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Monoclonal Antibody Development and Physicochemical Characterization by High Performance Ion Exchange Chromatography

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1. Introduction

Monoclonal antibodies (mAbs) represent a significant portion of products in the biopharmaceuticals market (Reichert, 2011; Scolnik, 2009). mAbs have been developed to treat a variety of indications to address significant unmet medical needs (Waldmann, 2003; Reichert & Valge-Archer, 2007; Ziegelbauer et al., 2008), and are generally target-specific and well tolerated with a relatively long half-life, contributing to the success of the molecule class for drug development (Scolnik, 2009). Of the classes of antibodies, or immunoglobulins, IgG1 is the most common immunoglobulin used for pharmaceutical and biomedical purposes (Reichert et al., 2005); however, other immunoglobulin types (e.g., IgG2, IgG4) and mAb-related products (e.g., Fc-fusion proteins, Fabs, etc.) are also being used for therapeutic purposes (Hudson & Souriau, 2003).

While a successful mAb product can generate upwards of a billion dollars or more in sales annually (Reichert, 2009), it takes a significant amount of time and resources to develop a new therapeutic mAb; current estimates indicate that it can take about 10-15 years (Dickson & Gagnon, 2004; DiMasi et al., 2003) and over $1 billion to bring a new biologic drug to market (DiMasi & Grabowski, 2007). Addressing bottlenecks and making improvements in the development process is essential to save time and money, expediting the delivery of new drugs to the clinic.

This chapter will briefly cover monoclonal antibody development, production and purification, and then focus on antibody characterization, particularly charge-species analysis using high performance ion exchange chromatography (IEC). Method development strategies, method robustness, validation and automation will be discussed. This chapter aims to be a reference text demonstrating the utility of IEC as well as providing strategies for developing rugged IEC methods for the characterization of therapeutic mAbs.

2. Monoclonal antibody development

Antibodies are physiological blood components that are produced by B lymphocytes, intended to bind to and neutralize foreign antigens and pathogens. Antibodies bind to a

corresponding antigen in a highly specific manner. Although not covered in this chapter, potential mechanisms of action for mAbs have been described (Green et al., 2000). Polyclonal antibodies are a combination of immunoglobulin molecules secreted against a specific antigen, each identifying a different epitope. In contrast, monoclonal antibodies are derived from a single cell line that are all clones of a unique parent cell.

The first monoclonal antibody product was approved in 1986 and was a murine antibody for the prevention of kidney transplant rejection; however, patients frequently developed antibodies against the mouse-derived mAbs, which limited their effectiveness (Jones et al., 2007; Kuus-Reichel et al., 1994; Shawler et al., 1985). Advances in antibody engineering yielded techniques for generating chimeric mAbs that contain sequences from both human and murine sources (Morrison et al., 1984; Reichmann et al., 1988). Many of the mAbs approved for commercialization in the 1990s and early 2000s were chimeric antibodies. Chimeric antibody products are superior to murine antibodies, but they still pose a risk of immunogenicity to patients from their residual murine components (Carter, 2001). New technologies were developed to produce humanized mAbs, which contain approximately 95% human components and 5% murine components (Carter & Presta, 2000, 2002). In these humanized mAbs, the CDRs of a human antibody gene were replaced by those from the CDR of a murine mAb gene (Figure 1). The resulting humanized antibody has the same antigen binding properties as the original murine antibody but contains minimal murine sequences (Co & Queen, 1991).
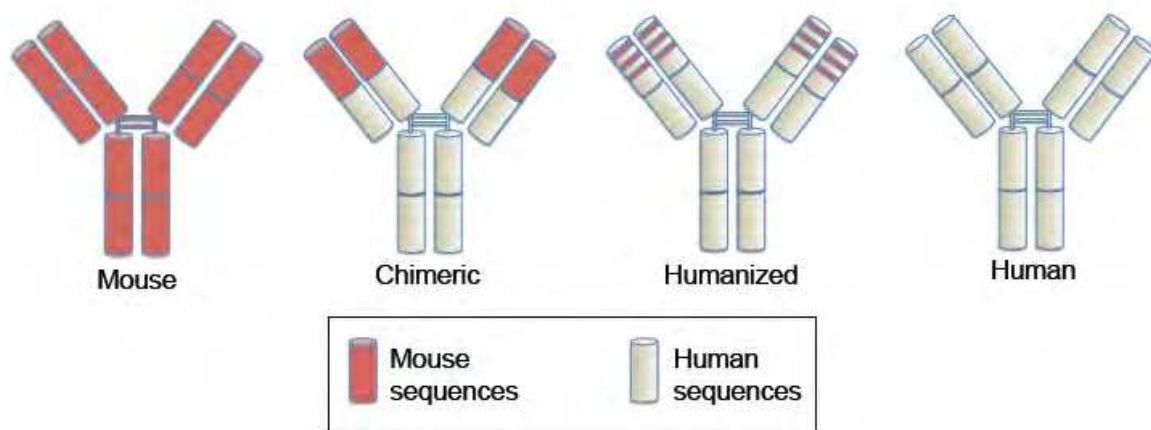

Fig. 1. Schematic representation of the sequence composition of murine, chimeric, humanized, and human antibodies.

More recently, less immunogenic therapeutic antibody products were developed by creating fully human mAbs. Several technologies exist to develop fully human antibodies, each falling into one of the two general classes—*in vivo* approaches using a murine system in which the immunoglobulin genes have been replaced by their human counterparts, or *in vitro* approaches such as phage display libraries containing millions of variations of antibody sequences coupled with a mechanism to express and screen these antibodies *in vitro* (Lonberg, 2005; McCafferty et al., 1990). Combining *in vivo* and *in vitro* discovery and molecular engineering technologies allows exquisite control of the antibody sequences and properties that was not possible when mAbs were first developed. Because of these

advanced antibody engineering technologies, almost all antibody products currently in development are humanized or fully human mAbs and their derivatives.

