A Universal Eluent System for Method Scouting and Separation of Biotherapeutic Proteins by Ion-Exchange, Size-Exclusion, and Hydrophobic Interaction Chromatography

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ABSTRACT: Characterization and quality control of biotherapeutic proteins commonly require the application of several orthogonal separation techniques in order to establish product identity and purity. Many of the techniques used rely on a buffered aqueous mobile phase system to maintain the native conformation of the protein and its variants. Optimal pH, buffer substance(s), and chromatography methods vary with each protein of interest and result in tedious method development for each new drug product. Linear controlled pH gradient systems from pH 5.6 to pH 10.2 has been shown to provide a global method for the separation of charge variants of monoclonal antibodies. This can be realized using two balanced zwitterionic buffer blends. The pH linearity of the resulting system, with a cation ion exchange column in place, can generate any pH value in this accessible pH range. This study expands the scope of this buffer system and demonstrates its application in conjunction with a quaternary HPLC pump for several analytical techniques: the pH optimization of salt gradient-based anion and cation exchange during method development, as well as performing pH gradient elution. In addition, the same universal buffers are used for hydrophobic interaction and size exclusion chromatography. This eluent system omits the need to prepare different buffers for each method and flushing of the HPLC system between method changes. The implementation of this concept is further demonstrated to allow an automated method scouting approach and selection of different methods that requires minimal manual intervention.

INTRODUCTION

Biotherapeutic proteins inevitably contain a certain degree of structural heterogeneity that is derived from their way of production and purification from a biological cell culture system, as well as their further storage and handling.1−3 During development, these protein variants need to be carefully analyzed to allow the risk-based assessment of their impact on efficacy and safety of the future drug product.5 Once characterized, this profile of heterogeneity can be used to establish product identity and lot-to-lot consistency and is thus the basis of many batch release test strategies. Furthermore, the requirements for biosimilar products to have no clinically meaningful differences in terms of safety, purity, and potency to their originator product center the biophysical characterization right at the heart of each biosimilar development program and are carefully regulated.5,6 The main source of protein heterogeneity comes from the variability in post-translational and chemical modifications, such as glycosylation, lysine truncation, deamidation, or oxidation.5,7 Examples of these modifications can cause changes in efficacy and immunological behavior and may also be indicative of product degradation.8,9 Many of these modifications create a direct change of the protein surface charge, such as a change in sialic acid units of the glycan structures, lysine truncation, or deamidation of asparagine and glutamine. Other modifications can cause a conformational change that affects the distribution and accessibility of surface charges, as seen for some oxidation sites or the incorrect formation of disulfide bridges.5,7,10 Given the vast number of modifications that cause a change in protein charge distribution, this product-specific profile of charge variants has become one of the key attributes to characterize the heterogeneity within the protein drug product.

Charge sensitive analytical techniques such as imaged capillary isoelectric focusing electrophoresis and ion exchange chromatography (IEC) are the methods of choice to facilitate this charge variant analysis (CVA).11−14 Hydrophobic interaction chromatography (HIC) is an orthogonal technique that addresses protein heterogeneity from a different angle, by looking into the differences in hydrophobicity between protein...
variants. Extent of aggregation and higher order molecular structure are an additional, generally critical, quality attribute of the drug product that needs to be carefully monitored. Size exclusion chromatography (SEC) is the predominant analytical technique applied for this. CVA/IEX, HIC, and SEC all operate under native conditions; that is, the protein remains in its functional form. A direct consequence is that the pH needs to be carefully optimized and controlled during method development for these separation techniques.

As each buffer of different pH tested needs to be individually prepared, this can amount to a substantial workload and a tremendous amount of time consumption. It also introduces a source of error, especially if the pH is adjusted by manual titration (Figure S1). The consequence could lead to a variability in the results and lack of method robustness. Buffer blending using a quaternary pump with the UHPLC system may be a tempting alternative; however, severe pH deviations can be introduced when standard buffer solutions are used and mixed online with salt. This also limits the use of pH blending software with salt containing buffers or solvents, as the pH calculated in the software is based on the acid to base ratio of the buffer without added components such as salt. This undermines the assumptions of the software. Using the approach herein described with a strong buffer system, the pH control chemistry works correctly and removes the reliance on software with coded-in assumptions. Furthermore, real-time monitoring of pH and conductivity during method development demonstrates the robustness of the finished method.

