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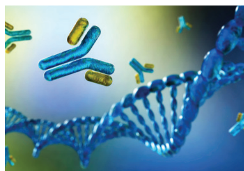
Workflow Solutions for Optimizing Native ADC and mAb Analysis

SEPTEMBER 2019



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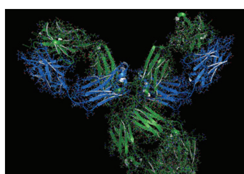

Preparation and Analysis of Released N-Glycans from Biotherapeutic Glycoproteins Using Two Labels: InstantPC and 2-aminobenzamide (2-AB)
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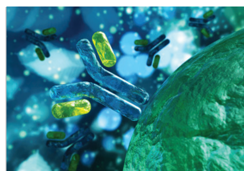

Applying High-Resolution and High-Efficiency Size Exclusion Chromatography Technology to Biotherapeutic Characterization
Veronica Qin, Yun Wang, and Zhengqi Zhang

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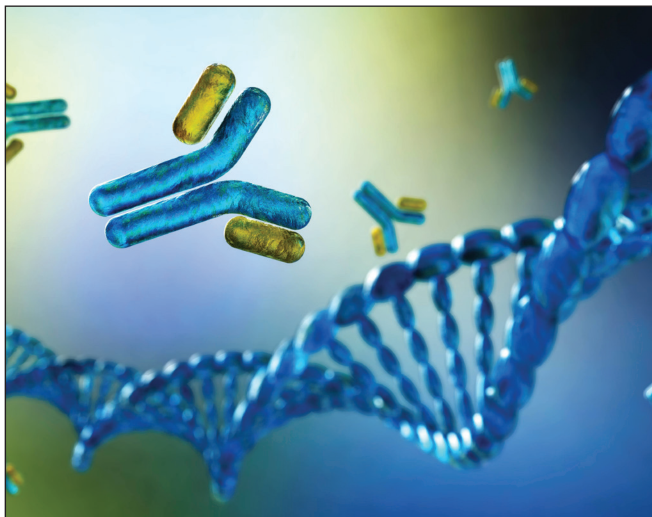

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Native Mode Analysis of mAbs and ADCs

Edward Ha and Sandeep Kondaveeti

Intact workflow solutions for characterizing biomolecules by HIC and SEC chromatography techniques.

Overview

Hundreds of therapeutic monoclonal antibodies (mAbs) and antibody–drug conjugates (ADCs) are currently in development, alongside notable examples approved in the market. Detailed analytical characterization of these biopharmaceutical proteins is a regulatory requirement. Aggregation, protein deamidation, oxidation, and glycosylation heterogeneity all affect the critical quality attributes (CQAs) of biologics. Size-exclusion chromatography (SEC) is the standard method used to quantify aggregation toward analytical and preparative processing of bioproteins. Hydrophobic interaction chromatography (HIC) is a powerful orthogonal chromatographic technique for separating proteins in order of increasing hydrophobicity using non-denaturing mobile phase buffers. Furthermore, the HIC technique is capable of resolving impurities caused by post-translational modifications (PTMs). Such impurities are often difficult to observe via other orthogonal chromatographic methods.

One possible improvement for measurements of biomolecules, which depends upon column optimization, is speed of analysis. Traditionally, 300-mm SEC columns of varying diameters have been used to achieve good separation of high-molecular weight species. Recent advances in column technology have allowed separation efficiencies on shorter length columns to be achieved, increasing sample throughput and accelerating drug discovery and process projects. SEC and HIC techniques for intact analysis of antibodies and ADCs, using the new Agilent suite of products, aid in the selection of optimal candidates for further CMC development and commercialization.

Analysis of mAbs and ADCs in their Native State

Biologic-based drugs, including mAbs, have become increasingly important in recent years. As the drug discovery process continues to evolve, biomolecules are becoming increasingly complex. Many of these molecules are produced using genetic engineering to develop recombinant methods of manufacture that result in some heterogeneity. With mAbs, many variants may arise during the manufacturing process. mAbs contain over 1,300 individual amino acids with numerous asparagine residues that could become deamidated and several methionine residues that could be oxidized. These impurities could have a significant impact on the efficacy and safety of the molecule, particularly if they are located in the antigen-binding region. Other variants, such as loss of C-terminal lysine from the heavy chains might be of less importance.

It is, therefore, necessary to prioritize the significance of these variants, which is done by determining critical quality attributes (CQAs). Many CQAs can be determined by various forms of liquid chromatography (LC). Some of the techniques, particularly reversed-phase LC, result in denaturation of the molecule during analysis. In contrast, analyses that employ Agilent's AdvanceBio SEC and HIC columns can be conducted under conditions that allow the molecule to remain in its native state.

SEC. SEC instruments excel at separating molecules based on large differences in size, for example, separation of monomers from dimers. Thus, it is the recommended technique for aggregation quantification. Proteins are very prone to aggregation under stress conditions such as changes in pH, concentration, and temperature, which may occur during purification. Separation with an AdvanceBio SEC column can be easily carried out under physiological conditions, roughly 150 mM salt concentration and pH 7–7.4, ideal conditions for maintaining protein integrity. Small amounts of aggregate as well as lower molecular weight impurities can usually be detected well below 1%.

For effective SEC analysis, a pore size should be selected that is appropriate for the analyte. In the case of mAbs and related molecules, the hydrodynamic size is typically around 5 nm. Dimers and higher order aggregates could be as large as 10 nm, which gives the optimum pore size for an SEC column of 300 Å. Good separation also requires a large available pore volume, since the sample will not be adsorbed onto the column. As such, SEC columns are usually at least 30 cm long, leading to long analysis times. The low flow rates and large column dimensions call for run times up to 20 minutes. This means, even with continual operation, it is only possible to run approximately 72 samples during a 24-hour period. A shorter column packed with high-efficiency particles combined with a faster flow rate may improve throughput by a factor of four. In regulated process and research environments, high throughput is essential.

It is imperative to consider the stationary phase chemistry when choosing a column, as interactions between the analyte and the stationary phase in the column must be avoided. SEC columns for protein separations are largely silica based. Silica has an acidic surface due to residual silanols, and most mAb-based proteins are somewhat basic, which can result in undesirable secondary interactions. More modern silica-phases have been developed, such as the AdvanceBio SEC columns, with a hydrophilic polymer coating applied to the silica particles to help to minimize secondary interactions. This column inertness becomes more important when looking at hydrophobic modified proteins such as ADCs, which are much more prone to undesirable secondary interactions.

HIC. HIC is an extremely powerful technique that uses non-denaturing conditions that keep proteins in their active conformation. It offers the separating power to resolve minor impurities that are exceedingly difficult to isolate by other LC approaches, including some of the more challenging variants such as oxidation. HIC is also a standard native technique to assay ADC drug-to-antibody ratio (DAR) and purity, and the new AdvanceBio HIC column with modified ZORBAX particle has a unique bonded phase designed specifically for high-resolution separation of ADCs.

Initially, the sample is dissolved in the mobile phase, however it is important to ensure the sample does not precipitate at this stage. The separation is performed under gradient elution conditions. Under the high salt conditions at the start of the gradient, the proteins will adsorb onto the stationary phase through hydrophobic interactions. The gradient runs from high to low salt concentration, thus allowing the proteins to elute in order of increasing hydrophobicity.

The stationary phase must be hydrophobic, but an ordinary reversed-phase column is not suitable, as it will likely result in phase collapse in the HIC eluent. Agilent's AdvanceBio HIC columns are not only hydrophobic but also capable of working in an aqueous high salt environment. Organic modifiers may still be used, if needed. These columns are versatile for a wide range of molecule types, from hydrophilic proteins to hydrophobic ADCs, reducing the demand for multiple columns.

Ammonium sulfate is typically the salt used for HIC, as it reduces protein solubility without causing denaturation and is transparent to the UV detector. It is readily soluble to the required level, often 2M, but this creates a rather viscous mobile phase (around 260 g/L). General good lab practices must be taken to avoid column damage caused by too much pressure. High salt concentration also promotes precipitation, making the use of Agilent's Bio-inert 1260 HPLC propitious, as it was expressly designed for operating under such conditions. The mobile phase should also include a dilute buffer to maintain the pH and stability of the sample during the separation.

