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Designing a New Particle Technology for Robust Charge Variant Analysis of mAbs

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APPLICATION BENEFITS

- Non-porous polymeric particles to minimize analyte band spread and enhance mAb separations
- Optimal sulfonic acid ligand grafting and hydrophilic surface chemistry for superior component recovery, peak shape, and resolution
- A 3 µm particle size affording columns with low backpressures amenable to use on UPLC™, UHPLC, or HPLC systems

WATERS SOLUTIONS

BioResolve™ SCX mAb Columns

mAb Charge Variant Standard

ACQUITY™ UPLC H-Class Bio System

Empower™ 3 Software

KEYWORDS

BioResolve SCX mAb, mAb charge variant standard, IEX, ion exchange, ACQUITY UPLC H-Class Bio, proteins, NIST mAb, monoclonal antibodies, charge variant analysis

INTRODUCTION

Ion-exchange chromatography (IEX) is commonly employed in the analysis of proteins to investigate charge heterogeneity. In particular, in the biopharmaceutical industry, IEX is routinely relied upon to characterize and monitor the charge variants of protein-based therapeutics such as monoclonal antibodies (mAbs), which can very often have implications on drug efficacy and accordingly be flagged as critical quality attributes.[1]

In some ways, there has been a paucity of analytical options for assaying charge heterogeneity. While both capillary electrophoresis and LC-based separations with ion-exchange stationary phases offer suitable means to gaining insights about charge variants, there has long been a need with both approaches to address resolution limitations and challenges related to method implementation and robustness. With the promise of facile fraction collection and multidimensional capabilities, it would seem that an LC-based approach warrants further development, particularly because of its capacity to facilitate both structure-function studies and detailed secondary assays.

The introduction of the BioResolve SCX mAb Column brings about improvements to LC-based charge variant analysis that make it more straightforward to reliably obtain higher resolution separations. This new column represents Waters' efforts to purposefully design a polymeric non-porous particle technology with an optimized surface chemistry expressly for robust, high resolution separations of mAbs. In this application note, we describe the design and performance of the BioResolve SCX mAb Column and its ability to deliver new levels of resolution along with loadability and unprecedented reproducibility.

EXPERIMENTAL

Sample preparation

NIST mAb (reference material 8671), adalimumab, trastuzumab, and infliximab were diluted with 18.2 MΩ water to a concentration of either 5 or 2.5 mg/mL (as specified below).

Salt gradient cation-exchange chromatography (Figures 1, 3, 5, 6, and 7)

Instrument:	ACQUITY UPLC H-Class Bio with Titanium (5 mm, 1500 nL) Flow Cell
Data management:	Empower 3

Method conditions

Columns:	Waters prototypes BioResolve SCX mAb, 3 µm (including 4.6 × 50 mm, p/n: 186009058)
	Competitor SCX, 3 µm
Mobile phase A:	100 mM MES hydrate
Mobile phase B:	100 mM MES sodium salt
Mobile phase C:	1 M sodium chloride
Mobile phase D:	18.2 MΩ water
Flow rate:	0.72 mL/min (for 4.6 mm I.D.)
	0.54 mL/min (for 4.0 mm I.D.)
	0.15 mL/min (for 2.1 mm I.D.)
Column temp.:	30 °C
Detection (UV):	20 Hz, 280 nm
Injection volume:	5 µL (5 mg/mL mAb samples)
Sample manager wash:	18.2 MΩ water
Seal wash:	10% HPLC-grade methanol/90% 18.2 MΩ water v/v (interval set to 0.5 min)

Gradient:

The following gradient was programmed using Auto•Blend Plus to deliver a pH 7 buffer system:

Time	%A	%B	%C	%D	Curve
Initial	2.2	17.8	1.0	79.0	Initial
10.0	2.2	17.8	20.0	60.0	6
10.5	2.2	17.8	70.0	10.0	11
12.5	2.2	17.8	1.0	79.0	11
20.0	2.2	17.8	1.0	79.0	6

Salt gradient cation-exchange chromatography (Figure 8)

Instrument:	ACQUITY UPLC H-Class Bio with Titanium (5 mm, 1500 nL) Flow Cell
Data management:	Empower 3