3. Monoclonal antibody production

Recombinant monoclonal antibodies are typically produced in mammalian cell lines under defined cell culture conditions. Commercial scale production processes vary depending on the mAb, but generally, cells are taken from a master cell bank and inoculated into small-scale bioreactors. The cell culture is transferred to increasingly larger bioreactors until it reaches the final commercial scale bioreactor. Currently, final scale reactors have volumes ranging from 12,000 L to 24,000 L (Gottschalk, 2009). The cells are cultured in a controlled environment for days to weeks, and then the cell culture fluid is harvested by centrifugation (Shukla & Kandula, 2008). In mammalian cells, the product monoclonal antibodies are secreted from the cells into the supporting fluid medium. Centrifugation separates the cells from the fluids and facilitates simpler recovery procedures downstream.

Commercial mAb production requires considerable preproduction effort to ensure that the cell line is stable and can produce commercially appropriate quantities of antibody. In addition, the commercial production process must produce a product that meets the quality expectations of regulatory authorities. In the past few years, improvements have been made in critical areas, such as cell line generation and large-scale cell culture production, to maximize specific antibody productivity from a given cell line and improve overall productivity in bioreactors. These advances include the use of new expression vectors and transfection technology, high-throughput, robust screening technologies to select the highest producing clones rapidly and more effectively, improvements in cell culture and optimized bioreactor processes (Li et al., 2010; Schlatter et al., 2005). As a result, the production of cell lines expressing multigram quantities of antibody per liter of culture medium is now routine.

The product quality and product heterogeneity of every mAb is highly dependent on its manufacturing process (Abu-Absi et al., 2010; Horvath et al., 2010). The ideal manufacturing conditions would have optimal production levels of product in conjunction with the desired product quality profile. Attributes that are typically deemed critical in selecting stable clones and cell culture conditions are the product titer and product heterogeneity, including charged species and aggregates. Production titers directly correlate to the costs of the process and are desired to be as high as possible with minimal impact to other quality attributes of the product (Kelley, 2009). Critical quality attributes of the product, such as the level of aggregation, are carefully monitored, as failure to control critical quality attributes may pose a safety risk to the patient (Rosenberg, 2006).

4. Monoclonal antibody purification and formulation

Once monoclonal antibodies are produced in cells, the mAbs must be recovered and purified. Recovery and purification processes vary widely depending on the manufacturing process and specific mAb characteristics, but generally, the isolation and purification of mAbs involve a centrifugation step to separate the cells from the cell culture fluid containing the mAb product, one or more chromatography steps, which can include affinity chromatography, cation or anion exchange chromatography, hydrophobic interaction chromatography (HIC) and displacement chromatography (Shukla et al., 2007), and

filtration or precipitation steps (Gottschalk, 2009). Many of the purification steps are designed to remove contaminants and adventitious agents (e.g., bacteria, fungi, viruses, and mycoplasma).

After elution from the final chromatographic purification step, a unit operation is required to exchange the components of the chromatography elution buffer with the chosen formulation components. The predominant technology that has been used in the industry for buffer exchange and concentration is ultrafiltration/diafiltration using tangential-flow filtration (Genovesi, 1983; Shiloach, 1988; van Reis, 2001). After this step, the drug substance is filtered and typically frozen as bulk for storage until filling occurs to produce the final drug product.

The formulation of the mAb therapeutic is chosen in part to ensure product quality during shelf life. Formulations are designed to minimize protein aggregation, decrease viscosity, and increase shelf life through preventing degradation (Shire, 2009). High protein concentration formulations are being developed to allow for subcutaneous or intramuscular delivery of mAb products (Shire et al., 2004). Historically, the most conventional route of delivery for protein drugs has been intravenous administration because of poor bioavailability by most other routes, greater control during clinical administration, and faster pharmaceutical development. Subcutaneous delivery allows for home administration and improved patient compliance. However, development of high protein concentration formulations involves unique manufacturing challenges compared to low concentration formulations, such as higher viscosities and necessary changes to unit operation steps.

5. Monoclonal antibody characterization and release testing

Biopharmaceutical manufacturing of monoclonal antibodies produces a heterogeneous product of structurally related species. Antibody speciation can occur throughout the manufacturing process at various steps, including cell culture, harvest, purification, formulation, filling and during shelf life. Full-length monoclonal antibodies are high molecular weight proteins (around 150,000 Da), and have highly complex secondary and tertiary structures, subject to post-translational modifications. Therefore, product characterization and quality control testing are required at critical points throughout clinical development and manufacturing to control for these species (Harris et al., 2004). Figure 2 depicts the structure of a monoclonal antibody compared to a small molecule drug, illustrating the increased complexity of a biologic compared to a small molecule therapeutic.

Antibodies can be characterized by many physicochemical properties including hydrated size (Stokes radius), molecular weight, charge, hydrophobicity, electrophoretic mobility, isoelectric point (pI), sedimentation velocity, glycosylation, and spectral properties. The nature of each species can be related to differences in their primary, secondary, tertiary, or quaternary protein structures. In addition, monoclonal antibodies are susceptible to chemical or enzymatic modification, particularly at sites that are exposed to the protein-liquid interface. Product heterogeneity can be caused by a number of modifications, such as C-terminal processing of lysine residues (Harris, 1995; Santora et al., 1999; Weitzhandler et al., 1998), deamidation (Di Donato et al., 1993; Hsu et al., 1998), glycation (nonenzymatic glucose addition) (Quan et al., 2008), amino acid sequence variations (Yang et al., 2010), and noncovalent complexes (Santora et al., 2001).

