High-Resolution Separation of Intact Monoclonal Antibody Isoforms

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Key Words
Protein heterogeneity, cation exchange, glycosylation, glycoforms, MAbPac SCX-10 column, CX-1 pH gradient buffer

Abstract
To develop a high-resolution, fast separation of intact protein sialylation isoforms using the Thermo Scientific™ MAbPac™ SCX-10 column and CX-1 pH gradient buffer on a Thermo Scientific™ Dionex™ UltiMate™ 3000 Dual Biocompatible Analytical LC system and an UltiMate 3000 BioRS LC system.

Introduction
Glycosylated proteins–including erythropoietins, monoclonal antibodies (mAbs), and various hormones–constitute a large portion of major approved therapeutic protein drugs. Sialic acid, usually attached at terminal positions of glycan molecules, is important to many biological processes such as cell recognition and migration. Sialic acids also have significant effects on the properties of therapeutic proteins, especially on their circulation half-life. For example, the circulation half-life of sialylated rhEPO is 5.6 h, whereas that of nonsialylated rhEPO is 1.4 min [1]. Thus, monitoring protein glycosylation, including sialylation, is important for both glycoprotein characterization and quality control purposes. Recently, an application note from Thermo Fisher Scientific showed good resolution of mAb sialylation isoforms by pH gradient ion-exchange chromatography using Thermo Scientific™ ProPac™ SCX-10 columns and self-prepared mobile phases [2]. The work described here shows a fast, reproducible, high-resolution, pH gradient ion-exchange chromatography approach for the separation of monoclonal antibody sialylation isoforms using the MAbPac SCX-10 column and CX-1 pH gradient buffer.

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Experimental Details

Consumables	Part Number
Deionized (DI) water, 18.2 MΩ-cm resistivity, generated from the Thermo Scientific™ Barnstead™ GenPure™ Pro UV-TOC Water Purification System	50131948
CX-1 pH gradient buffer A (pH 5.6), 250 mL, Thermo Scientific	085346
CX-1 pH gradient buffer B (pH 10.2), 250 mL, Thermo Scientific	085348
Neuraminidase, Fisher Scientific™	P0720S

Sample Handling Equipment
UltiMate 3000 Dual Biocompatible Analytical LC system*, including:
DGP-3600BM Biocompatible Dual-Gradient Micro Pump
WPS-3000TBFC Thermostatted Biocompatible Pulled-Loop Well Plate Autosampler with Integrated Fraction Collection
TCC-3000SD Thermostatted Column Compartment
DAD-3000 Diode Array Detector with 13 µL flow cell
UltiMate 3000 BioRS system, including:
LPG-3400RS Quaternary Rapid Separation Pump
WPS-3000TRS Rapid Separation Wellplate Sampler, Thermostatted
TCC-3000RS - UltiMate 3000 Rapid Separation Thermostatted Column Compartment
DAD-3000RS - UltiMate 3000 Rapid Separation Diode Array Detector
Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System (CDS) software version

* This application was successfully evaluated on the two UltiMate systems listed.

Preparation of Mobile Phases	
Mobile Phase A	10-fold dilution of CX-1 pH gradient buffer A (pH 5.6) with DI water
Mobile Phase B	10-fold dilution of CX-1 pH gradient buffer B (pH 10.2) with DI water

Sample Preparation
The mAb sample [5 mg/mL] was kindly provided by Shanghai National Engineering Research Center of Antibody Medicine Co., Ltd. (Shanghai, China)
Neuraminidase Digestion Procedure This procedure follows vendor recommended reaction conditions with minor modifications. Combine 4 µL (equal to 20 µg) of mAb, 31 µL water, and 5 µL of 10X G1 Reaction Buffer to make a 40 µL total reaction volume. Add 10 µL neuraminidase and incubate at 37 °C for 1 h.