Method conditions

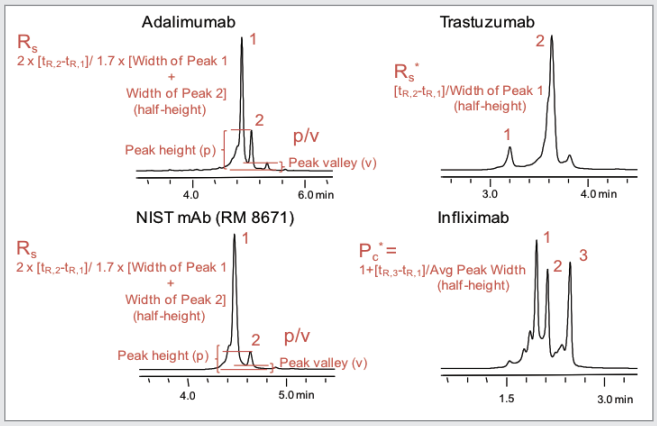
Columns:	BioResolve SCX mAb, 3 µm, 4.6 × 50 mm (p/n: 186009058)
Mobile phase A:	20 mM MES pH 6.0
Mobile phase B:	20 mM MES pH 6.0, 1 M NaCl
Flow rate:	0.96 mL/min
Column temp.:	30 °C
Detection (UV):	20 Hz, 280 nm
Injection vol.:	4.8 µL (2.5 mg/mL NIST mAb)
Sample manager wash:	18.2 MΩ water
Seal wash:	70% HPLC-grade 2-propanol/30% 18.2 MΩ water (interval set to 0.5 min)

Gradient:

Time	%A	%B	%C	%D	Curve
Initial	99.0	1.0	0.0	0.0	Initial
7.50	80.0	20.0	0.0	0.0	6
7.51	70.0	30.0	0.0	0.0	11
8.50	99.0	1.0	0.0	0.0	11
13.00	99.0	1.0	0.0	0.0	11

Calculations and data analysis

Four different mAbs (adalimumab, NIST mAb [RM 8671], trastuzumab, and infliximab) were selected for use in this study because of their diverse pI values and unique retention behavior. Each of these mAbs also has a distinct charge variant profile to allow for different types of resolution measurements to be made during the evaluation of a separation. For adalimumab and NIST mAb, resolution of the most abundant basic variant was monitored by way of a peak-to-valley number (p/v) or a USP Half-Height (HH) measurement (R_s). Trastuzumab separations afforded an abundant acidic variant for which peak shape suggested it to be fairly homogenous. A pseudo resolution measurement (R_s*) was accordingly employed to track the resolution of this species against the main peak, which itself could not be subjected to peak width measurements on account of a partially resolved pre-peak/shoulder. Lastly, infliximab was separated by ion exchange into numerous peaks, among which the most predominant were the main peak and two subsequent basic variants corresponding to equimolar lysine variants of the molecule. Using these three peaks of infliximab, chromatographic resolving power was evaluated via measurements of effective peak capacity (P_c*). The following figure shows each of these measurements in greater detail (t_R denotes retention time):



RESULTS AND DISCUSSION

BASE PARTICLE MORPHOLOGY, SIZE, AND COMPOSITION

It is imperative that chromatographic tools for the analysis and characterization of monoclonal antibodies be rigorously developed so that they are fit-for-purpose and capable of robust, reproducible performance. In pursuit of a new stationary phase for charge variant analysis, numerous insights have been made about what makes for the best optimization of an ion-exchange sorbent.

Many ion-exchange columns for protein separations have been commercialized and, for good reason, are based on the use of non-porous, polymer-based particles. A non-porous particle eliminates intra-particle diffusion and minimizes the band spread of large molecules. With the advent of specialized ion-exchange ligands having well-defined loading capacities, there has seemed to be little need to research alternative technologies. The effective utilization of a 3 µm, non-porous, polymeric particle as an ion-exchange stationary phase can be attributed to work performed three decades ago.[2] What has been more topical as of late has been the continued optimization of protein charge variant separations and considering whether benefits might come from miniaturizing the particle to a diameter less than 3 µm.

To that end, two prototype stationary phases were prepared with comparable ionic capacity, their only difference being particle diameter (3.1 versus 1.7 µm). Each was accordingly applied to a separation of adalimumab and the resulting chromatograms are provided in Figure 1.

