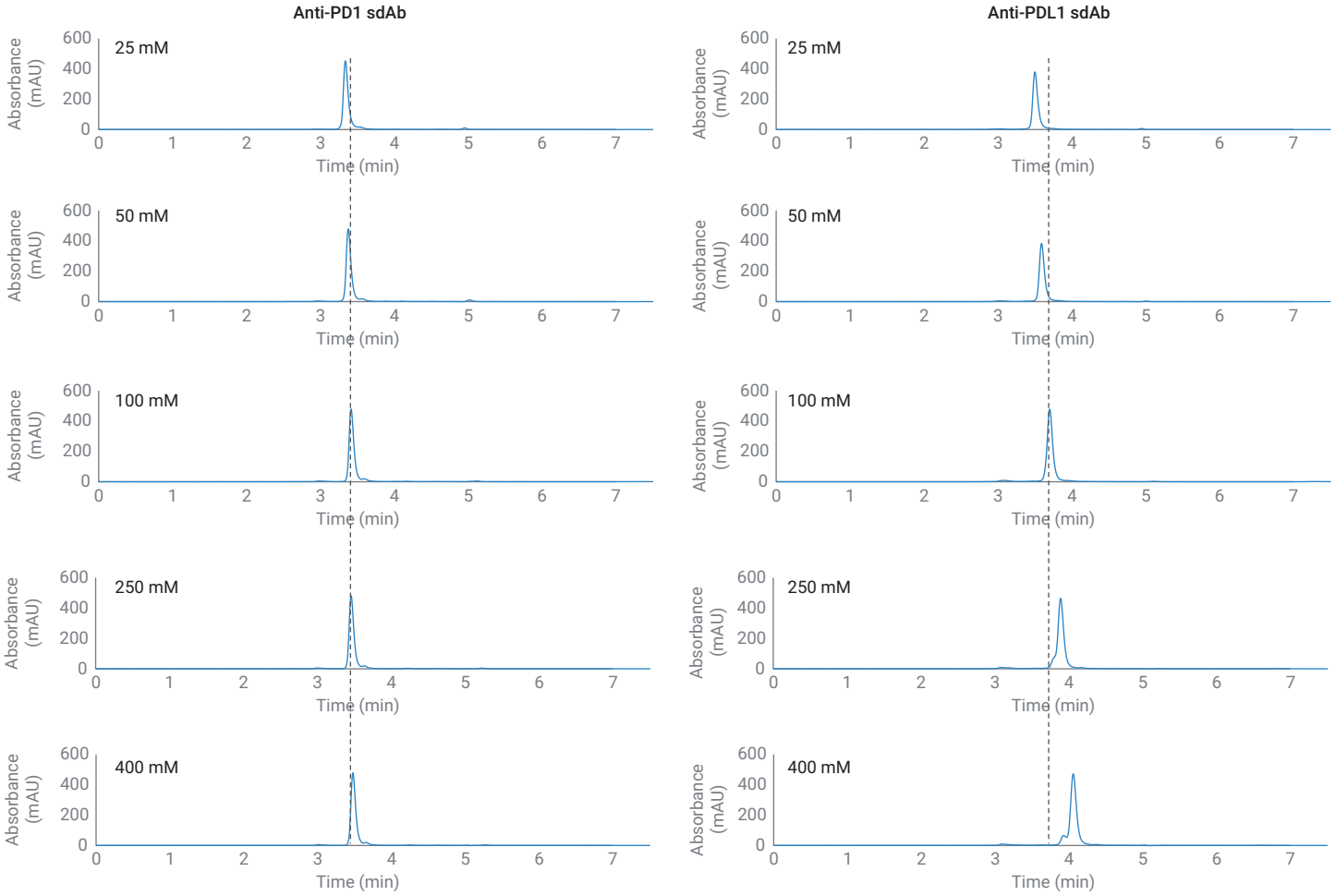

Figure 3. Salt plot studies of anti-PD1 (left) and anti-PDL1 (right) single-domain antibodies using the Agilent AdvanceBio SEC 1.9 µm 120 Å column.