There are various pH effects seen during IEX chromatography that can affect method robustness and should be considered during method development. Here we elaborate in more detail on an alternative approach that overcomes limitations caused by pH effects with conventional buffer blending. Separation in IEX can be driven by either applying a salt or pH gradient. Buffer systems that produce a truly linear pH gradient allow easy to use pH-dependent elution, suitable as a multiproduct method for CVA. However, a strongly buffered system that can produce linear pH gradients can also be used to dial in any pH value within its buffering range. The efficient pH control negates the effect of salt gradient introduction and thus provides a tool for pH optimization of any salt-based method. This eliminates the requirement for multiple buffer preparations and minimizes pH variances of manually prepared buffers, directly translating into method robustness. pH optimization can be automated using a sequence of injections set up to automatically produce and screen the complete pH range of the buffer system. We describe the application and usability of a strong pH gradient buffer system, as a tool for pH optimization in the range of pH 5.6–10.2 and additionally as a universal eluent system for IEX, HIC, and SEC separations. The resulting universal eluent system can automatically prepare the correct eluents for cation exchange with salt or pH gradients, yet also be used for anion exchange salt gradients, HIC, and SEC. This allows for a walk-up system to be configured with column selection valves, where the correct eluents and column are determined by the method program. This saves time on eluent preparation and allows ease of use and flexibility of the UHPLC systems.

■ MATERIALS AND METHODS

Chemicals. Biosimilar drug substances of adalimumab, bevacizumab, rituximab, trastuzumab, secukinumab, and a proprietary mAb were kindly provided by a collaborating biopharmaceutical company. BSA and ovalbumin were purchased from Sigma-Aldrich. All proteins were provided as lyophilized powder and reconstituted in ultrapure water to stock concentration of 5 mg/mL if not otherwise indicated. Sodium chloride (99.5%) and ammonium sulfate (99.999% trace metals basis) were purchased from Sigma-Aldrich. Thermo Scientific CX-1 pH gradient buffer concentrates A and B were obtained from Thermo Fisher Scientific (Sunnyvale, CA, USA).

Chromatography Instrumentation and Data Processing. A Vanquish Flex UHPLC system (Thermo Fisher Scientific) was used, equipped with quaternary Vanquish pump F, dwell volume 679 μL, Vanquish split sampler FT, Vanquish column compartment with active preheater, and Vanquish diode array detector H. A PCM-3000 pH and conductivity monitor was connected via the shared board of an UltiMate 3000 VWD-3400 variable wavelength detector. Chromeleon 7.3.1 chromatography data system (Thermo Fisher Scientific) was used for instrument control and analysis of the chromatographic data.

Cation Exchange Chromatography. The four lines of the quaternary pump were set up to deliver ultrapure water (line A), 1.00 M NaCl stock solution in ultrapure water (line B), and the undiluted 10X CX-1 pH buffer concentrate pH 5.6 and pH 10.2 in lines C and D, respectively. The combined proportion of the two CX-1 buffers was maintained at 10%, corresponding to a 1:10 dilution (1× final concentration). A discrete ratio between CX-1 buffer pH 5.6 and pH 10.2 was set and held constant to establish a given pH value during the separation. The salt gradient was established via the pump by linearly increasing the proportion of the NaCl solution in line B: 0–1 min, 0% B; 1–16 min, 0–20% B; 16.1–17 min, 50% B; 17.1–24 min, 0% B. A MABPac SCX-10, 4 mm × 150 mm or ProPac Elite WCX, 4 mm × 150 mm column was operated at 30 °C (still air) and a flow rate of 1 mL/min. The UV absorption at 280 nm, as well as the pH and conductivity of the eluting eluent, was recorded.

Anion Exchange Chromatography. The same eluents and eluent line configuration as for CEX chromatography were used. Protein charge variants were separated on a ProPac SAX-10, 4 mm × 150 mm column with following gradient: 0–1 min, 2% B; 1–21 min, 2–50% B; 21–24 min, 50% B; 24.1–32 min, 2% B. The SAX column was operated at 30 °C (still air) with a flow rate of 1 mL/min. UV absorption at 280 nm was measured for protein.