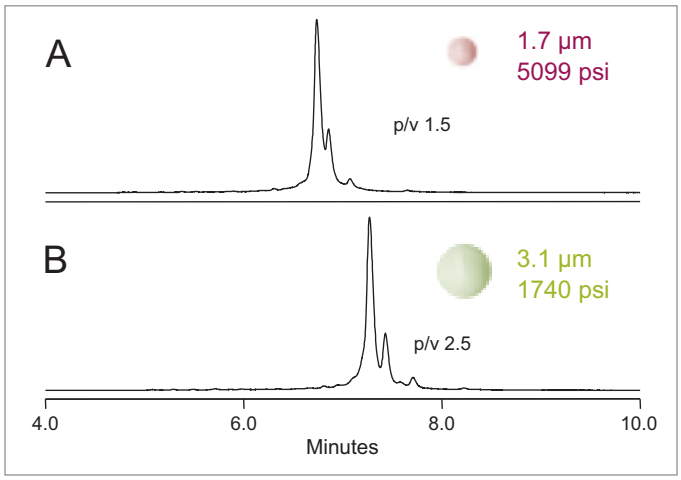


Figure 1. Salt gradient separations and UV chromatograms of adalimumab as obtained with early 2.1 × 50 mm IEX prototype columns packed with either a (A) sub-2-µm or a (B) a 3.1 µm stationary phase. Column pressures generated during these separations are noted along with the peak-to-valley (p/v) ratio measured for the separation of the first major basic variant from the adalimumab main peak.

The 3.1 µm stationary phase produced a slightly improved separation versus the 1.7 µm prototype when both were used on a low dispersion LC system (<12 µL band spread). In sum, even though mAbs are 150 kDa proteins with very low diffusion coefficients, it appears there is little to no value to miniaturizing a non-porous particle, seeing how its morphology already addresses potential mass transfer limitations. Moreover, a very sizable difference in column pressure was observed, with the 2.1 × 50 mm column containing 3.1 µm particles exhibiting a maximum pressure of 1740 psi and the 1.7 µm column showing 5099 psi. It is reasonable to propose that this pressure difference had some effect on the separation. In separate experiments, wherein flow restrictors were added to the separation of infliximab, it was indeed observed that peaks broadened slightly with added pressure (data not shown). Albeit protein dependent, pressurization can begin forcing the hydration of interior protein domains and thereby temporarily perturb conformational states.[3] As such, it was preferred to pursue the development of an approximately 3 µm non-porous stationary phase which appears to be more optimal for mAb separations and facilitate separations on multiple LC platforms, regardless of their pressure limitations.

This 3 µm non-porous stationary phase could be predicted to most frequently be employed at pressures ranging between 1000 and 9000 psi. However, its capacity to withstand excess pressure was essential for ensuring column bed stability. If the polymeric composition of the particle were to be poorly optimized, it might fracture or become malleable at high linear velocities and thereby cause premature column failures. For this reason, numerous base particle polymer compositions were explored and subjected to mechanical strength testing. With this testing, short columns were tested with methanol flowing at constant pressures up to 21,000 psi. Flow rates observed during these tests are provided in Figure 2 and can be interpreted as indicators of particle strength and mechanical bed stability. An ideal column would show a one-to-one correspondence between pressure and flow rate. Where some compositions, like Composition A, showed flow rate deviation as low as 5000 psi, others, like Composition C, showed reasonably good mechanical properties up to and beyond 10,000 psi. It is this latter result that is representative of the newly designed base particle and its amenability to relatively high linear velocities and higher flow rate method conditions.

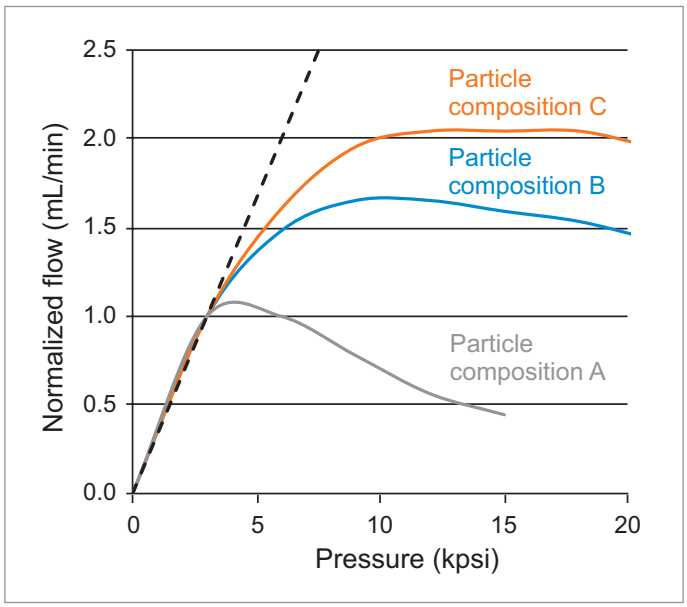


Figure 2. Mechanical strength testing of non-porous base particles of varying chemical composition. Specialized columns (2.1 × 20 mm) were flushed with methanol at increasingly higher pressures and the resulting flow rates were measured. Deviations from the theoretical flow rate (dashed line) indicate perturbation of either the particles and/or the packed bed.