Antibody products are characterized by physicochemical, immunochemical, and biological methods. Guidance documents have been issued by regulatory agencies and industry representatives recommending approaches for protein characterization (International Conference on Harmonisation of Technical Requirements for the Registration of Pharmaceuticals for Human Use (ICH), 1999; Schnerman et al., 2004). These orthogonal assays include potency, identity and purity assays, which evaluate "critical quality attributes" such as size and charge heterogeneity. These critical quality attributes are part of the overall target product profile, which is based on the desired clinical performance. The extent of characterization is linked to the level of risk associated with each phase of drug development. For example, while there may not be sufficient time or resources for extensive characterization of an antibody during early stage development, it is expected that the molecule will be well-characterized before the Biologic License Application (BLA) is submitted to the regulatory agencies.
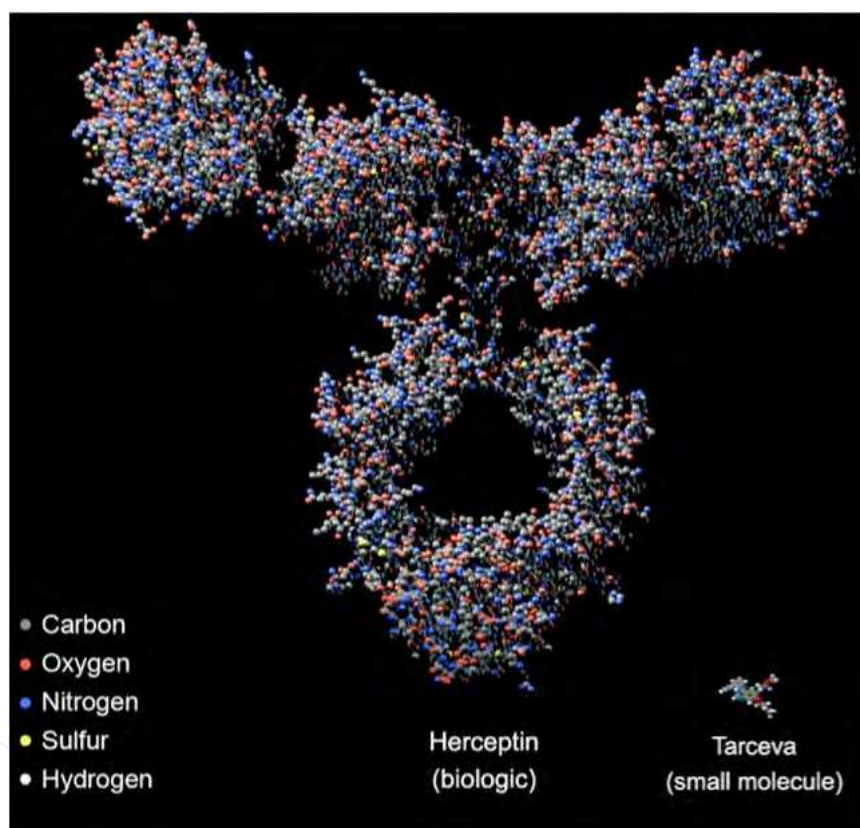

Fig. 2. Comparison of the structures of a mAb (Herceptin) and a small molecule therapeutic (Tarceva).

Many of the recommended protein characterization assays are based on liquid chromatography methods, such as ion exchange chromatography (IEC) for charge heterogeneity analysis, size exclusion chromatography (SEC) for size heterogeneity, and reversed-phase high performance liquid chromatography (RP-HPLC) for peptide mapping (Chirino & Mire-Sluis, 2004). The remainder of this chapter will primarily focus on ion exchange chromatography methods for analyzing charge heterogeneity for characterization and support of formulation and process development, as well as for lot release testing of drug substance and drug product (Schnerman et al., 2004).

5.1 Analyzing mAb charge heterogeneity using IEC

As mentioned previously, monoclonal antibodies are large proteins that are quite complex. While the light chain and heavy chain sequences of a particular mAb may be known, a number of modifications can introduce heterogeneity in the product. Thus, it is important to develop appropriate analytical methods to resolve the minor forms of the product. Analytical biochemists routinely use IEC for resolving charge variants of the protein. The scientist must then utilize orthogonal analytical methods to characterize the separated peaks of the ion exchange chromatogram. The characterization of a mAb is particularly important if the modifications occur in the complementarity-determining regions (CDR), as modifications in the CDR can affect the binding activity and potency of the mAb.

A strategy for the assignment of peaks from a weak cation exchange (WCX) mAb separation using a salt gradient has been published (Harris et al., 2001). Seven forms of a therapeutic recombinant antibody were resolved by cation-exchange chromatography. The peak fractions were collected, and structural differences were assigned by peptide mapping, which involves digesting the mAb with an enzyme and injecting the digest onto a reverse-phase column coupled to a mass spectrometer, and by hydrophobic interaction chromatography (HIC) after papain digestion. The peaks in this particular case were attributed to deamidation, isomerization, and succinimide intermediates. Other orthogonal analytical methods were used to characterize the IEC peaks; one of these methods—potency testing—determined that one minor peak demonstrated much lower potency than the main peak.

In another study, a recombinant humanized monoclonal IgG1 antibody with different states of glycosylation on the conserved asparagine residue in the CH2 domain was analyzed by cation exchange chromatography (Gaza-Bulseco et al., 2008). Two major peaks were observed and were further characterized by enzymatic digestion and mass spectrometry. It was found that this recombinant monoclonal antibody contained three glycosylation states—zero, one or two glycosylated heavy chains. The peak that eluted earlier on the cation exchange column contained antibodies with two glycosylated heavy chains containing fucosylated biantennary complex oligosaccharides with zero, one or two terminal galactose residues. The peak that eluted later from the column contained antibodies with zero, one or two glycosylated heavy chains. The oligosaccharide on the antibodies that eluted in the later peak was composed of only two GlcNAc residues. These results indicate that conformational changes, caused by different types of neutral oligosaccharides as well as the absence of certain oligosaccharides, can be differentiated by cation exchange column chromatography.