NaCl concentration used. This is evident from the similar chromatographic peak shapes and retention times at each mobile phase condition. In contrast, anti-PDL1 sdAb had slightly deteriorated peak shape with delayed retention time at higher salt concentration mobile phase, indicative of potential hydrophobic interaction between analytes and the column. Interestingly, at 250 mM NaCl and above, a secondary (shoulder) peak became evident. This phenomenon is commonly encountered with challenging proteins that contain highly hydrophobic motifs or which have extreme isoelectric points. As nanobodies contain only the variable domain of an antibody, the amino acid sequence varies significantly when comparing one nanobody to another, potentially causing large differences in protein physicochemical characteristics. The method demonstrated here is useful for fast screening of SEC mobile phase conditions to determine the optimum conditions for analyzing different samples. The data inform the use of lower salt concentration (50 mM or below) in the mobile phase for anti-PDL1 sdAb.

To further characterize the two nanobodies, native SEC-LC/MS experiments were conducted (Figure 4). Results showed that the AdvanceBio SEC 1.9 µm 120 Å column was suitable for SEC-MS, where low concentrations of volatile aqueous buffer (i.e., 50 mM ammonium acetate) are used. Excellent ion chromatograms can be seen for both sdAb samples, together with high-resolution mass spectra. The deconvoluted MS results showed accurate molecular weight measurement for both samples: anti-PD1 sdAb (16,528 Da)

Native SEC-LC/MS Conditions	
Column	Agilent AdvanceBio SEC 1.9 µm 120 Å, 4.6 × 300 mm (p/n PL1580-5250)
Mobile Phase	50 mM ammonium acetate, pH 7.0
Flow Rate	0.35 mL/min
Column Temperature	25 °C
Injection Volume	20 µL, 1 mg/mL
Total Run Time	15 min
MS Detection	Min range: 300 *m/z* Max range: 7,000 *m/z* Ion polarity: Positive
MS Source Parameters	Gas temperature: 325 °C Gas flow: 5 L/min Nebulizer: 30 psi Vcap: 5,500 V Fragmentor: 250 V Skimmer: 65 V Octopole RF peak: 750 V