HIC columns can be short lengths; efficient separations are possible on 10-cm long columns with a 2.1–4.6 mm ID; and in some cases, shorter columns may be used. AdvanceBio HIC columns also offer 3.5 µm particle diameters for reduced operating pressures. The high-viscosity mobile phase used for HIC creates significant back pressure with sub–2-µm particles. The 3.5-µm particle diameter allows for acceptable back pressure and, together with the larger frits used in the column assembly, reduces the potential for clogging. Moreover, the wider pore size (e.g., 450 Å) achieves good mass transfer. Impurity identification may require larger injection volumes or scale up to semi-preparative column dimensions; a fully porous ZORBAX particle provides that capability. Thus, Agilent AdvanceBio HIC columns deliver higher sample capacity but maintain rapid mass transfer.

Available column dimensions are 4.6 x 100 mm for higher resolution separations and 4.6 x 30 mm for faster separations. As in SEC, HIC separations may take a great deal of time to perform due to pressure restrictions and mobile phase viscosity. Using a shorter column with a higher flow rate greatly reduces the analysis time. Throughput can be tripled by switching from a 10-cm column to one that is 3 cm in length with a higher flow rate.

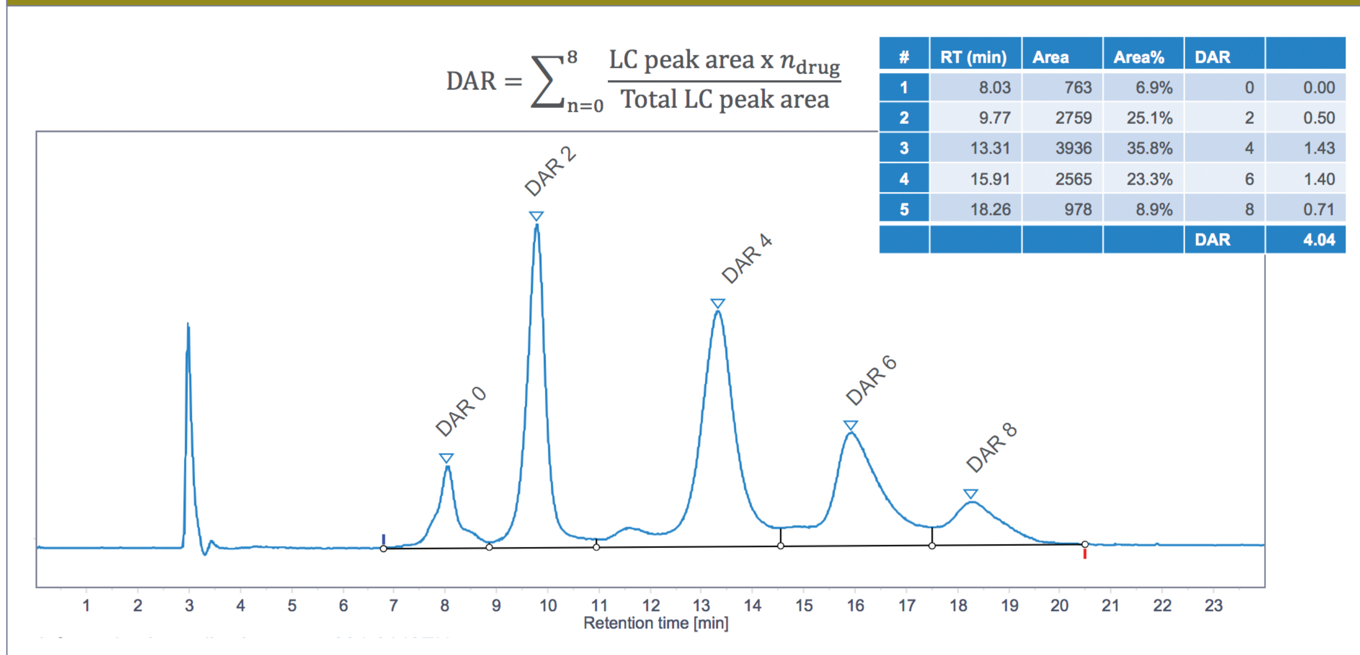
One challenge in making mass measurements from HIC and SEC separations is that their mobile phases used comprise high concentrations of salts that are incompatible with mass spectrometry. This can be addressed via offline fraction collection before analyzing each fraction by a mass spectrometry (MS)-compatible technique such as reversed-phase LC. The Agilent 1260 Infinity II Bio-Inert Fraction Collector is designed for this application. Alternatively, 2D-LC/MS is the simplest approach to accomplish the goal of mass measurement from high salt first dimension separations.

Monitoring oxidation in mAbs. HIC is often used for separation of challenging variants, including those caused by oxidation. NIST mAb used in this study contain 16 or so methionine residues distributed in the heavy and light chains. HIC can separate out oxidized variants, although it does not provide the ability to determine which methionine residues have been oxidized. Determination of oxidation locations would require an alternative technique, such as peptide mapping coupled with MS instruments.

Measuring DAR values. mAbs for therapeutics act as a delivery/targeting vehicle for small cytotoxic drugs. By conjugating several drug molecules to each mAb molecule, a higher specific therapy can be created. It is particularly important to determine the average number of drug molecules per mAb (i.e., DAR). Typically, up to eight drugs can be conjugated to a mAb before problems occur; the drug changes the hydrophobicity of the mAb, and too many drug moieties will cause poor bioavailability and increased *in vivo* toxicity.

Conjugating a linker-drug to the antibody can be achieved via multiple methods.

Figure 1: HIC separation of brentuximab vedotin and determination of DAR.



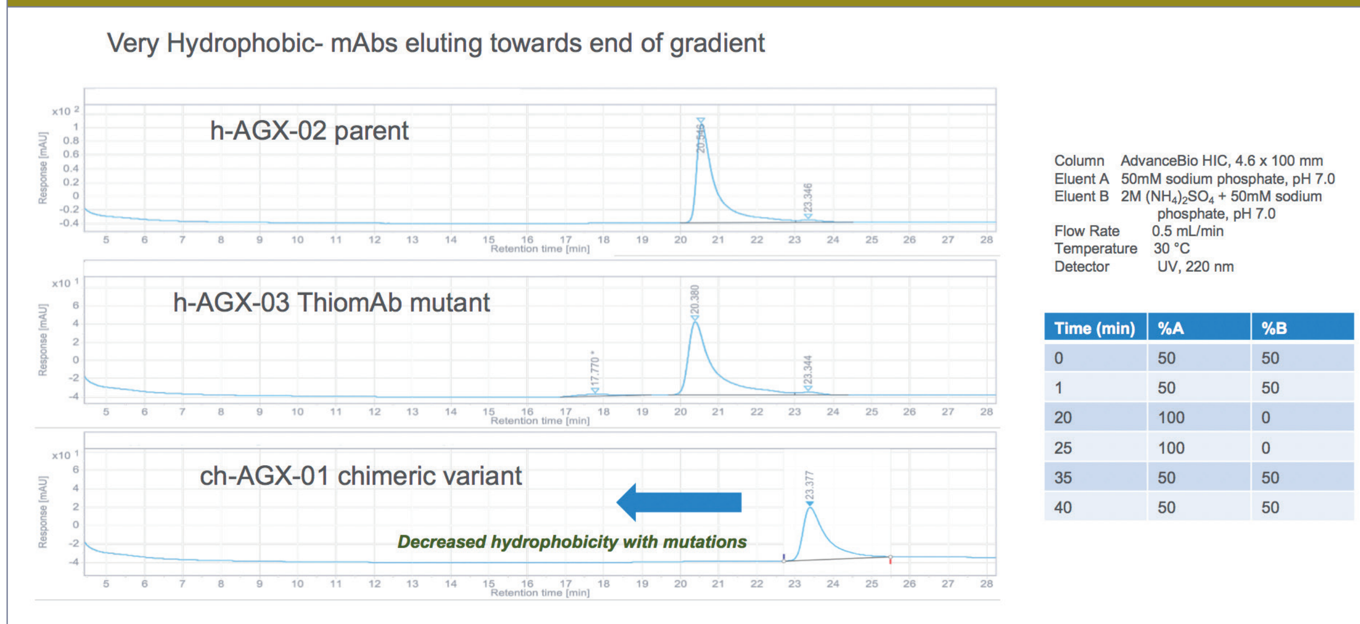
$$\text{DAR} = \sum_{n=0}^{8} \frac{\text{LC peak area x } n_{\text{drug}}}{\text{Total LC peak area}}$$