TAILORING A SURFACE CHEMISTRY FOR mAbs

Protein interactions are intricately dependent on many different factors. In the form of chromatographic adsorption during ion exchange, this correctly predicts the significance of tuning the surface chemistry of a sorbent. In the case of developing a chemistry optimized for mAb charge variant analysis, it must first be considered that these are native aqueous separations with a requirement to be free of hydrophobic secondary interactions. The surface of the new base particle was accordingly modified to increase and optimize its hydrophilicity. To this point, modification of the base particle with three varying extents of hydrophilicity yielded three unique prototypes, each subsequently prepared with the same ion exchange ligand.

Chromatograms resulting from these prototypes and the separation of adalimumab are displayed in Figure 3A. It is with an intermediate extent of modification that resolution was found to be optimized. With too little hydrophilic modifier, it can be assumed that deleterious hydrophobic secondary interactions were encountered. With too much hydrophilic modification, the separation not only showed poor resolution but also inordinately high backpressure. Knowing the range of optimal hydrophilicity has led to precise guidelines for this novel BioResolve particle to be robustly manufactured.

Equally careful consideration was paid to the optimization of the ion-exchange ligand grafting. Numerous rounds of synthesis optimization were guided by a design of experiments approach and the observed performance of many different prototypes. First and foremost, it was reasoned that it would be most valuable to develop a cation exchanger, since most mAbs exhibit a basic pI value.[4] The chemical properties of the ion-exchange grafting were next considered in order to afford an optimized ionic capacity, an effective distribution of ligands, and control of swelling that can occur because of charge-charge repulsion. Preliminary work established that a sulfonic acid based, strong cation-exchange ligand showed the most promise for developability. Among several reasons, it was observed that a sulfonic acid ligand showed faster equilibration times versus a carboxylic acid moiety when used for pH gradient chromatography. A focus was thus made to explore the effectiveness of prototypes prepared with sulfonic acid graftings of varying chemical compositions. Six example chromatograms that are representative of a much broader set of prototypes are provided in Figure 3B. Based on separations of adalimumab, it could be seen that peak shape, peak sharpness and resolution were affected by these different chemical compositions. For instance, Grafting C produced an adalimumab profile with pronounced retention yet poorer comparative resolution. Meanwhile, Grafting F yielded the lowest comparative retention, highest resolution, and a chromatogram replete with fine structure. It is this latter prototype that defines the desired chemical composition of an ion-exchange ligand. In their sum, each of the above optimizations forms the basis of the BioResolve SCX mAb stationary phase, which is best summarized as a 3 µm, non-porous sorbent made from specialized polymerization reactions and a finely tuned sulfonic acid grafting (Figure 4).

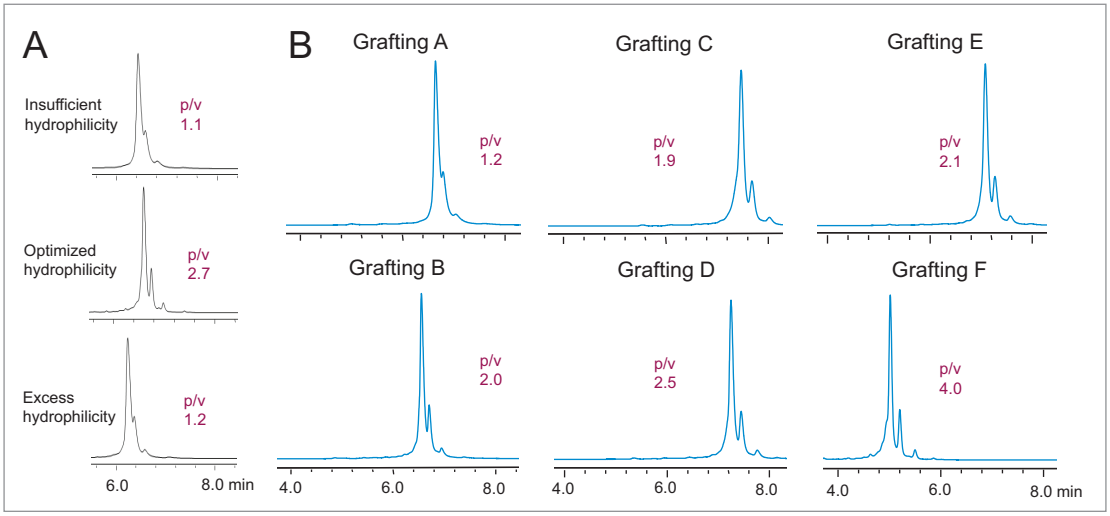


Figure 3. Salt gradient separations and UV chromatograms of adalimumab as obtained with 4.6 × 50 mm IEX prototype columns packed with stationary phase prepared with (A) varying levels of hydrophilicity and (B) varying compositions of sulfonic acid grafting. Peak-to-valley (p/v) ratios are reported as measured for the separation of the first major basic variant of adalimumab.