5.2 Lot release testing of mAbs

Once the mAb is purified and formulated, the resulting drug substance must be tested prior to lot release. A set of tests and acceptance criteria are established based on mAb characterization and regulatory requirements in order to ensure product quality (Food & Drug Administration (FDA), 1999). These tests typically include appearance, identity, purity, protein concentration, potency of the molecule, microbial limits or bioburden, and bacterial endotoxins (Table 1). IEC is one of the most frequently used lot release methods for purity for mAbs (Schnerman et al., 2004). Once these tests are performed and the results

meet the established acceptance criteria, a Certificate of Analysis (COA) is generated and the lot is released for use. Finally, adequate stability studies should be performed on the mAb drug substance (e.g. frozen bulk for storage) and drug product (e.g. final vial) according to regulatory guidelines (Food & Drug Administration (FDA), 2003).

Attribute	Test Name
Appearance	Color, Opalescence and Clarity
Identity	Peptide Mapping by RP-HPLC (Reverse-Phase HPLC), or
	MALDI (Matrix-Assisted Laser Deionization) Mass Spectrometry, or
	UV Spectroscopy (2nd Derivative)
Purity	Limulus Amebocyte Lysate (Endotoxin)
	Size Exclusion Chromatography (SEC)
	CE-SDS (Capillary Electrophoresis-Sodium Dodecyl Sulfate)
	IEC (Ion Exchange Chromatography) or icIEF (Imaged Capillary Isoeletric Focusing)
	Glycosylation Profile
	Peptide Mapping by RP-HPLC
Potency	Potency (ELISA/Cell-Based Assay)
Strength	UV Spectroscopy
General Tests	Osmolality
	pH
	Surfactant Concentration (e.g. Polysorbate 20)

Table 1. Commonly used tests found on a Certificate of Analysis for lot release; a selected subset is used for stability testing of mAbs.

6. Mechanism of ion exchange chromatography of mAbs

Ion exchange chromatography (IEC) has been a platform for monoclonal antibody purification and characterization for many years. For the analysis of charged species of proteins, IEC is a popular method due to the fact that it preserves the native conformation and maintains bioactivity of the protein, is relatively easy of use, is supported by the maturity of the equipment and consumables market, and has widespread use in the biopharmaceutical industry (Rea et al., 2010).

Charge-based methods are an integral component of characterization studies and quality control strategies because they are sensitive to many types of modifications. Charge profiling of intact antibodies can resolve species related to protein conformation, size, sequence species, glycosylation and post-translational modifications (Gaza-Bulseco et al., 2008; Harris et al., 2001; He et al., 2010). Although IEC can be used to track specific species, it is common to group all species not associated with the main peak and report them as either acidic or basic species (Figure 3). In addition, fractions collected from an IEC run can often be directly injected onto orthogonal columns for further analysis, such as reverse-phase and size exclusion chromatography columns, or submitted for potency testing.

IEC separates proteins based on differences in the surface charge of the molecules, with separation being dictated by the protein interaction with the stationary phase. The two main categories of ion exchange chromatography are cation exchange (CEX) and anion exchange (AEX). Cation exchange chromatography retains biomolecules by the interaction of the negatively-charged resin with histidine (pK ~ 6.5), lysine (pK ~ 10) and arginine (pK ~ 12) in the protein. Anion exchange chromatography primarily retains biomolecules by the interaction of the positively-charged resin with aspartic or glutamic acid side chains, which have pKa of ~4.4. In addition to the amino acid residues, cation exchange columns can also separate deamidated, glycated and other charged variants. Anion exchange columns have also been useful for separating phosphorylated and hydroxyl modified amino acids. When the pH equals the pI value of the protein, the net charge on the molecule is zero. However, significant retention can occur for proteins even when the pH of the mobile phase is equal to the pI of the molecule; despite an overall net charge of zero, only a portion of the mAb molecule will interact with the stationary phase, and there will be a net charge on that portion of the molecule because of an uneven distribution of charged groups throughout the molecule (Vlasak & Ionescu, 2008). Thus, it is possible to separate proteins having very similar charge (Figure 4), or even structural isomers with identical pI values, by ion exchange chromatography.
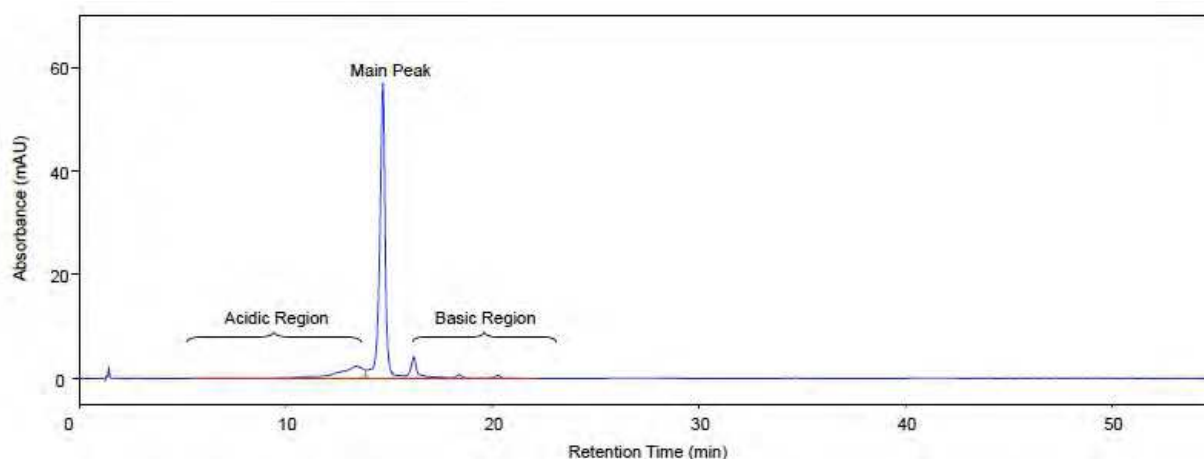

Fig. 3. Typical cation exchange chromatogram for analytical characterization of a mAb. Integration is shown, and main peak, acidic and basic regions are denoted.