#	RT (min)	Area	Area%	DAR	
1	8.03	763	6.9%	0	0.00
2	9.77	2759	25.1%	2	0.50
3	13.31	3936	35.8%	4	1.43
4	15.91	2565	23.3%	6	1.40
5	18.26	978	8.9%	8	0.71
				DAR	4.04

One approach is to use the intra-chain hinge-cysteine linkages which naturally limits the maximum number of drugs that can be conjugated. Currently approved ADCs employ conjugation at cysteines in the hinge region or at different engineered sites on the antibody backbone. ADCs can also be conjugated via the lysine sites of the antibody. Cysteine-linked ADCs results a more fragile molecule however, a separation of the different DAR species under the non-denaturing conditions used for HIC is still possible. These ADCs typically demonstrate a DAR value of around 4, compared to lysine conjugates which have a DAR of around 2.7. Reliable LC instrumentation is required to monitor and control the conjugation process. Agilent Bio-inert 1260 HPLC and 1260 Infinity II Bio-Inert Fraction Collector coupled to AdvanceBio 6545XT Q-ToF MS are ideal for this application.

For example, different levels of cytotoxin conjugation in native ADCs may be investigated using HIC technique. The conjugates are eluted in order of increasing number of cytotoxin molecules. This is an orthogonal technique to reversed-phase LC, which is used for the characterization of denatured ADCs. With the correct method gradient, it is possible to use the HIC peak area results to calculate the average DAR value. In **Figure 1**, the calculated DAR of 4.04 for commercially available brentuximab vedotin is in close agreement with the published value. While this characterization includes an assessment of the conjugation, peptide maps or intact/subunit analysis via reversed-phase

Figure 2: HIC screening – hydrophobic index.



Time (min)	%A	%B
0	50	50
1	50	50
20	100	0
25	100	0
35	50	50
40	50	50

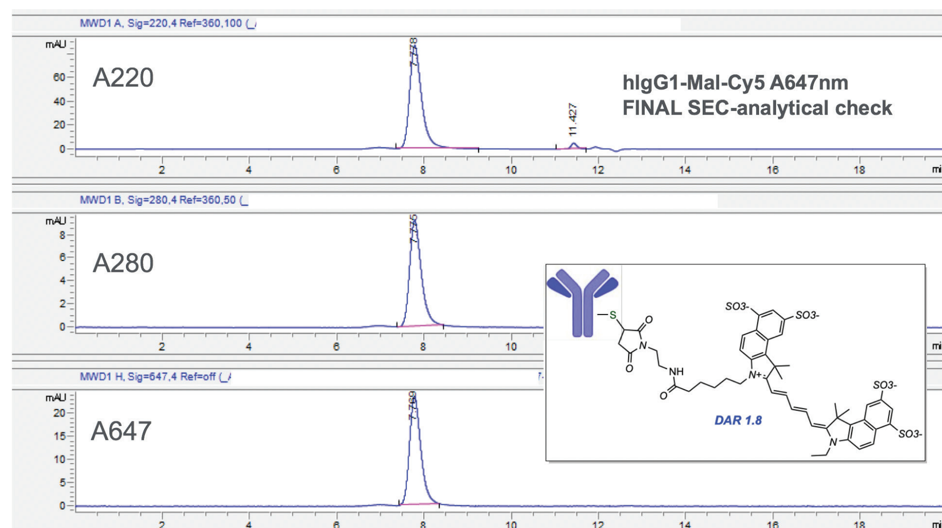
LC–MS analysis must be performed to identify the sites of conjugation.

Method Optimization and Applications

The first step of antibody analysis is triage, and a small Design-of-Experiments (DOE) is used to optimize the analytical method. Investigation of high molecular weight species, fragments and monomer is critical to determine if the antibody is monomeric enough to make an ADC. Buffer concentration, pH, mobile phase, and analyte concentration are among the factors that must be considered in the DOE. Agilent's method optimization software, Buffer Advisor, automatically generates the DOE and blends the buffers, which eliminates any errors that may result from manual blending. Peak shapes and resolution are optimized during this automated method development stage.

It typically takes about a day to scout out an investigational ADC in terms of the scope of conjugation with various bio-reduction agents as well as alkylation with the linker. Initially, stability of the modified antibody should be determined by SEC and HIC. Hydrophobic payloads can induce aggregation, valuable information to know in the early stages of drug development. A quick high-throughput SEC screen to look for aggregation is time well spent. Hydrophobic index screening can be readily performed using HIC, as shown in **Figure 2**. The chimeric form of mAb, represented in the bottom chromatogram, is most hydrophobic, with hydrophobicity decreasing in the human form, and slightly more so with a

Figure 3: hIgG1-Cys-Maleimide-Cy5 Conjugation Confirmation by SEC-UV.



SEC method

- 4.6mm x 300mm Agilent AdvanceBio SEC
- PLUS 50mm Guard column
- 0.350ml/min
- PBS 7.4 1x Gibco from Gibco 10x
- 15minute run
- 220, 260, 280 & 647 nm

- hIgG1 does not absorb at 647nm & maleimide-Cy5 dye absorbs at 647nm
- hIgG1-Maleimide-Cy5 absorbance overlap confirms alkylation
- Quantify via CoE's and UV-vis 280/647 ratio

mutagenized human form. Hydrophobicity of the mutant can also be compared with benchmark antibodies such as Herceptin in similar experiments. Antibodies are engineered to be less hydrophobic so that when joined with hydrophobic linker payloads, the overall conjugate will not precipitate out of the buffer. Forced degradation via oxidation can also be studied at this stage. Unexplained peaks should be investigated using mass spectrometry. For deeper characterization, antibodies can be digested and separated by HIC or reversed-phase techniques for the analysis of the fragments.

For site-specific homogeneous conjugation, a commercially available dye linker payload is recommended for initial conjugation studies as it is inexpensive and safe to use. Conjugation can be confirmed with SEC by overlapping chromatograms from the various wavelengths. Dye linker conjugated to antibody can be detected at a different wavelength illustrated in **Figure 3.** If the extinction coefficients of the antibody and linker payload are known, a rough DAR calculation may be obtained using areas under the curves. In addition, purified DAR fractions can be collected for *in vitro* studies and offline analytics such as digestion and peptide mapping.

Figure 3 demonstrates the utility of HIC for reduction/alkylation time studies; it indicates that the reactions of a NIST mAb with Cy5-dye are complete after 30 minutes. Mobile phase screening

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Figure 4: HIC method development — mobile phase screening, 4.6x30mm AdvanceBio HIC column.