Separation Conditions		Part Number
Columns:	MAbPac SCX-10, 10 µm, 4 × 250 mm MAbPac SCX-10, 5 µm, 4 × 150 mm	074625 085198
Mobile Phase:	A: 10-fold dilute CX-1 pH gradient buffer A (pH 5.6) B: 10-fold dilute CX-1 pH gradient buffer B (pH 10.2)	
Gradient:	For MAbPac SCX-10 column (10 µm): 0–30 min: 10–35% B	
	For MAbPac SCX-10 column (5 µm): 0–12 min: 18–40% B	
Flow Rate:	1.0 mL/min for the columns with 10 µm particle size 0.8 mL/min for the columns with 5 µm particle size	
Injection Volume:	50 µL for the columns with 10 µm particle size 25 µL for the columns with 5 µm particle size	
Column Temperature:	30 °C	
Detection:	UV, absorbance at 280 nm	

Results

Separation of a Highly Sialylated mAb on a MAbPac SCX-10 Column

A new generation of ion exchange column, the MAbPac SCX-10 column, has been shown to provide better resolution of mAb charge variants compared to earlier generation columns [3]. Figure 1 shows that when using the CX-1 pH gradient buffer, the MAbPac SCX-10 column resolved 19 mAb peaks, many of which are likely sialylation isoforms with this highly sialylated mAb. The MAbPac SCX-10 column not only provided better resolution between major peaks compared to the ProPac SCX-10 column, but also resolved more minor peaks. The MAbPac SCX-10 column resolved some small peaks that are not observed in the ProPac SCX-10 column chromatogram [2]. Under these optimized conditions (column, mobile phase, and narrow pH range), the pH gradient ion-exchange chromatogram is impressive in its resolution of the large number of isoforms present in this mAb, many of which are putative sialylation isoforms (glycoforms).

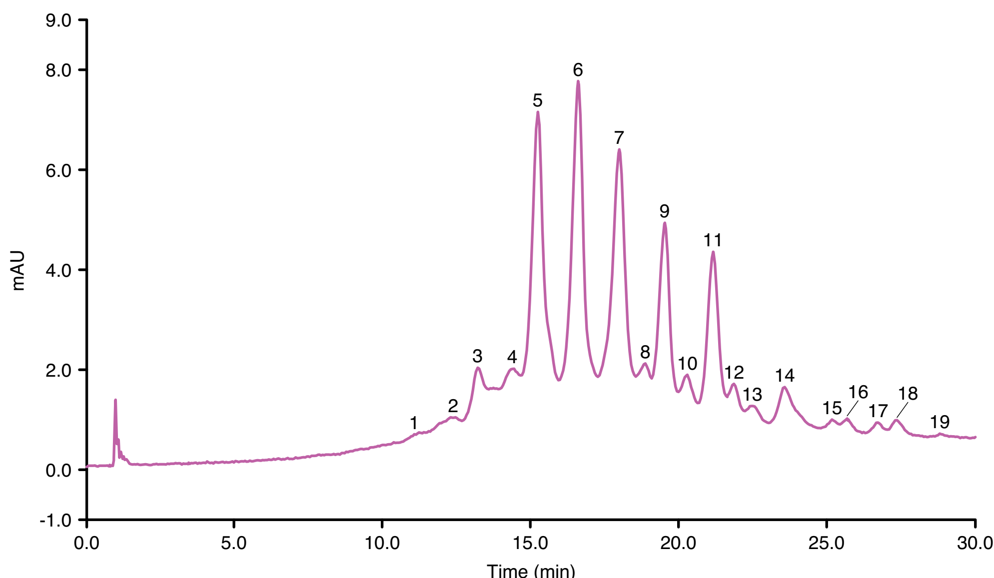

Figure 1: Chromatogram of mAb charge variants separated using the CX-1 pH gradient buffer solutions and a MAbPac SCX-10 column, 4 × 250 mm, 10 µm