Hydrophobic Interaction Chromatography. A 4.00 M NaCl stock solution (line B) was used to establish hydrophobic interaction in order to avoid potential pH buffering interference from the more commonly used ammonium salts. A nonporous, polymeric MABPac HIC-butyl, 4.6 mm × 100 mm was operated at 30 °C (still air) with a flow rate of 1 mL/min using the following salt gradient: 5–0 min, 90% B; 0–1 min, 90% B; 1–15 min, 90–0% B; 15–20 min, 0% B. UV absorption at 280 nm was measured.

Size Exclusion Chromatography. 150 mM NaCl was used by selecting 15% of the 1.00 M NaCl in line B. The pH was set to 6.5 using 8% of line C and 2% in line B. The flow rate was set at 0.3 mL/min using a 4 × 300 mm MABPac SEC-1 column. UV absorption at 280 nm was measured for protein.
RESULTS AND DISCUSSION

Setup of the Universal Eluent System. Salt gradient-driven methods, as applied during ion exchange and HIC, require careful pH control of the mobile phase for the optimal separation of the protein variants of interest. Screening for the optimum pH values requires the identification of an appropriate buffer substance for each pH value and the manual preparation of the corresponding buffers. Errors in preparation can be a source of retention and selectivity changes (Figure S1). Variances in pH and ionic strength can directly affect the selectivity of the method. Traditional buffer-blending using a quaternary UHPLC pump helps to dramatically reduce the work load on buffer preparation; however, the introduction of salt into an aqueous buffer system will result in pH changes that may not be sufficiently controlled to produce the intended pH value, especially over a wider pH range if only one buffer substance is used. A viable alternative is the use of a linear pH gradient buffer system made up of several buffer components that not only allows the formation of pH gradients but also can be used to create, maintain, and stabilize any pH value within its dedicated pH range.

The generation of a linear pH gradient requires a careful blend of buffering substances with overlapping effective pH ranges to control and stabilize the pH. An estimate for this is within one pH unit from the pK of the buffering group. A concentration sufficiently high to provide good buffering capacity is a further requirement for effective pH control. The addition of an ion exchange column adds additional buffering to the HPLC system. Ion exchange resins contain functional groups that can constitute a buffering system themselves, with a buffering capacity related to the amount of resin packed in the column housing. Often overlooked is that the surface ion exchange phase itself needs to be effectively buffered by the mobile phase. This, however, is difficult to achieve when the buffer substance has the opposite charge of the stationary exchange phase, creating repulsive electrostatic interactions.

Zwitterionic buffer substances overcome this obstacle and are thus the buffer class of choice for this study. The CX-1 pH gradient buffer formulation is commercially available and provides sufficient buffering capacity in the range of pH 5.6–10.2 to minimize pH deviations caused by the introduction of salt to the system. This was utilized to create a universal buffer system for automated method scouting and walk-up method selection.

Figure 1 shows a system configuration to allow mixing of the two pH buffers and includes a salt stock solution, in addition to the two undiluted CX-1 buffer concentrates and water. This allows the generation of a salt gradient while maintaining a set pH defined by the constant ratio of CX-1 buffers pH 5.6 to pH 10.2. The use of different method programs in a run sequence allows each pH value to be created automatically from the ratio of the buffer concentrates in the method. The pH can be monitored online after the column with the pH and conductivity detection system. The system as shown can automatically screen a set of pH values for a given separation without the need for manual intervention. The addition of column change valves allows automated column selection to screen columns for the best selectivity. Other methods critical in therapeutic protein characterization also use salt containing buffers at a set pH. Size exclusion chromatography and hydrophobic interaction chromatography fall into this category and can also be set up to run in this system configuration with walk-up method selection.

The pH gradient buffer system provides sufficient buffering capacity in the range of pH 5.6–10.2. To minimize pH deviations caused by the introduction of salt to the system. Blending of the CX-1 pH gradient buffer solutions generates a pH value following the theoretical equation.

$$\text{pH} (25^\circ C) = 0.046\% \text{ B} + 5.6$$

The accuracy of this calculation is confirmed in Figure 2, which shows the measured pH values eluting from an ion exchange column by stepwise increases in the proportion of pH buffers in the final buffer blend.