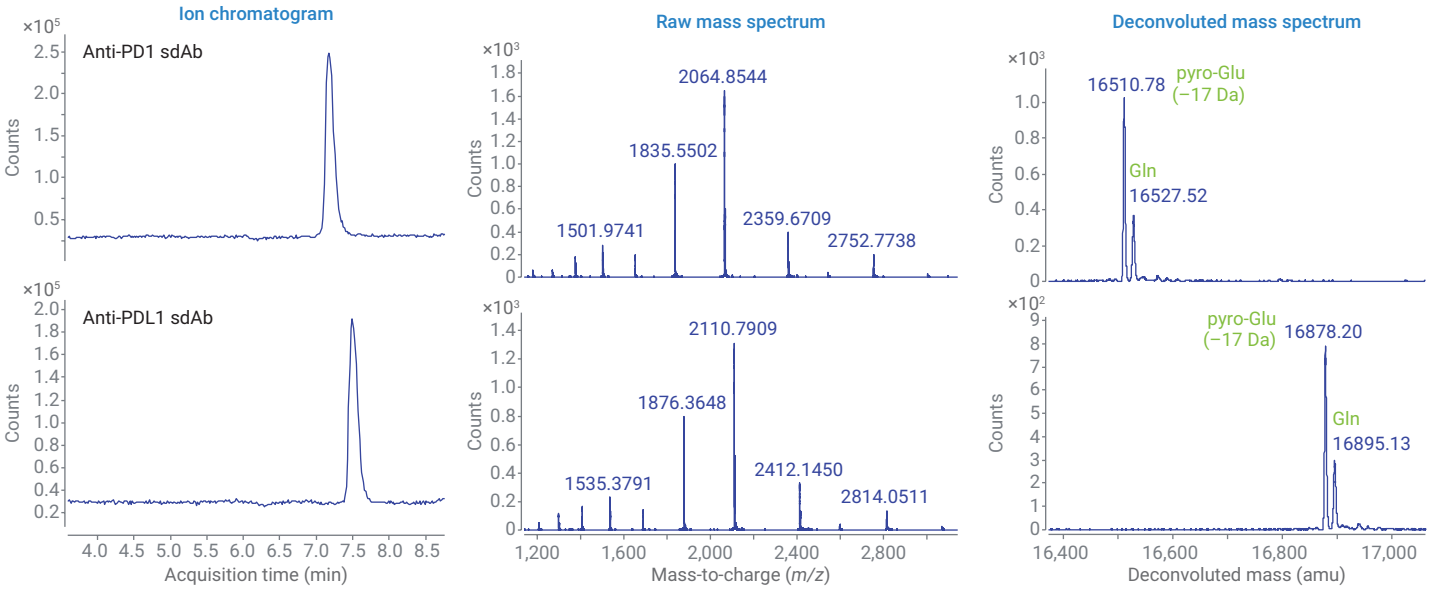

Figure 4. Native SEC-LC/MS analysis of anti-PD1 (top) and anti-PDL1 (bottom) single-domain antibodies.

and anti-PDL1 sdAb (16,895 Da). Interestingly, MS results revealed a large amount of a –17 Da modification on both samples. This is a typical mass shift associated with cyclization of N-terminal glutamine (Gln) to form pyro-glutamic acid (pyro-Glu).[5] The Gln/pyro-Glu conversion commonly occurs at the heavy chain variable domain of the antibody; thus, it is not surprising to observe this PTM in nanobodies (V_{HH} fragment). Both sdAb samples analyzed here were produced in bacteria (*E. coli*). It has been well documented that production in prokaryotic systems may result in proteins being recovered as inclusion bodies, thus leading to unusual PTMs.[5] Because the N-terminal Gln residues of V_{HH} are near the complementarity-determining region (CDR), pyro-Glu formation can potentially have significant impact on target binding.[1] Therefore, careful characterization and documentation of this PTM is typically required.[5]

To characterize protein Gln/pyro-Glu conversion, chromatographic methods such as ion-exchange chromatography and HIC can be used. Here, HIC was chosen because, in addition to PTM analysis, it also provided an assessment of hydrophobicity for the two sdAb samples. HIC uses a salting-out mechanism to separate intact, native proteins based on hydrophobicity under near physiological conditions. Figure 5 shows the HIC separation of anti-PD1 and anti-PDL1 sdAbs and an IgG mAb using a generic ammonium sulfate gradient method. For both Nbs, two chromatographic peaks were well resolved, indicating that both samples contained two species of different hydrophobicity. This result was consistent with the LC/MS data showing the Gln/pyro-Glu conversion. In addition, HIC data suggested that the anti-PDL1 sdAb was very hydrophobic. The anti-PDL1 sdAb retention time was much longer than anti-PD1 sdAb

HPLC Conditions (HIC analysis)	
Column	Agilent AdvanceBio HIC, 4.6 × 100 mm (p/n 685975-908)
Mobile Phase	A) 2 M ammonium sulfate in 50 mM sodium phosphate, pH 7.0 B) 50 mM sodium phosphate, pH 7
Gradient	0 min: 50% B 2 min: 50% B 17 min: 100% B 20 min: 100% B 22 min: 50% B 32 min: 50% B
Flow Rate	0.4 mL/min
Column Temperature	30 °C
Injection Volume	5 µL, 0.8 mg/mL (mAb) 5 µL, 1.0 mg/mL (sdAb)
Detection	UV at 214 nm