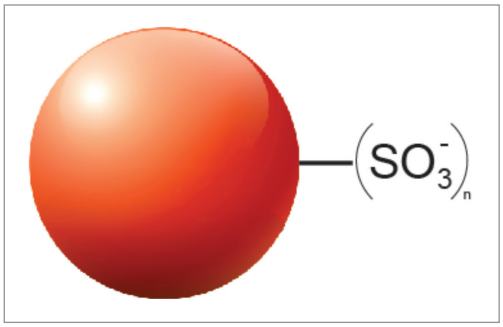


Figure 4. Schematic depicting the particle and surface technology of the BioResolve SCX mAb stationary phase.

PERFORMANCE, ROBUSTNESS, AND LIFETIME

Every new analytical tool for the discovery, development, and potential QC of biopharmaceuticals should deliver improved performance along with rigorous robustness. Therefore, testing of BioResolve SCX mAb Columns was regularly performed on four different monoclonal antibodies, namely infliximab, trastuzumab, NIST mAb, and adalimumab using a fast MES-based pH 7 salt gradient. Resolution metrics unique to each separation were devised to assess resolving power and details on each calculation can be found in the experimental section. Seeing through stationary phase development with this feedback ensured that high performance separations could be universally achieved and that we would avoid optimizing selective performance for only a limited subset of mAbs. Chromatograms acquired with two BioResolve SCX mAb Columns and two different batches of packing material highlight this universal capability and are displayed in Figure 5A along with chromatograms obtained with a leading alternative column technology. The BioResolve SCX mAb Column showed higher resolving power, as measured in the form of either peak capacity (P_c), resolution (R_s) or peak to valley (p/v) ratios. One visually captivating example of improved resolution can be seen in the example of the NIST mAb charge variant separation. It was only with the BioResolve SCX mAb Columns that the acidic variant front shoulder of NIST mAb could be teased apart from its main peak. Of benefit to their usability, it was also determined that the BioResolve Columns exhibited lower injection-to-injection carryover and equivalent or lower pressures (Figures 5B and C).