Characterizing mAb Charge Variants

The pattern of evenly distributed mAb charge variants is similar to sialylation profiling by capillary electrophoresis [4]. To investigate whether the mAb charge variants separated on the MAbPac SCX-10 column are due at least in part to sialylation, mAb was desialylated with neuraminidase. Figure 2 shows the mAb sample with and without neuraminidase treatment. The chromatogram without neuraminidase treatment in Figure 2a has ten times less sample than the chromatogram in Figure 2b, so the minor peaks are not observed. After neuraminidase treatment, peaks 1–3 completely disappeared and peak 4 decreased, while peaks 5 and 6 increased. This strongly suggests that neuraminidase treatment has removed the sialic acids on the glycans resulting in a neutral glycan pattern on the mAb and increasing some of the peaks, which are presumably the original neutral glycan variant peaks. There are also several new peaks such as a, b, and c that appear. This can also be explained by desialylation of other charged variants that the pH gradient has separated, reducing the negative charge and so giving longer retention time on the SCX chromatogram. These new isoform peaks are more easily seen now due to the individual isoform originally being split into several different sialylation forms at low levels that have now added together as a single neutral glycan variant at a higher amount.

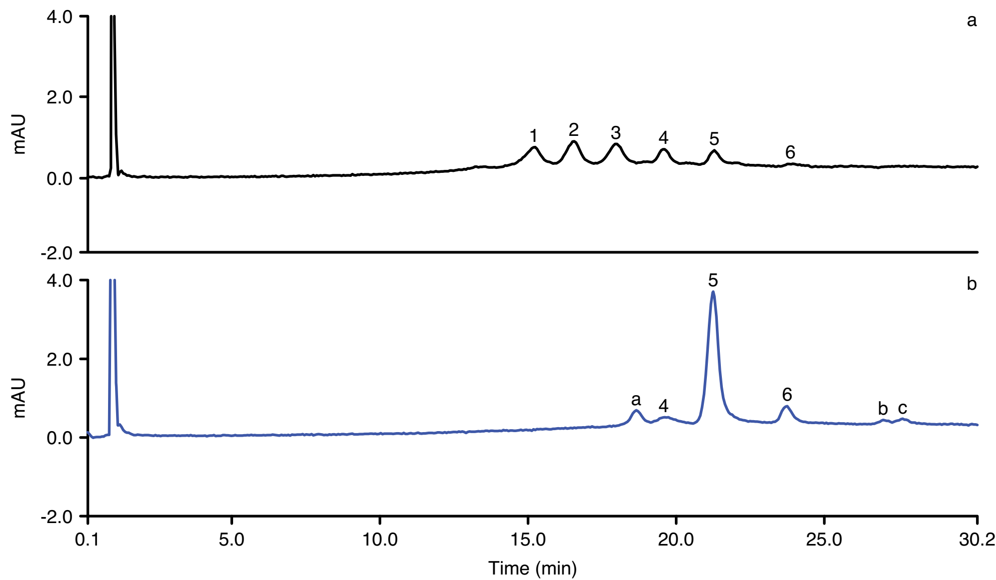

Figure 2: Chromatograms of a mAb (a) without neuraminidase treatment, and (b) with neuraminidase treatment. The chromatogram was generated under the same conditions as in Figure 1.

Comparison of MAbPac SCX-10 Column Separations using Different Particle Sizes

All the above chromatograms were obtained with 10 µm particle size columns in a 4 × 250 mm format. Although the pH gradient ion-exchange chromatograms achieved remarkable resolution, 50 min run times are too long when a fast analysis is needed. To reduce analysis time, a 5 µm particle size column, 4 × 150 mm was evaluated. As shown in Figure 3, the 5 µm column resolved 14 peaks in 11 min (total run time of 16 min). Peaks 6, 8, 10, and 11 can be identified in the chromatogram, but with compromised resolution.

The 5 µm column showed good reproducibility. Overlaid chromatograms of mAb sialylation variants obtained by five consecutive injections are shown in Figure 4. RSDs of main peak (peak 3, 4, 5, 7 and 9) retention times are less than 0.2%, and the RSDs of these peak areas are less than 1.5%.