There are two ways to elute the protein from the IEC column: 1) increasing salt concentration with time or 2) by varying the mobile phase pH value as a function of time. Increasing the salt concentration elutes the protein by increasing the ionic strength of the mobile phase, thus affecting the charge interaction of the mAb and the stationary phase. A pH gradient elutes the protein by changing the charge on the molecule, thus affecting the binding of the molecule to the stationary phase. While conventional salt gradient cation exchange chromatography is regarded as the gold standard for charge sensitive antibody analysis (Vlasak & Ionescu, 2008), method parameters such as column type, mobile phase pH, and salt concentration gradient often need to be optimized for each individual antibody. A recent publication described a multi-product pH gradient IEC method for the separation of mAb charge species for a variety of mAbs using a single method (Farnan & Moreno, 2009). The following sections will discuss both salt-gradient and pH-gradient based elution methods, and the combination of the two modalities (hybrid methods).
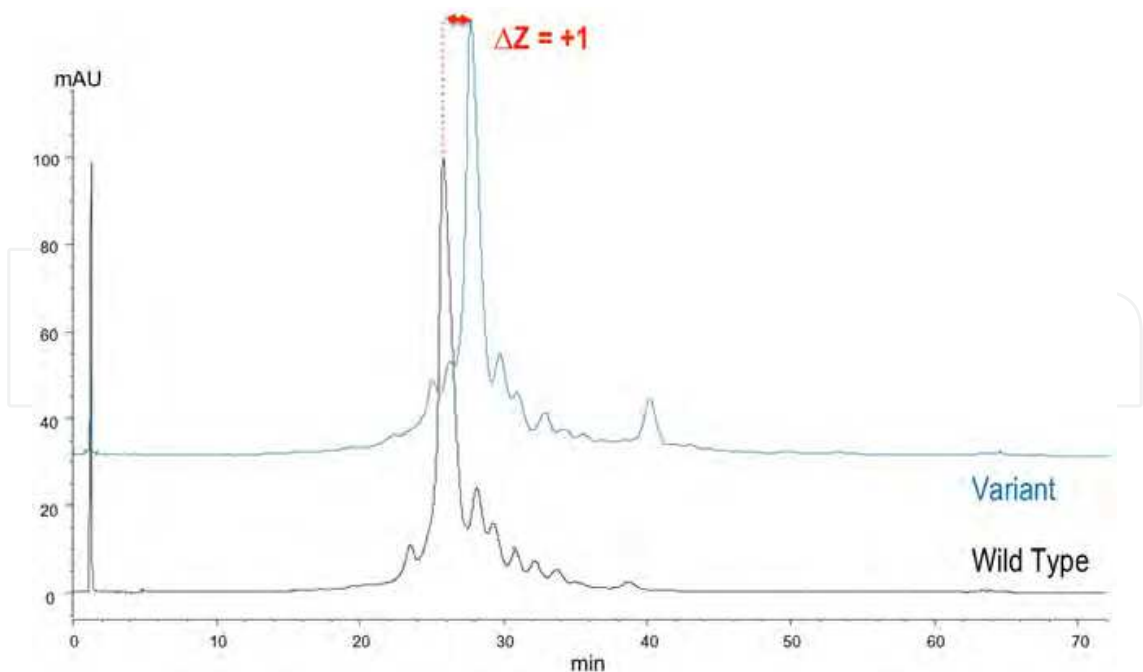
Fig. 4. Separation of mAbs differing by only one charge, a single amino acid change to primary structure. The elution buffer (0.5 M NaCl in 20 mM Tris, pH 7.3) was increased linearly on a ProPac WCX-10 column (4 x 250 mm), which was held at 50 °C and had a flow rate of 1 mL min^{-1}.

7. Developing a salt-based IEC method

Salt-based IEC separations are developed by choosing a cation or anion exchange column and varying the buffer system, mobile phase pH value, and ionic strength gradient of the elution buffer. Figure 5 shows a typical development workflow for salt-based IEC and pH-based IEC development, and can serve as a guide for initial IEC method development. The following sections will cover in more detail the outputs to consider when screening various parameters during development. More general considerations regarding HPLC method development can be found in various texts (Kastner, 2000; Snyder et al., 1997).
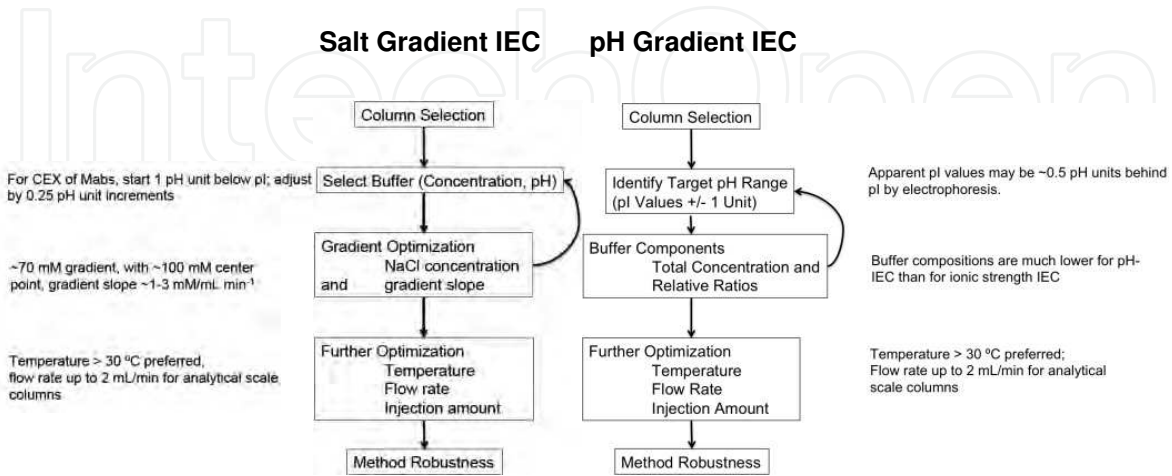