Table 1 lists the theoretically expected and actual pH values, measured at the detector outlet. The values of the calculated and measured pH are in good agreement with the biggest difference at 0.19 of a pH unit. A sharp linear pH change is observed in response to the gradient changes as shown in Figure 2.

**Figure 1.** Simplified flow diagram for the universal eluent system (autosampler unit not shown).
optimize the pH used for salt gradient charge variant analysis of several monoclonal antibody drug substances using WCX and SCX chromatography. The optimization of the pH and the column selection can both be achieved automatically with different methods in the run sequence.

Figure 3 shows the effect of different pH values on the salt gradient separation of charge variants from secukinumab and pertuzumab with a strong and a weak cation exchange column. The same experiment was performed with rituximab, trastuzumab, adalimumab, bevacizumab, and NIST mAb, which can be found in the Supporting Information Figure S2. The columns compared were both new generation ion exchange columns of similar particle size. Analysis of the chromatography from each experiment was manually performed to judge which method produced the best resolution, peak shape, and number of charge variants. This comparison could be automated with method scouting software such as ChromSword. In these experiments the optimum starting conditions for separations with rituximab were SCX, pH 7.5, trastuzumab WCX, pH 8.0, adalimumab SCX, pH 8.0, bevacizumab SCX, pH 6.0, and NIST mAb SCX at pH 6.5 (Figure S2). Figure 3 shows that the optimum conditions for secukinumab was using an SCX column at pH 7.5 and pertuzumab, a WCX column at pH 7.5. Further optimization of the gradient slope conditions for each drug product can be quickly achieved from this information. An important point to note is that obtaining the optimum pH and column conditions is the most time-consuming to complete and would normally require the manual preparation of several buffers and flushing of the system between buffer and column changes. The comparative data in this case were easily completed automatically in 1 day with the optimization of 6 monoclonal antibody drug products, on two different columns. In addition, the methods will be inherently stable due to the firm control of the pH during the chromatography. Once the final conditions for each mAb are determined, this system configuration can be used for routine analysis of any drug product optimized in this way. This allows walk-up analysis for all the products and simple management of the laboratory without any need for buffer preparation. The cost of the buffers needs to be considered, which would be reduced with the use of 2 mm columns.

**pH Scouting for Salt Gradient Anion Exchange.** The pH range of 5.6–10.2 of the described buffer system also gives applicability to salt gradients for acidic proteins. An example for ovalbumin is given in Figure 4; the pH stability during the separations showed good control as expected, with the anion exchange chromatography salt gradients. The pH scouting for the anion exchange separation of ovalbumin showed the optimum conditions to be pH 5.6 for the ProPac SAX-10 column. Ovalbumin has two phosphorylation sites which can be influenced during this pH range due to one of the pK\(_a\) values close to neutral pH.\(^{29−31}\) There are also sialic acid residues on the glycan structures which will be influenced by the pH changes in addition to the charged groups within the protein itself.\(^{15}\)

**Hydrophobic Interaction and Size Exclusion Chromatography.** Hydrophobic interaction chromatography (HIC) is another salt based chromatographic technique which in this case uses a reverse salt gradient under pH control. The majority of HIC methods use ammonium sulfate
as the salt ion, which would also act as a strong buffer at these high concentrations. However, sodium chloride can prove to be a good choice of eluting salt for resolution as the salt concentration change required to change retention is greater with sodium chloride than it is for ammonium sulfate. This gives better control over resolution, and so it is not a compromise to include HIC in the repertoire for this universal eluent system. NaCl will not act as a buffer to prevent pH optimization in this system. However, the amount of NaCl needs to be increased to 4 M to utilize the system with HIC methods. This is demonstrated in the upper panel of Figure 5. Good resolution of hydrophobic variants from the rituximab biosimilar drug product is achieved in its native form. The same chromatographic method has been applied to the other mAb samples in our laboratories [not shown] indicating additional applicability for the HIC method using a sodium chloride reverse gradient at pH 5.6 on the same universal gradient system.