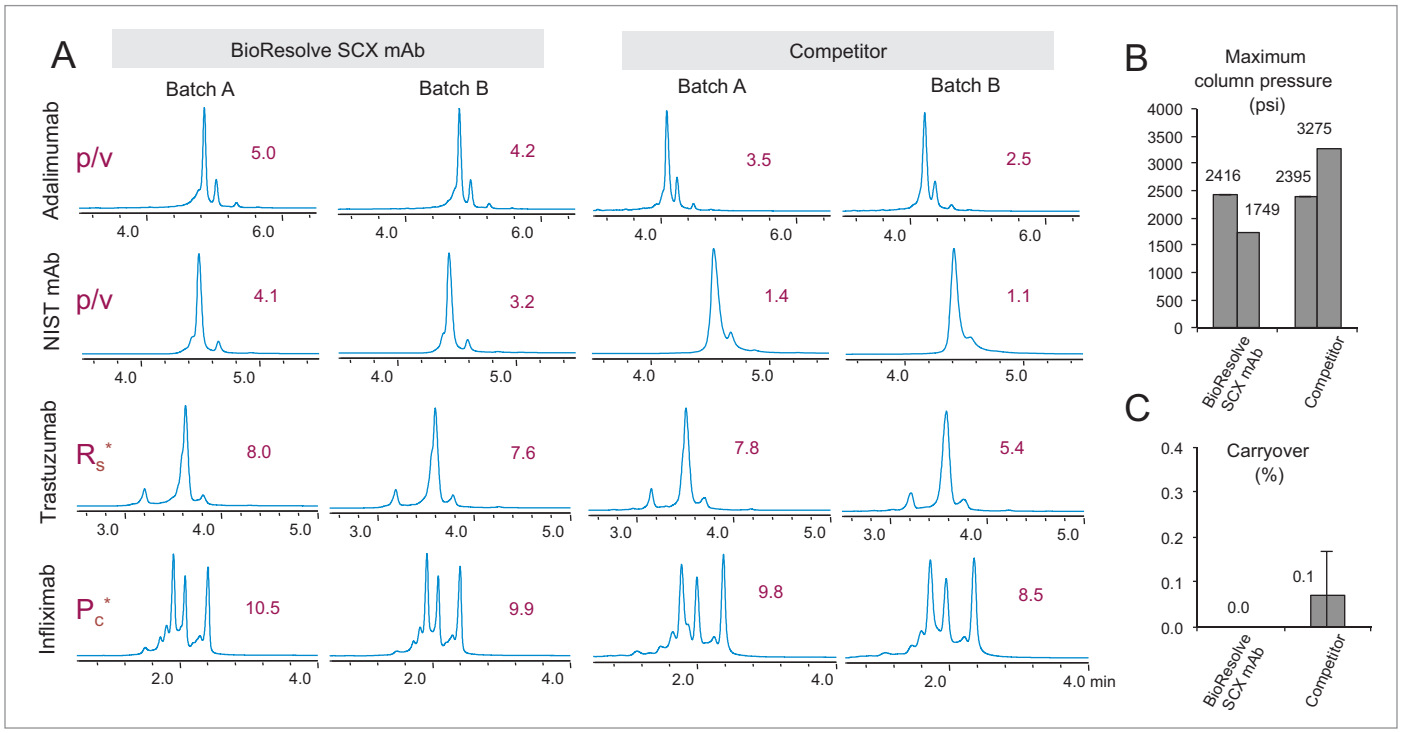


Figure 5. Salt gradient separations of adalimumab, NIST mAb (RM 8671), trastuzumab, and infliximab as performed with BioResolve SCX mAb, 4.6 × 50 mm Columns or competitor SCX, 3 µm, 4.0 × 50 mm columns packed with unique batches of stationary phase. (A) UV chromatograms and measured resolution metrics, including peak-to-valley (p/v) of the major basic variant, a pseudo resolution value for the separation of an acidic variant (Rs), and effective peak capacity (Pc*). (B) Maximum column pressures during use at the same linear velocity. (C) Percent carryover of NIST mAb observed during a repeat gradient.*

Similar testing was performed at an even broader scale to assess the reproducibility of seven different batches of BioResolve SCX mAb stationary phase. Figure 6 displays the chromatograms obtained during this analysis. Retention and resolution metrics corresponding to the average performance have been compiled into the charts displayed on the right. Each charted value is marked with two error bars that are indicative of reproducibility. The first error bar represents the observed column-to-column standard deviation and is derived from a study of seven columns packed with the same batch of stationary phase. The corresponding RSD values for column-to-column reproducibility, marked with a superscript C, were found to be 0.9% or less for retention times and 2.4% or less for resolution metrics. In addition, a second error bar is provided that corresponds to the observed batch-to-batch reproducibility derived from seven unique batches of stationary phase. The RSD values for this variation, marked with a superscript B, were found to be 3.0% or less for retention times and 4.9% or less for resolution metrics. In turn, high confidence can be placed in a BioResolve SCX mAb Column to know it can produce both high resolution and the reproducibility needed to facilitate method qualification as well as the on-going implementation of a new assay.