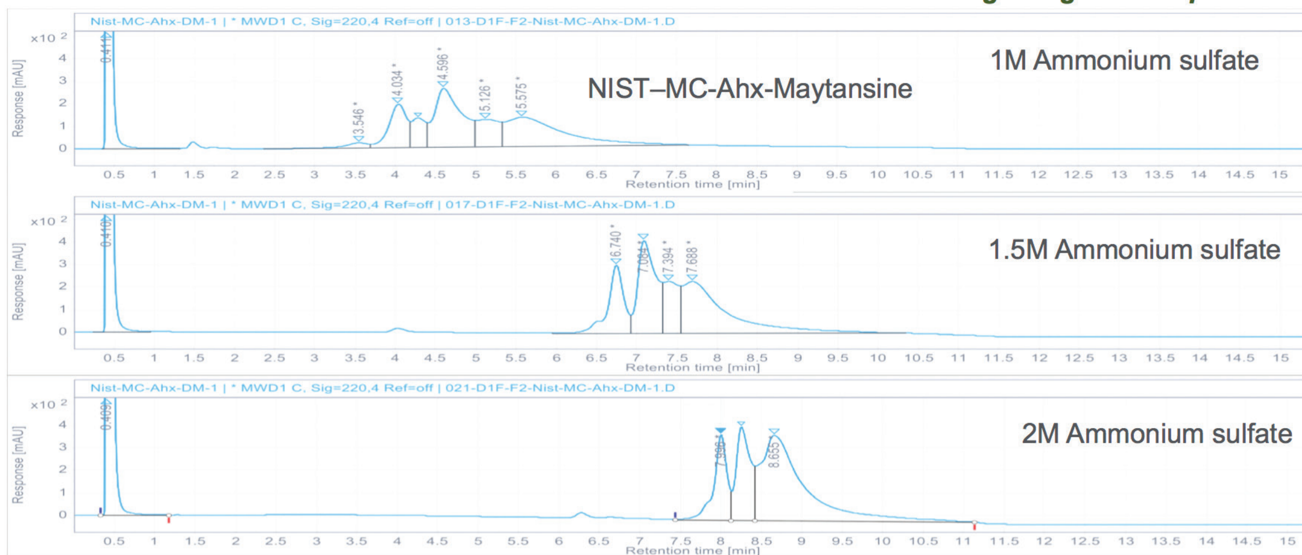
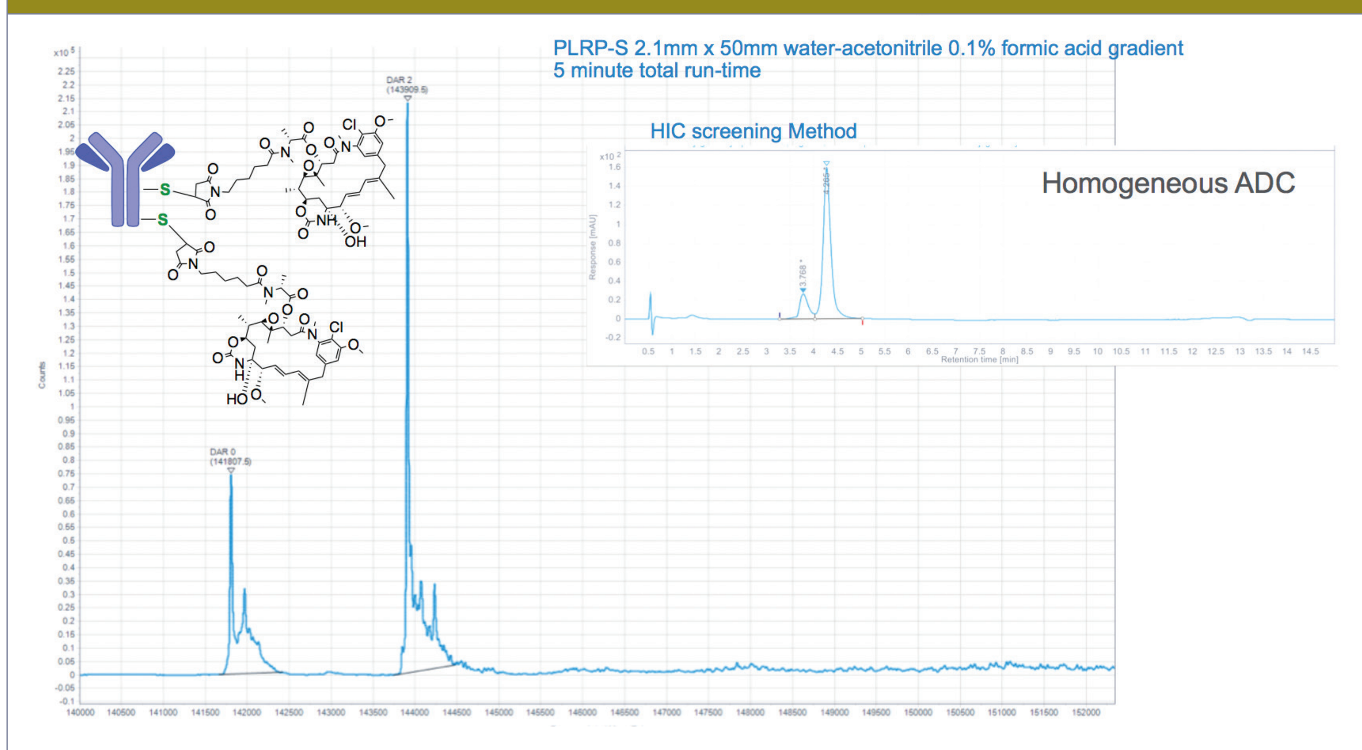


Figure 5: NIST-MC-Ahx-DM-1 random conjugation — effect of Propan-2-ol on ADC separation.

Time	%A	%B	%C
0	20	75	5
15	(a) 90 (b) 85 (c) 80	0	(a) 10 (b) 15 (c) 20
20	(a) 90 (b) 85 (c) 80	0	(a) 10 (b) 15 (c) 20
21	20	75	5
26	20	75	5

Figure 6: Native Q-Tof 6545XT LC–MS characterization of Angiex-ThiomAb-ADC.



for stochastic MC-Ahx-Maytansine conjugation is illustrated in **Figure 4.** In this site-specific homogeneous conjugation scheme, a full reduction followed by re-oxidation of the hinged disulfide left the engineered cysteine available for conjugation. Interestingly, lower salt concentration led to better resolution on the AdvanceBio HIC column. A short column was used for this experiment simply for speed; a longer column would enhance separation with better resolution

Since the presence of the hydrophobic drug molecule significantly changes the overall hydrophobicity of the ADC, isopropanol (IPA) is added to the mobile phase to ensure all DAR variants are eluted. In addition, a low amount of linker payload may produce odd species that may be hidden from detection under certain chromatographic conditions. A sufficient amount of IPA or acetonitrile modifier is necessary to reveal these hidden peaks. **Figure 5** illustrates the effect of IPA concentration on the resolution of closely eluting constituents. This is highly important, as it is vital to discover all conjugated biomolecule targets early in the development process, rather than discovering them as the project heads into the chemistry, manufacturing, and controls (CMC) endgame.

Mass spectrometry also provides essential information toward ADC

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development. Sole reliance on LC methods may be insufficient; employment of MS in tandem with LC ensures more complete characterization of the analyte. MS is commonly used to identify unexpected peaks from the chromatography that should be included in the CQAs of the ADC. The result of homogeneous ADC conjugation is shown in **Figure 6**, which contains both HIC and MS data. HIC screening revealed the partial conversion of the mAb to the conjugate, which was confirmed by MS analysis of the native species.

The intact analyte can also be digested to get the F(ab)2 fragments, a full reduction can be performed to discern the heavy/light chains, and peptide mapping can confirm where the alkylation occurred on the linker payload. MassHunter software from Agilent automates much of this to facilitate rapid data interpretation.

Conclusion

AdvanceBio SEC and HIC columns coupled to the Bio-Inert LC systems deliver a native workflow solution for high resolution separations of mAbs and ADCs. With ZORBAX fully porous particles and proprietary bonding technology, AdvanceBio HIC columns provide new levels of hydrophobicity and selectivity to address particularly challenging molecules such as mAbs and ADCs. Accordingly, the new AdvanceBio HIC column enables identification of post-translation modifications that are difficult to characterize using other chromatographic techniques. The optimized column particle chemistry produces better peak shape and resolution with lower back pressure.

> "AdvanceBio SEC and HIC columns coupled to the Bio-Inert LC systems deliver a native workflow solution for high resolution separations of mAbs and ADCs."

Designed for biopharma, Agilent's 6545XT AdvanceBio Q-TOF MS enables deeper interrogation of native investigational ADCs for more comprehensive understanding of biopharmaceutical molecules. Exceptional performance of the AdvanceBio suite of products leads to better understanding of CQAs, improved accuracy and speed of quantitation, and increased confidence in biopharmaceutical data.

Edward Ha is a principal scientist at Angiex. Sandeep Kondaveeti was an application scientist at Agilent, Inc.