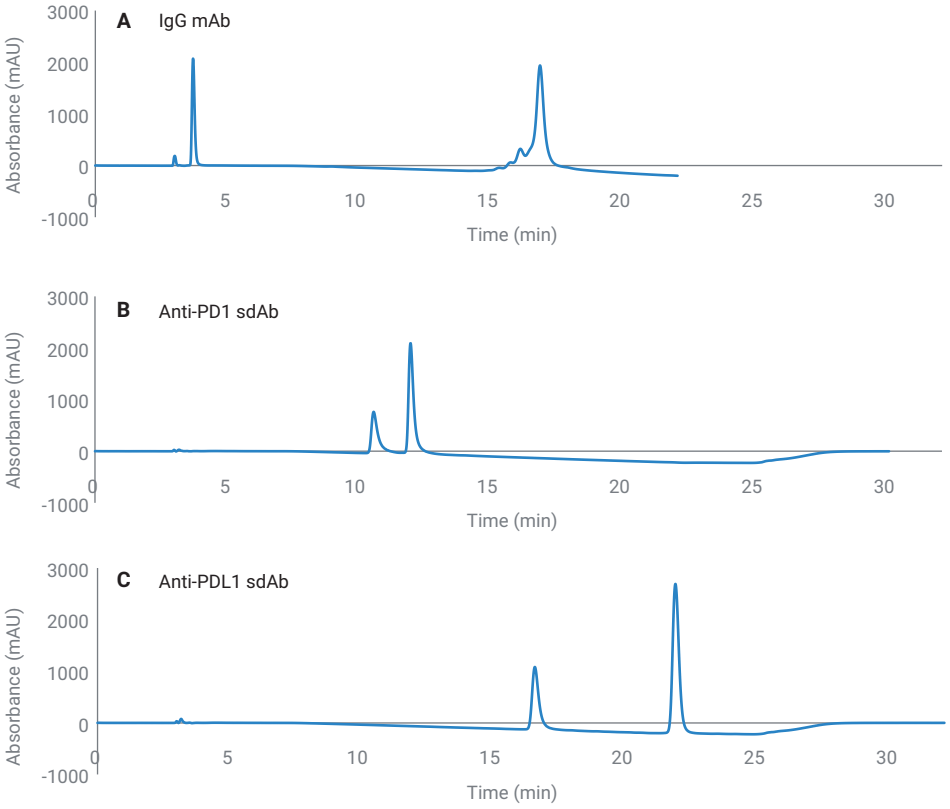

Figure 5. Hydrophobic interaction chromatograms of (A) an IgG mAb, (B) anti-PD1 single-domain antibody, and (C) anti-PDL1 single-domain antibody.

and the IgG mAb (with a molecular weight that is 10 times larger). These results help explain the observations from the SEC salt plot study (Figure 3), suggesting major physicochemical property differences between the two single-domain antibodies.

To further confirm whether the two peaks separated using HIC were indeed sdAb species that contained Gln or pyro-Glu, a biochemical approach was developed using glutaminyl-peptide cyclotransferase (QPCT) (Figure 6). The enzyme is known to catalyze the conversion of N-terminal glutaminyl residues of proteins to pyroglutamyl groups.[6] Indeed, treatment of both sdAb samples with QPCT resulted in chromatograms containing only the pyro-Glu peak. The experiments presented here using the AdvanceBio HIC column demonstrated excellent selectivity for nanobody PTM analysis.

Conclusion

Nanobodies are revolutionary, new biotherapeutic modalities that offer many advantages over conventional mAb therapy. For research and development of this novel class of biologic, it is of utmost importance to characterize and document quality attributes that can be formed or changed during the process of production and storage. These attributes have been shown to impact drug potency, pharmacokinetics, immunogenicity, and safety. Reliable and robust analytical tools and methods are needed. This application note presents SEC, HIC, and SEC-LC/MS techniques that can successfully be applied to nanobody characterization. Important critical quality attributes (CQAs) such as HMW aggregates and LMW fragments can be characterized using SEC in a high-resolution and high-throughput manner. Subtle changes on the molecules such as post-translational Gln/pyro-Glu conversion can be detected using HIC and SEC-MS approaches. The methods described here offer guidance for careful analysis of nanobodies in native, non-denaturing modes.