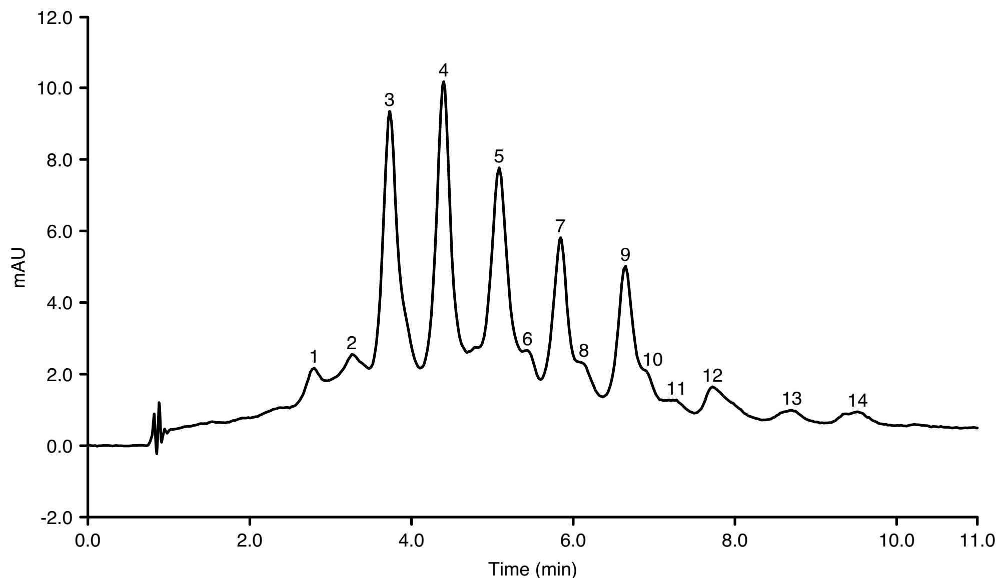

Figure 3: Chromatogram of mAb sialylation isomers separated using the CX-1 pH gradient buffer solutions and a MAbPac SCX-10 column with 5 µm particle size

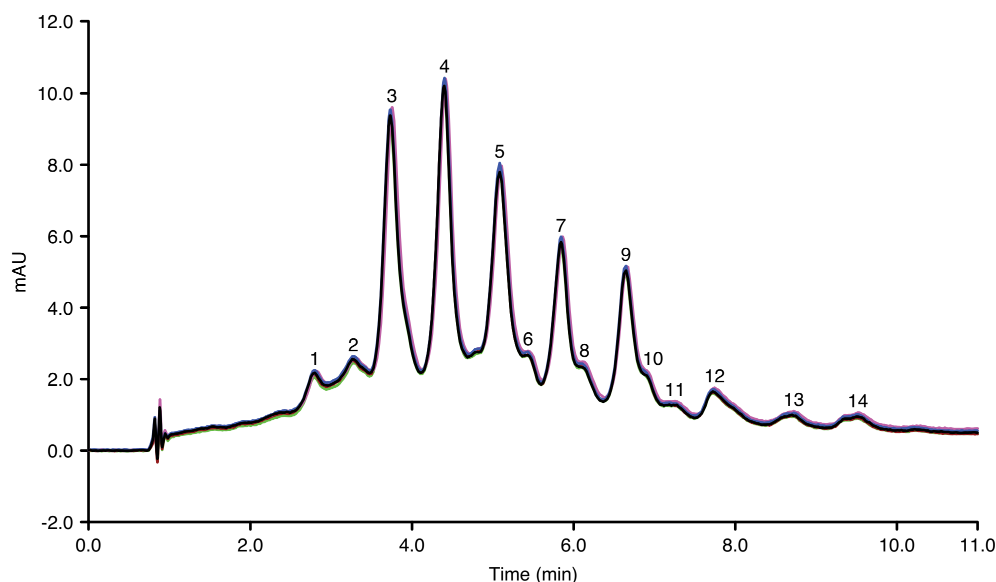

Figure 4: Overlay of chromatograms of five consecutive injections of mAb sialylation isomers separated on a 5 µm MAbPac SCX-10 column

Conclusion

This application note shows that pH gradient-based strong cation-exchange chromatography on a MAbPac SCX-10 column can provide excellent resolution for mAb charged variants including different glycan isoforms of sialylated mAbs. The CX-1 pH gradient buffer approach provides high resolution, reproducibility, and convenience. Fast separation could be achieved by using short columns with 5 µm particles and an UltiMate BioRS HPLC.

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