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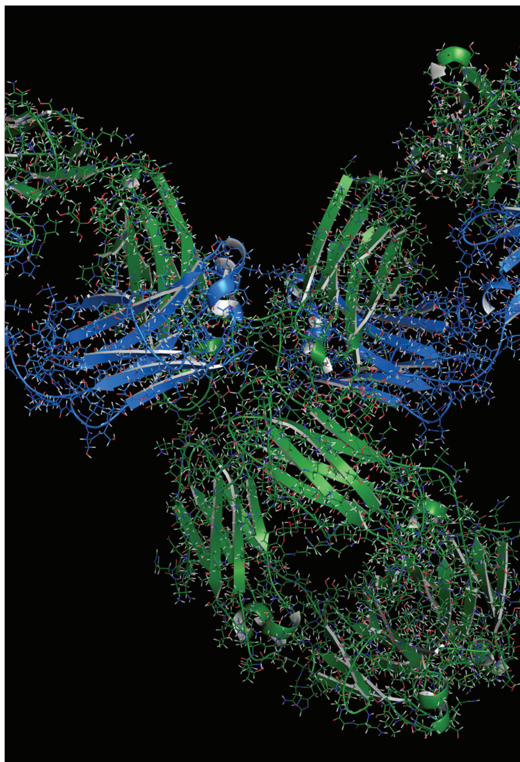

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Preparation and Analysis of Released N-Glycans from Biotherapeutic Glycoproteins Using Two Labels: InstantPC and 2-aminobenzamide (2-AB)

John Yan and Aled Jones

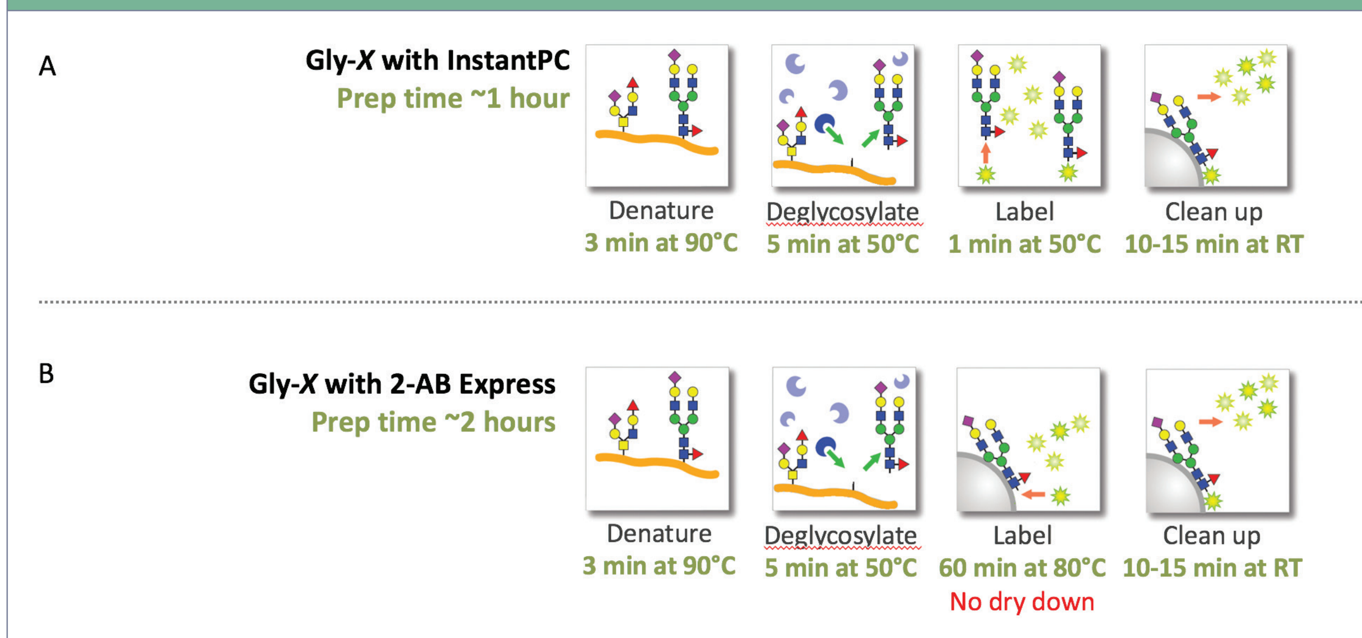
N-glycan analysis is vital to the development and production of biotherapeutics, as glycosylation can influence the therapeutic function of the final drug product.

Introduction

The characterization of N-glycans is an essential part of the biotherapeutic development process, as the structure of N-linked glycans can influence the function of glycosylated biotherapeutics, frequently making glycosylation a critical quality attribute (CQA) (1). N-glycan analysis often involves the labeling of released glycans with a tag to allow for detection by fluorescence and to enhance ionization for mass spectrometry (MS), followed by N-glycan separation, detection, and relative quantitation. Many frequently used fluorescent tags, such as 2-AB (2), are limited with regard to MS sensitivity compared with recently introduced dyes such as InstantPC. Additionally, pre-existing N-glycan sample preparation workflows can be time consuming (3). However, 2-AB has been used for over 20 years, so is well-established in the literature and in many laboratories.

This application note presents streamlined workflows for preparation of InstantPC and 2-AB labeled N-glycans, coupled with analysis using Agilent LC/FLD/MS instrumentation. **Figure 1** shows all the reagents for the Gly-X N-glycan sample prep kits for InstantPC or 2-AB Express, including: denaturation, deglycosylation, labeling, and sample clean-up.

Figure 1: Gly-X N-glycan sample prep. A) InstantPC workflow with in solution deglycosylation and labeling followed by on-matrix cleanup; B) 2-AB workflow with deglycosylation in solution, followed by on-matrix labeling and cleanup.



N-Glycan Sample Preparation

In this experiment, Agilent AdvanceBio Gly-X N-glycan prep with InstantPC and Gly-X 2-AB Express Kits were used to prepare labeled N-glycans from monoclonal antibody rituximab and Fc fusion protein etanercept, 40 µg protein per preparation. Both InstantPC and 2-AB labeled samples were prepared by standard manual protocols.

InstantPC and 2-AB labeled N-glycans were separated by HILIC using an Agilent AdvanceBio Glycan Mapping column, 2.1 x 150mm, 1.8 µm with an Agilent 1290 Infinity II UHPLC system with in-line fluorescence detection, coupled to an Agilent 6545 LC/Q-TOF mass spectrometer.

HILIC Separation of InstantPC and 2-AB N-Glycans

HILIC separation of labeled N-glycans from rituximab and etanercept labeled with InstantPC or 2-AB results in well-resolved peaks for major glycan species with the 60-minute method used.

Rituximab **(Figure 2; Figure 4A, 2-AB)**, an IgG, has an N-glycan profile typical of monoclonal antibodies with one N-glycosylation site in the Fc region produced in Chinese hamster ovary (CHO) cells: predominantly neutral biantennary complex N-glycans with core fucose, some Man5, and a relatively low proportion of sialylated glycans.

The N-glycan profile of etanercept **(Figure 3B; Figure 4B, 2-AB)**, an

Figure 2: Comparison of InstantPC glycosylamine labeling and traditional reductive amination with 2-AB.

Glycoprotein
N-Glycanase
InstantDyes: Glycosylamine Labeling ($-NH_2$)
$CH_2O(R)$
O
OH
Glycan
NH_2
NHAc
InstantPC™
H_2O
NH_3
Reductive Amination: Free reducing end labeling (-OH)
OH
NH
H_2N
2-AB

Fc fusion protein, contains a higher level of sialylated glycans because of two additional N-glycosylation sites in the fusion partner: TNF-α receptor (TNFR) extracellular domain and the single N-glycan site in the Fc portion (5).

The HILIC retention time of 2-AB N-glycans is shorter than for InstantPC N-glycans, although the elution order of N-glycan species is comparable. Critical pairs such as G0F/Man5 and Man5/G1, that are often monitored during the development process of biotherapeutics, are well-separated with both InstantPC and 2-AB labels, leading to confident determination of relative percentage composition. G1F isomers G1F[6] and G1F[3] are also separated. Relative percent areas, standard deviation, and relative standard deviation are reported in the tables alongside **Figures 3A and B**; **Figure 4A and B**, and they show a low degree of variability between the four sample prep replicates. This variability rises for lower abundance glycans.