This expands the broad applicability of this universal buffer system beyond ion exchange chromatography, and a further extension to SEC can also be achieved (Figure 5, lower panel). SEC is already regarded as a universal method for mAb samples. A common eluent system for this chromatography is 150 mM NaCl with buffering at pH 6.0−7.5. This is also easily achieved using the eluent system we have described here.

The universal eluent system in combination with column selection valves enables a range of analytical techniques commonly used in mAb characterization. This allows charge variant analysis, aggregates, and hydrophobic variants to be characterized with one system setup. The charge variant analysis can be achieved with anion and cation exchange using salt gradients and cation exchange with pH gradient elution. An example of how a simple walk-up system could be configured is shown in Figure 1. The columns can be selected by the valve position on the 7 port−6 position valves. This includes a bypass line to equilibrate the capillaries to any new eluent and waste lines for equilibration. The methods in the chromatography control software can then select the column as well as the standard method conditions. Any equilibration of the system capillaries can also be included in a method included in the sequence, in front of a column change.

**CONCLUSION**

This manuscript highlights the universal applicability of a versatile linear pH gradient buffer system for several separation methods used in protein characterization. These methods are all commonly used for the characterization and quality control analysis of protein-based biotherapeutics. These include different modes of ion exchange chromatography as well as HIC and SEC. The defining features here are the true linearity and pH control of the buffering system based on the use of several zwitterionic buffer substances in the formulation. The use of pH gradients for charge variant analysis of mAbs has become more accepted in the fields because of its simplified requirement for method development and applicability as a multiproduct method. This alone translates to time and cost saving during method development. The fast method cycle times possible with pH gradients allow for high throughput applications, further adding to the time saving and applicability. This is the designed purpose of the CX-1 pH buffers and can also be implemented using this system.

In cases where a salt-gradient may result in more suitable separation performance during IEX, method scouting for the optimum pH can be automated. The tight control of pH using this method results in robust, high-resolution methods that can be used routinely. If required, once the optimum quaternary conditions have been found, the buffer conditions can be easily converted to a binary system and the appropriate buffering component used. The commercial availability of the buffers increases the attractiveness of this solution to many routine laboratories. The system configuration can be used with any...
column or in-house prepared buffers and salt systems to suit the user, but care must be taken to ensure the strict pH control available with the system described herein.

The universal applicability of the CX-1 buffer system demonstrated here allows HIC and SEC methods to be implemented without compromise. Simple method selection can be easily configured and the UHPLC used as a multimethod walk-up system. With the universal eluent system in place on a quaternary HPLC system with column selection valves, the majority of ion exchange, HIC, or SEC methods can be selected to run automatically for comprehensive product characterization. This would also have applicability in automated process or high throughput environments.

Current limitations are the restricted pH range of 5.6–10.2 and its intrinsic incompatibility with direct MS coupling due to the buffer formulation and of course the actual salt present in the system. As the accessible pH range covers the alkaline region, caution should be given to the tolerable pH range when a silica-based column is used, as too alkaline conditions may damage the column. The latter is generally not a problem when polymeric columns are used as in this manuscript; these are readily available for IEX and HIC chromatography. Only the pH gradient elution for charge variant analysis of mAbs uses the high pH 10.2 for short periods in the methods for protein characterization. As all the eluents are specific for the column selected in the method, we do not consider this as a significant problem.

We believe the universal buffer system described here can help to minimize the efforts and costs for method development and aid analytical laboratories by providing easier to use, robust methods with fewer eluent related problems.

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.2c03531.

Figure S1 showing an example of chromatography reproductibility by preparing buffers by pH titration and gravimetrically; Figure S2 showing online pH and column scouting for charge variant analysis with the universal eluent system for five different mAb drug products using an SCX and a WCX column (PDF)

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**Author Contributions**

**A.B.S. and J.B. contributed equally to the manuscript. A.B.S. and J.B. acquired data, did the data analysis, and reviewed and edited the manuscript. S.L. and C.A.P. provided experimental guidance and reviewed and edited the manuscript. K.C. provided some experimental work and guidance and wrote, reviewed, and edited the manuscript.**

**Notes**

The authors declare the following competing financial interest(s): All of the authors were employees of Thermo Fisher Scientific at the time of writing this manuscript.

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