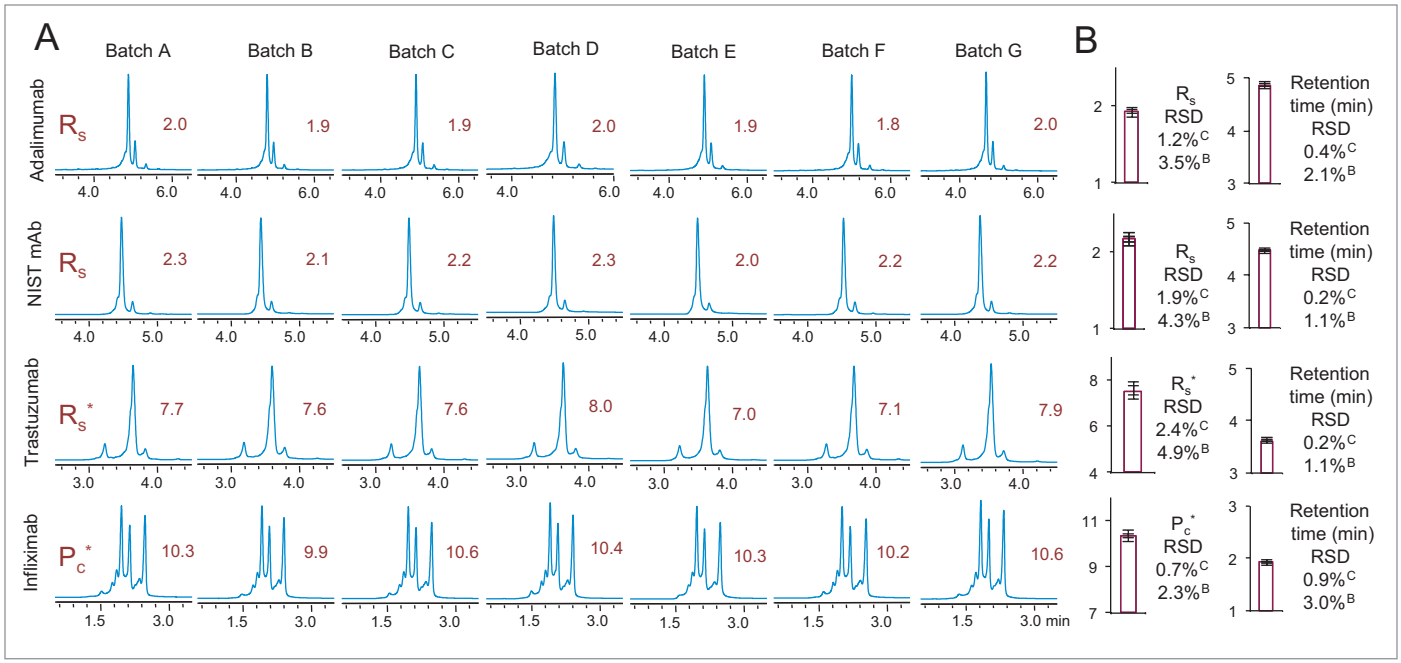


Figure 6. Reproducibility of the BioResolve SCX mAb stationary phase as observed with salt gradient separations of adalimumab, NIST mAb (RM 8671), trastuzumab, and infliximab. (A) UV chromatograms collected using BioResolve SCX mAb, 4.6 × 50 mm Columns packed with seven unique batches of stationary phase. (B) Average resolution metrics and retention time data shown along with RSD values collected from either seven columns packed with the same stationary phase batch (column-to-column variability)[C] or seven columns packed with seven unique batches of stationary phase (batch-to-batch variability).[B]

Also of significance to the implementation of an IEX column technology is its loadability, particularly since charge variant separations are used to acquire fractions for structure function studies and thereby address regulatory concerns. With a BioResolve SCX mAb, 4.6 × 50 mm Column, the separation of NIST mAb was studied as a function of increasing mass load, from 1 to 500 µg. Chromatograms collected from separations of 4, 16, 63, and 250 µg mass loads are provided in Figure 7A, while Figures 7 B–D show plots on peak area, basic variant resolution, and relative response. Across this range of mass loads, excellent peak area linearity was observed (R^2 = 0.9999) and only a slight decrease in resolution was encountered at the higher end mass loads of 250 and 500 µg. This column technology should thereby lend itself to applications requiring elevated mass loads and fractionation. Based on these data, it is likely for a 4.6 mm I.D. column to maintain noteworthy resolution up to 1 or even 2 mg mass loads depending on sample properties.