Figure 3: HILIC-UHPLC fluorescence profile of A) Rituxan and B) Enbrel N-glycans labeled with InstantPC. N-Glycan relative percent areas are shown in the inset tables, n = 4.

A) Rituxan, InstantPC

	Average Rel % Area	Standard Deviation	%cv
G0F-N	0.75	0.01	1.55
G0	1.47	0.02	1.18
G0F	46.82	0.07	0.15
Man5	1.21	0.01	0.83
G1[6]	0.75	0.02	2.67
G1F[6]	31.21	0.11	0.35
G1F[3]	9.27	0.05	0.54
G2F	7.04	0.04	0.51
G2FS1[6]	0.67	0.02	2.29
G2FS1[3]	0.37	0.06	15.98
G2FS2	0.45	0.03	6.67

B) Enbrel, InstantPC

	Average Rel % Area	Standard Deviation	%cv
G0	1.10	0.02	2.09
G0F	19.36	0.16	0.84
Man5	5.08	0.03	0.52
G1[6]	0.48	0.00	0.00
G1F[6]	10.48	0.04	0.39
G1F[3]	3.97	0.01	0.25
G2	2.08	0.01	0.55
G1FS1	1.84	0.05	2.49
G2F	4.26	0.09	1.99
G2S1[6]	1.18	0.01	0.49
G2S1[3]	13.91	0.04	0.31
G2FS1[6]	0.89	0.00	0.00
G2FS1[3]	20.54	0.08	0.37
G2S2	4.26	0.01	0.14
G2FS2	10.54	0.08	0.78

Response Units vs. Acquisition Time (min)

An added benefit of InstantPC is the separation of isoforms G2S1[6]/[3] and G2FS1[6] from etanercept compared to 2-AB, using above described chromatography conditions. Analysis with fluorescence detection of InstantPC and 2-AB labeled N-glycans from biotherapeutics rituximab and etanercept results in comparable relative percent areas for major glycoforms G0F, G1F[6]/[3], G2F, G2S2, and G2FS2.

FLD and MS Detection of InstantPC and 2-AB N-Glycans

InstantPC displays higher fluorescence and MS signal compared to 2-AB when using the same amount of glycoprotein starting material (40 µg) and injecting the same relative volume for HILIC separations (1 µl of 100 µl kit eluent). Individual spectra for InstantPC and 2-AB labeled Man5 illustrates the higher MS signal of InstantPC.

Figure 4: HILIC-UHPLC fluorescence profile of A) Rituxan and B) Enbrel N-glycans labeled with 2-AB. N-Glycan relative percent areas are shown in the inset tables, n = 4.

A) Rituxan, 2-AB

	Average Rel % Area	Standard Deviation	%cv
G0F-N	0.78	0.09	11.94
G0	1.64	0.05	3.12
G0F	44.89	0.39	0.87
Man5	1.54	0.14	8.83
G1F[6]	31.39	0.09	0.27
G1F[3]	10.40	0.14	1.34
G2F	7.52	0.16	2.10
G2FS1	1.17	0.03	2.13
G2FS2	0.67	0.02	3.58

B) Enbrel, 2-AB

	Average Rel % Area	Standard Deviation	%cv
G0F-N	0.32	0.02	7.44
G0	1.27	0.07	5.34
G0F	20.18	0.45	2.22
Man5	5.50	0.34	6.17
G1[6]	0.45	0.02	3.89
G1F[6]	10.35	0.33	3.18
G1F[3]	3.92	0.17	4.39
G2	2.21	0.15	6.78
G2F/G1FS1	7.00	0.25	3.63
G2S1	15.19	0.17	1.09
G2FS1	20.10	0.32	1.59
G2S2	4.19	0.25	5.95
G2SF2	9.35	0.74	7.93

Response Units vs. Acquisition Time (min)

Conclusion

Glycosylation is a feature of many biotherapeutic proteins and is often a CQA that must be monitored. N-glycan analysis is, therefore, important in the development and production of therapeutic proteins. Gly-X N-glycan sample preparation workflows enable five-minute release of N-linked glycans suitable for labeling both by glycosylamine labeling with InstantPC and reductive amination chemistry with 2-AB. These workflows allow for instant glycosylamine labeling with InstantPC or no dry-down, on-matrix reductive amination labeling with 2-AB. Glycan species were profiled by relative fluorescence peak area percentage and peak assignments confirmed by high-resolution MS. Compared with 2-AB,

InstantPC labeled glycans display higher fluorescence signal and greater MS ionization efficiency in positive mode, allowing for confident detection of low-abundance glycan species. Although the performance benefits of InstantPC are clear, 2-AB is an N-glycan label that has been used for many years, so a rapid 2-AB workflow enables continuity with historical 2-AB N-glycan data sets.

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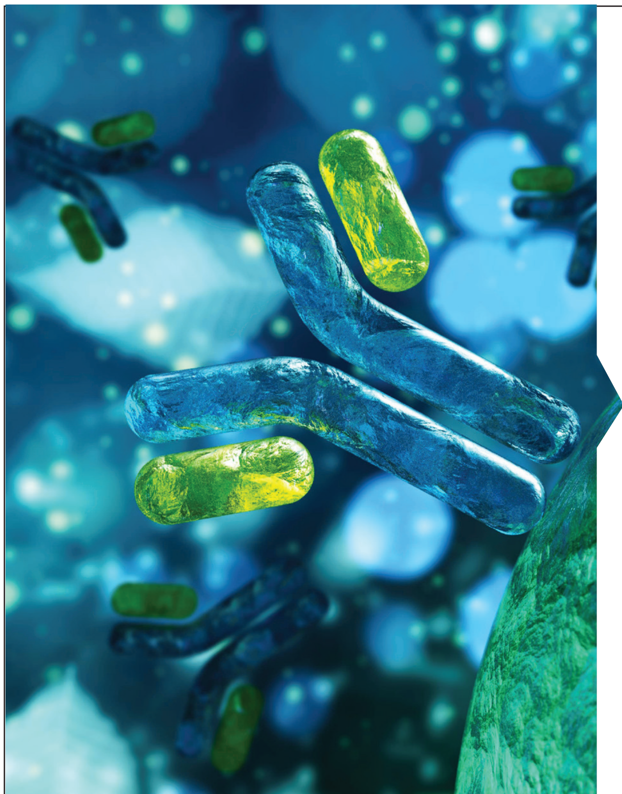

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Applying High-Resolution and High-Efficiency Size Exclusion Chromatography Technology to Biotherapeutic Characterization

Veronica Qin, Yun Wang, and Zhengqi Zhang

More efficient and accurate analysis of potential biotherapeutics at an early stage of development can speed up successes and reduce failures.

Overview

Scientists use size-exclusion chromatography (SEC) to study many biomolecules. SEC columns with sub-2 µm particles can afford high resolution and efficiency separations for characterizating size variants of biotherapeutics. SEC can also be used in two-dimensional (2D) liquid chromatography–mass spectrometry (LC–MS) separation and with MS detection to support structural characterization of biotherapuetics. This summary discusses some of the challenges and solutions for getting the most accurate data out of an SEC separation.

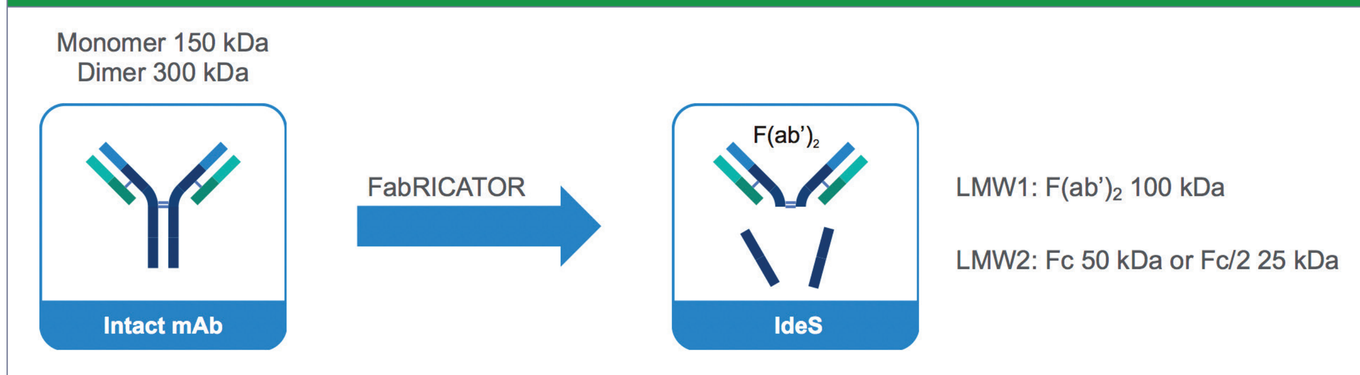
Column Features

Over time, particle sizes in SEC columns have become smaller, thus offering columns with higher efficiencies. Additionally, optimized surface coatings helped to improve the separation characteristics.