Fig. 5. Sequential salt-gradient IEC and pH-gradient IEC method development and optimization work flow.

7.1 Column selection, buffers and operating parameters for salt gradient IEC

Column selection is perhaps the most subjective part of the optimization process; picking between the different vendor offerings and functionalities can be difficult. Prior experience, data in the literature or unpublished results within the organization are often the best starting points.

Analytical ion exchange chromatography of proteins is typically carried out using mobile phases that are relatively neutral in pH values, 5.5 to 8.5 This general practice is recommended because at pH extremes, the protein is more likely to degrade. The selection of whether to use anion or cation exchange chromatography is also driven by the isoelectric point of the protein (pI) and the species to be resolved, e.g., phosphorylated species, C-terminal lysine variants, etc.

If the pI value of the mAb is greater than 8, a CEX column is evaluated at pH 6-7 initially. CEX primarily retains mAbs by the interaction of acid groups on the CEX resin with lysine, arginine and histidine side chains on the mAb. Since mAbs are positively charged at a mobile phase pH below their pI, the mAb species would likely be retained and resolved on a CEX column under the recommended mobile phase pH range.

If the pI value of the mAb is less than 6, an AEX column is evaluated at a pH above 6 initially. AEX primarily retains biomolecules by the interaction of amine groups on the ion exchange resin with aspartic or glutamic acid side chains. Since mAbs are negatively charged at a mobile phase pH above their pI, the mAb species would likely be retained and resolved on an AEX column.

For intermediate pI values of 6-8, both CEX and AEX are evaluated because of the possibility that the portion of the mAb that interacts with the stationary phase, typically the side chains that are exposed to the mobile phase, has a different charge than the pI would suggest, e.g., the surface charge of the mAb is positive despite the entire mAb having an overall negative charge. Ultimately, the species of interest that are to be resolved determine whether CEX or AEX is chosen for molecules with intermediate pI's; the separation mode that better separates the species of interest is usually the one that is chosen for mAb analysis.

Figure 6 shows CEX and AEX chromatograms of a Fab (mAb fragment) reference sample and thermally stressed sample. In this case, the Fab molecule has a nominal pI value for the main species of 7.6. It should be noted that the separations on the AEX and CEX columns were each optimized independently for column type, pH value and salt gradient. It should also be noted that the terms "strong" and "weak" (in SAX, strong anion exchange, and WCX, weak cation exchange) refer to the extent of variation of ionization with pH due to the functional groups on the resin and not the strength of binding. Strong ion exchangers are completely ionized over a wide pH range whereas with weak ion exchangers, the degree of dissociation and thus exchange capacity varies much more markedly with pH. For this example, SAX results in significantly more peaks and much better resolution of the charge species in comparison to the WCX chromatogram. Particularly interesting is that the difference between the WCX and SAX elution profiles are much more vivid for the stressed samples than for the reference materials. We have seen examples where the converse is true and the CEX separation is better than that observed on the AEX. This contrast between the AEX and CEX profiles highlights an important feature of IEC that electrophoretic methods don't exhibit, which is the ability to magnify particular aspects of the protein structure and accentuate the separation of species relating to particular motifs (Vlasak & Ionescu, 2008).
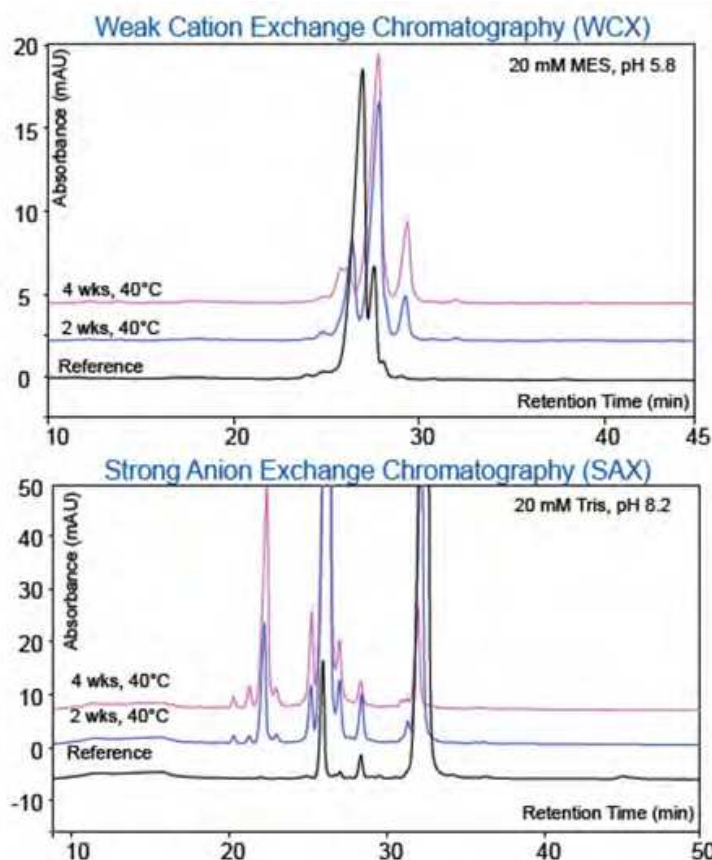
Fig. 6. Separation of Fab charge species using a weak cation exchange column (WCX) and a strong anion exchange column (SAX). Thermally stressed samples are labeled by incubation time and temperature of incubation.