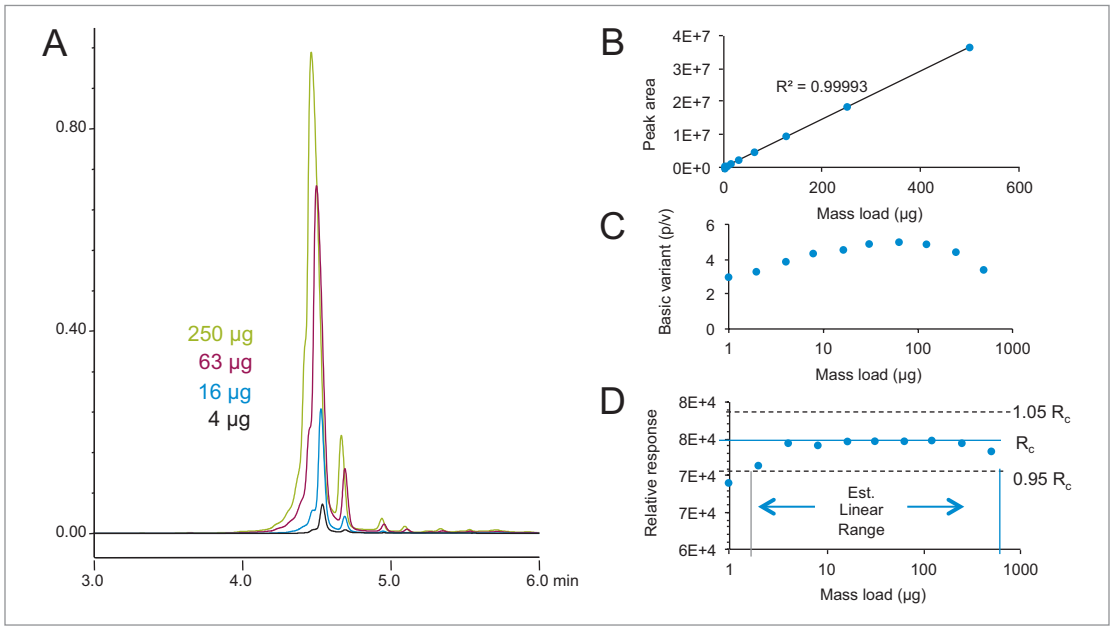


Figure 7. Loading capacity of a BioResolve SCX mAb, 3 µm, 4.6 × 50 mm Column as studied with increasing mass loads of NIST mAb (RM 8671). (A) UV chromatograms of NIST mAb as obtained with 4, 16, 63, and 250 µg injections. (B) Peak area. (C) basic variant resolution. (D) relative response (Rc) as a function of mass load.

In an examination of robustness, a BioResolve SCX mAb, 4.6 × 50 mm Column was also subjected to lifetime testing, where repeat separations of NIST mAb were performed for 500 injections at a reasonably high flow rate of 0.96 mL/min. Figure 8A displays chromatograms from the 4th and 500th injections of this testing, and it can be observed that the quality of the separation was effectively preserved. The partial resolution of an acidic variant pre-peak is seen in both cases as is characteristic of the NIST mAb charge variant profile. As quantified in Figure 8B, consistent column performance can also be confirmed by the fact that retention times, resolution, and pressure were essentially unchanged across the duration of lifetime testing.

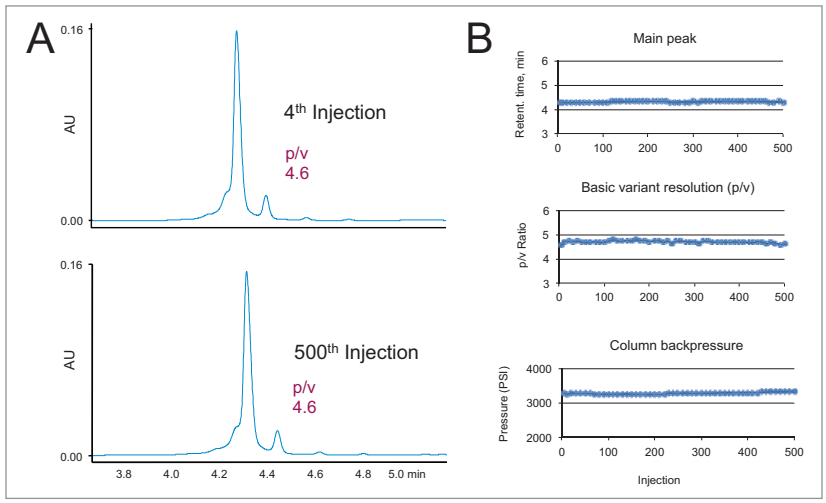


Figure 8. Lifetime testing of a BioResolve SCX mAb Column using a salt gradient separation. (A) UV chromatograms obtained for NIST mAb (RM 8671) with a 4.6 × 50 mm column and flow rate of 0.96 mL/min. (B) Retention time, p/v resolution, and column pressure across 500 injections.

CONCLUSIONS

A new IEX column technology based on a 3 µm, non-porous sorbent, specialized polymerization reactions and a finely tuned sulfonic acid grafting has been developed to improve charge variant analyses of monoclonal antibodies. Purposeful selection and optimization of particle size, particle morphology, and surface chemistry has produced a column with superior properties compared to other leading technologies. Its performance has been confirmed to be universally applicable to multiple mAbs, and it has been proven to be reproducible and robust by way of observations on minimal column-to-column and batch-to-batch variation as well as column lifetime. In addition, this column provides a high degree of loadability that can be used to advantage when purifying charge variants for characterization and structure-function studies. With its reproducibility and improved performance, it is believed that this next generation of ion-exchange column technology will better serve biopharmaceutical scientists dealing with even the most perplexing investigations and even the most rigorous QC monitoring of mAb charge variants.

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