It is critical to choose an SEC column with the correct pore size that would allow the molecule of interest to permeate the pores while having the maximum amount of pore volume to help resolve other size variants. SEC separation is based on the size of the molecules rather than their molecular weight. However, since most proteins are globular in nature, resolving ranges is often expressed in terms of molecular weight.

A column with sub-2 µm particles, such as the Agilent AdvanceBio SEC

Figure 1: Target application: mAb monomer, dimer, and LMW fragments.



200 Å 1.9-µm columns, increases the resolution of monomers and other fragments with low molecular weights. In this case, the 200 Å pores are designed to analyze molecules with a hydrodynamic radius of 6.7 nm or less, which works well with monoclonal antibody (mAb) monomers and dimers as well as separating low molecular weight (LMW) molecules. It can also reduce non-specific interaction and increase the life of the column. For example, AdvanceBio SEC 200 Å 1.9-µm columns provide better resolution at a relatively low pressure and a reduced risk of clogging in the column, which allow for faster separations.

Sub-2 µm Applications

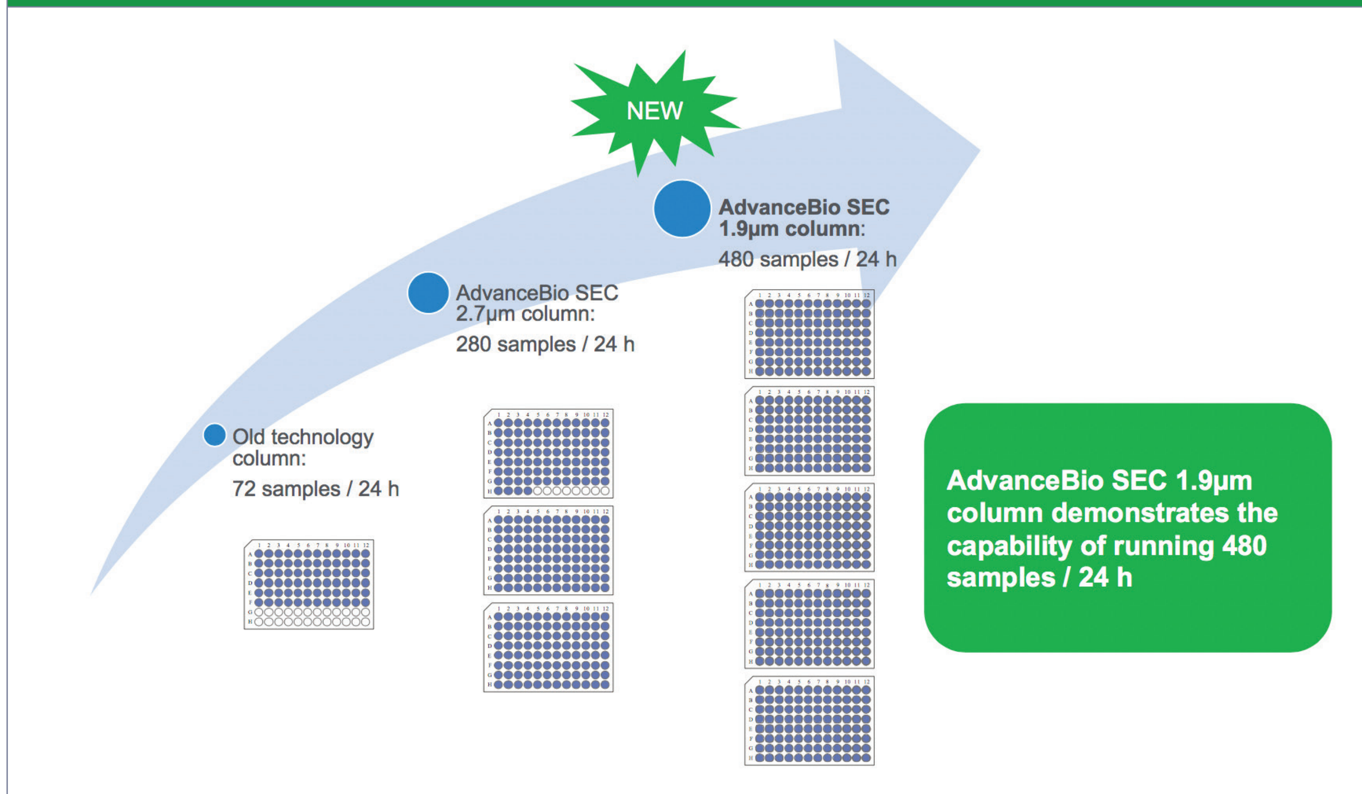
Using smaller particles, especially ones less than 2 µm in diameter, provides many benefits. For example, the high resolution makes quantification possible. In characterizing biotherapeutics, this technology can be used in four ways:

1. LC with ultraviolet (UV) detection can be used to quantify high molecular weight (HMW) dimers and LMW fragments;
2. LC–MS in native or denaturing modes can be used in various ways, including analyzing protein-ligand and protein-protein interactions;
3. With 2D-LC, scientist can analyze aggregate and free drugs, plus other forms of analysis; and
4. Bioprocessing, such as monitoring antibody aggregation.

With antibodies, as shown in **Figure 1**, analysis can involve separations of various components, including mAb dimers, monomers, and fragments, including antigen-binding (Fab)2 and fragment crystallizable (FC) fragments. When separating these mAb components, the resolution depends on particle size, in addition to other factors that affect the column performance.

The surface of the particles, for instance, impacts non-specific interactions. As an example, AdvanceBio SEC 200 Å 1.9 µm particles are inert due to unique phase chemistry, a hydrophilic coating, and

Figure 2: Column length increases throughput.



optimized bonding density. Also, using a highly crosslinked phase makes the column chemically stable. Together, this results in a sharp monomer peak and well-resolved fragment peak. Under similar conditions, a sample performed on other vendor columns observed poor performance with split peak. This might be because of high hydobophocity of sample causing secondary interation with column media.

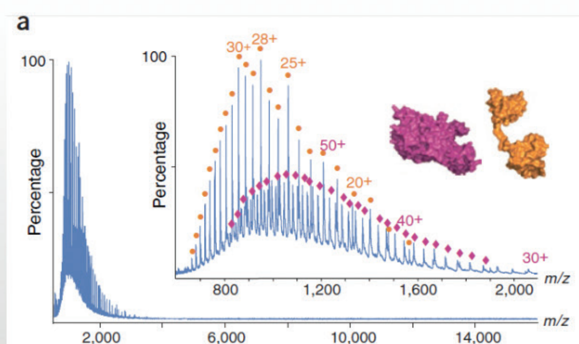
Looking for a Long Life

In addition to achieving desirable separations, scientists want a column that can be used as long as possible with consistent peak areas and stable column backpressure over hundreds or even thousands of injections.