In general, we have observed that for the separation of mAb variants using ion-exchange chromatography, the optimized chromatogram has a relatively shallow gradient over a narrow range of salt concentration. A typical method results in 100 mM NaCl as the center point of the gradient, with salt concentration increasing over 70 mM NaCl in a linear gradient. It is recommended to perform iterative gradient optimizations to narrow the NaCl gradient down to around 2 mM/mL min^{-1}. Iterative cycles are quicker and more predictive than performing a very long shallow gradient.

Chromatograms obtained during the mobile phase pH value optimization for a mAb with a pI value around 9.5 are shown in Figure 7. Buffer species and buffer concentration for salt-gradient IEC are generally not significant factors, but should be chosen considering target pH and buffer pKa.

Although temperature does not significantly affect electrostatic interactions, it often affects the pH value of the mobile phase. This is particularly of concern for a Good's buffer system (group of buffers described in the research of Dr. Norman Good et al. in 1966, often used for IEC and other biochemistry applications) (Good et al., 1966), which can exhibit a change in pH value of around 0.02 per °C temperature change. This sensitivity creates a need to control the column temperature carefully. A column compartment is always used, typically set at a value greater than 30°C to ensure good temperature stability in compartments that can only apply heat. Above 30°C, temperature control within +/- 1°C is readily achievable with commercially available equipment.
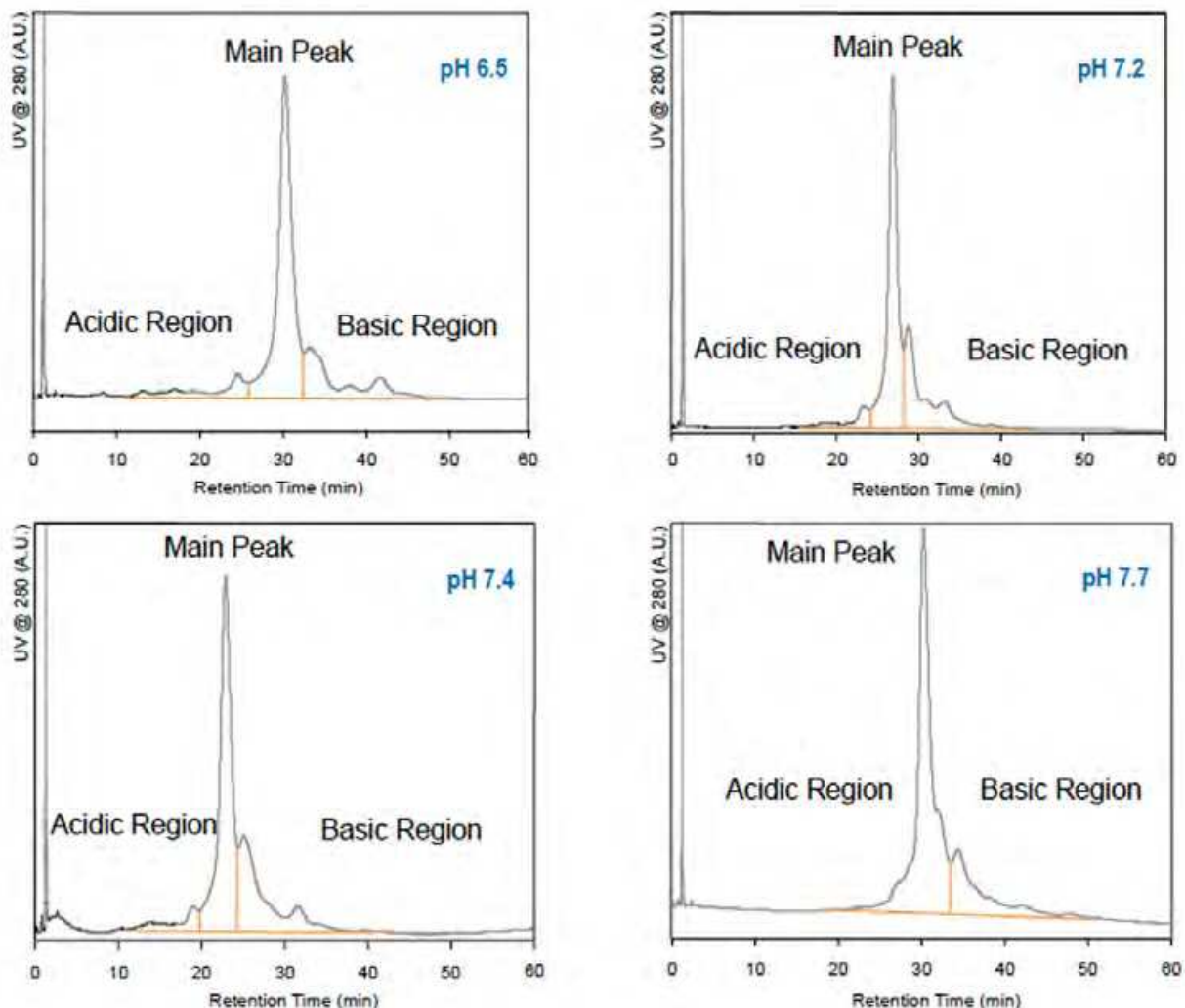
Fig. 7. Effect of mobile phase pH on mAb separation by WCX. The elution buffer (0.5 M NaCl) was increased linearly at 1 mM min^{-1} at a flow rate of 1 mL min^{-1} on a ProPac WCX-10 column (4 x 250 mm), which was held at 30 °C. Different initial salt concentrations were optimized for each pH value. Integration is shown, and main peak, acidic and basic regions are denoted.