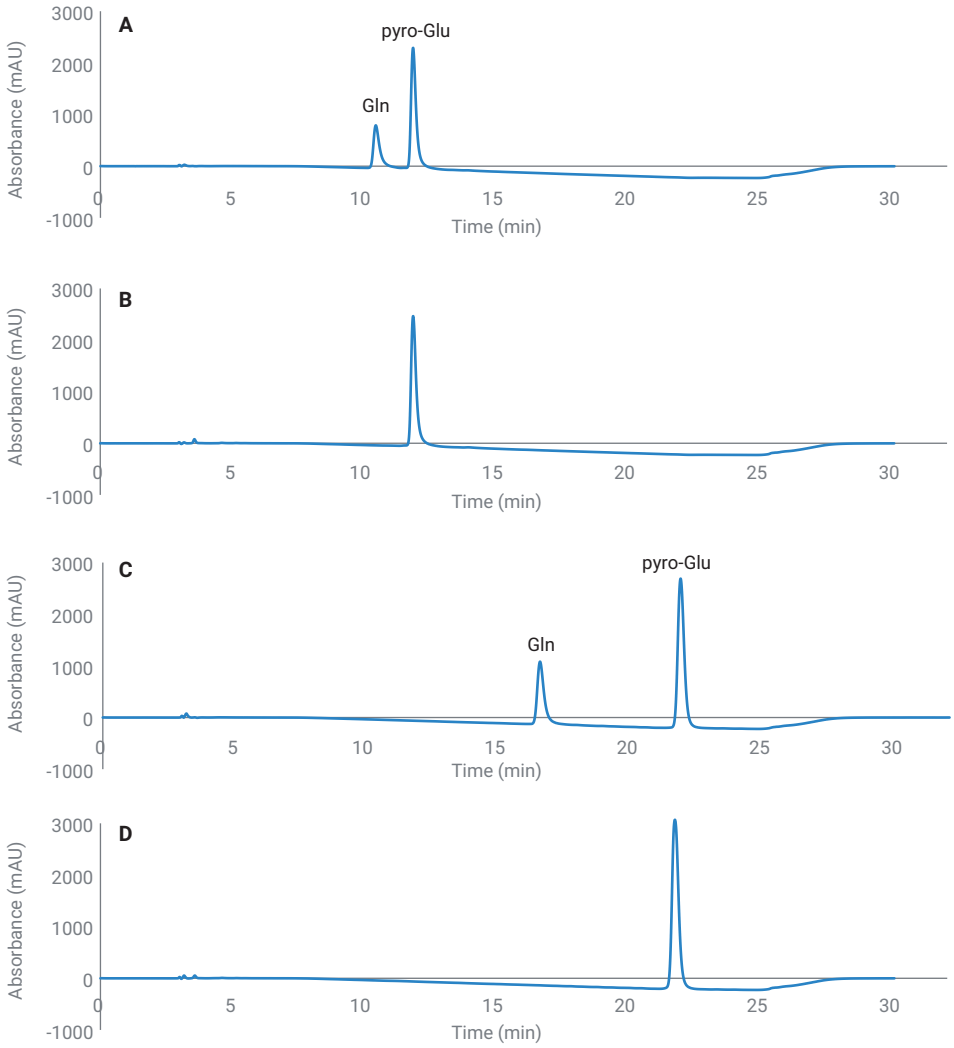

Figure 6. HIC analysis of (A) anti-PD1 sdAb, (B) anti-PD1 sdAb + QPCT, (C) anti-PDL1 sdAb, and (D) anti-PDL1 sdAb + QPCT. For enzyme treatment, 20 µL of sdAb (1 mg/mL) was incubated with 4 µL QPCT at 37 °C for 16 to 18 hours.

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