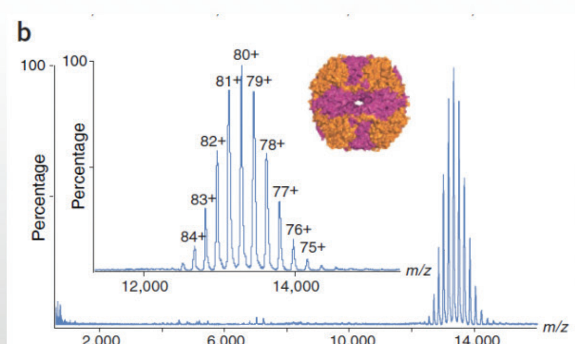
Agilent's R&D team has designed a stop-and-run method to understand the mechanical strength of the particle and probe for column lifetime stability. In this instance, flow is started, completely stopped, and then started again. This represents the use of a column that is not constantly running. Some columns show reduced efficiency after 200 injections by this method. With an AdvanceBio SEC 200 Å 1.9 µm column, the performance stayed the same for at least 400 injections in one comparison.

Figure 3: Challenge of native analysis.

1. Separation condition may be incompatible with MS condition.
   Mobile Phase, pH, Salt concentration, Flow rate etc.
2. Ionization could also lead to denature.
3. High m/z range and low analytical sensitivity



Protein complex under denatured condition



Protein complex under native condition

The length of the column also plays a role in performance (**Figure 2)**. For example, reducing the length of a column by half cuts the runtime in half and doubles the throughput. The reduced length also produces less back pressure, which allows for higher flow rates.

In one example, the run time was reduced to three minutes—compared to previous SEC columns taking 20 minutes to run. As **Figure 2** shows, this increases the throughput from 72 samples in 24 hours into 480.

Characterizing Biologics

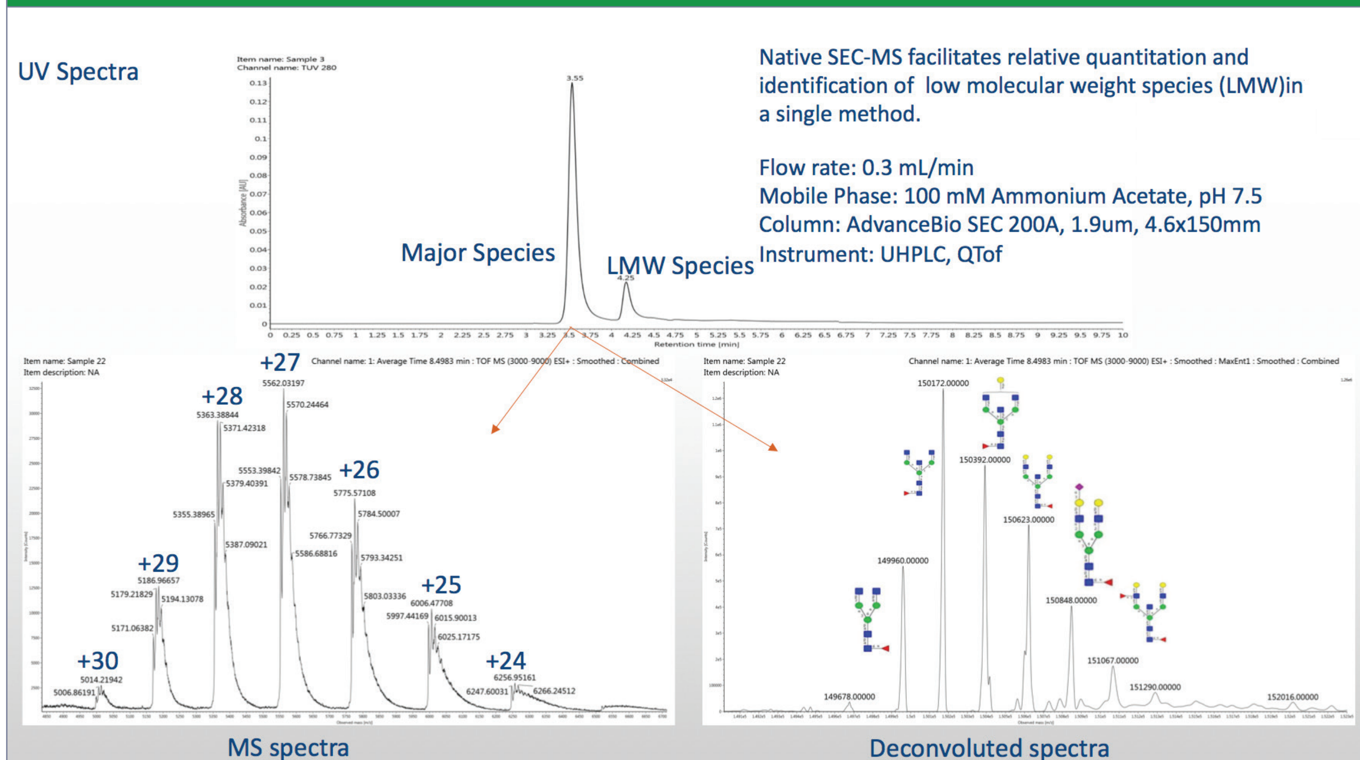
High-efficiency SEC makes an effective tool for characterizing biology-based therapies, which are often called biotherapeutics or biologics. An example application is stability characterization.

For product quality, pharmaceutical scientists assess a biologic's chemical and physical properties. Running these studies early in the drug-development process reduces late-stage failures. In short, early protein characterization reduces risk in chemistry, manufacturing, and controls developent to bring the final pharmaceutical product to the market.

Native Analysis

Scientists at Frontage Laboratory analyze large molecules for many features: charge variants, size variants, as well as the glycan profile, binding potency, and host-cell proteins. Some of these scientists also

Figure 4: Case Study 1: Characterization of mAb using native MS.



use native MS on proteins in their native condition, which means that the folding is intact. To maintain a protein's natural structure, no denaturing solvent is used during LC and only soft ionization is used in the MS stage.

The pharmaceutical industry uses native MS for many reasons, such as analyzing protein–protein and protein–ligand interactions. This method can also be used to assess how much of a large-molecule sample is oligomer versus monomer.

Despite many technological advances, **Figure 3** shows why native MS remains challenging. One challenge is caused by normal salt buffers used in SEC being incompatible with MS. Another challenge includes potential denaturation of proteins caused by ionization.

In a study using an MS-compatible mobile phase, 100 millimolar ammonium acetate at pH 7.5, in the AdvanceBio SEC 200 Å 1.9 µm columns, UV detection showed good separation. Then, using this mobile phase and column, scientists at Frontage Laboratory characterized a mAb with native MS, as shown in **Figure 4**. The UV spectra (top) shows two peaks: the major species and a LMW species. The zoomed-in MS spetra (lower left) shows charge states from 24 to 30, and deconvolution (lower right) shows six major glycoforms of the intact mAb, around 150 kDa. The scientists were able to identify different glycoforms

of the intact mAb. For the LMW species, its mass is about 64 kiloDaltons, as revealed after deconvolution (not shown here), which matches the molecular weight of the transition from the hinge region of the mAb. The key here is that one method can be used for relative quantification and identification of the components.

For looking at oligomerization, the company's scientists analyzed bovine serum albumin (BSA). The results of SEC and a UV spectra showed two major peaks—presumably one being the monomer and the other the dimer. The MS spectra and deconvoluted spectra for the major and minor peaks showed molecular weights that matched the calculated weights for the monomer and dimer, respectively.

Such studies of oligomerization give an idea of how SEC-MS can be used to study binding between proteins or proteins and ligands. This technology can also be used to analyze cysteine-conjugated antibody–drug conjugates (ADCs).

In-Practice Pointers

In all applications of a sub-2 µm SEC column, several elements of the system should be considered. One is to use tubing with a 0.12 millimeter internal diameter, which reduces the extra-column volume and minimizes band broadening.

To extend a column's lifetime, the flow of the mobile phase should be increased gradually. As an example, start at 0.1 mL/min and slowly increase the flow in increments of 0.1 mL/min. Continue the slow increase to the desired flow rate.

Bad connections can also reduce the quality of data. High data collection rates are also essential to accommodate the sharp peaks that sub-2µm columns can provide.

Conclusion

Scientists can use SEC to characterize biotherapeutics for a wide range of applications. This technology can also be combined with UV or MS detection to quickly analyze a range of samples—from protein mixtures or mAb and fragments, to more complex structures such as a mAb bound to an antigen. These techniques can provide pharmaceutical scientists with the information from early stages of drug development that can prevent costly late-stage failures.

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