Subtle variations in selectivity with temperature may result from temperature-induced changes in mobile phase pH value (Figure 8). In Figure 8, the elution profile changes in two distinct regions as a function of temperature. Below 40°C, subtle changes in elution profile and retention times are observed consistent with minor changes to the mobile phase pH value as a function of temperature. However, above 40°C, the profiles exhibit much more radical changes with increasing temperature. This is interpreted to be related to the mAb having lost higher order structure at those elevated temperatures due to protein denaturing. For the mAb in Figure 8, it is clear that moderately elevated temperatures are not possible while maintaining the higher order structure; in general for IgG1 mAbs, chromatography at temperatures up to 55°C is readily possible. In summary, while mobile phase temperature does not affect protein charge directly, temperature can affect mobile phase pH and the structure of the protein, which can affect chromatographic separations. Thus, column temperature should be optimized considering these temperature effects.
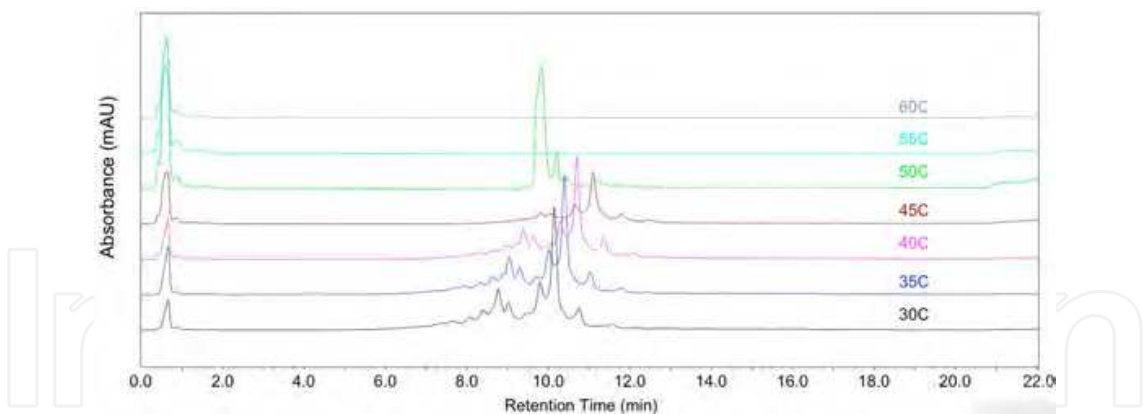
Fig. 8. Effect of temperature on mAb separation by CEX. The elution buffer (0.2 M sodium sulfate) was increased linearly on a ProPacWCX-10 column (4 x 250 mm).

8. Developing a pH gradient-based IEC method

Despite good resolving power and robustness, salt-based ion exchange separations are usually protein-specific and time-consuming to develop. A novel pH-based separation of proteins by cation exchange chromatography that was multi-product, high-resolution, and robust against variations in sample matrix salt concentration and pH was recently reported (Farnan & Moreno, 2009). A pH gradient-based separation method using cation exchange chromatography was also evaluated in a mock validation and deemed highly robust (Rea et al., 2011). Figure 9 depicts the separation of 16 mAbs by pH gradient IEC (pH-IEC). Each mAb was injected sequentially, demonstrating that in contrast to salt-based IEC, pH-IEC can be used to analyze multiple mAbs with a single method.
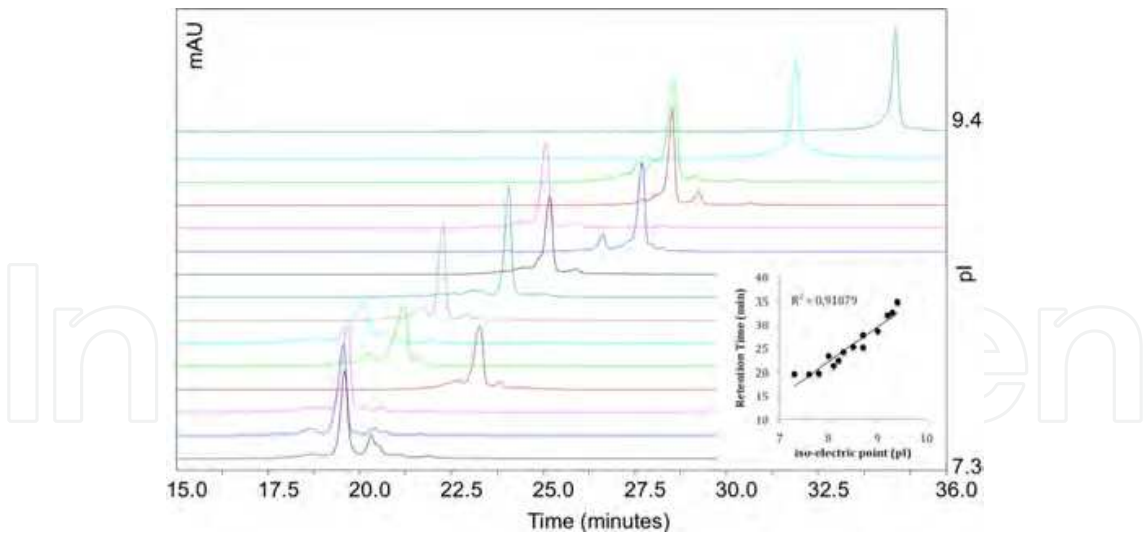

Fig. 9. Separation of 16 mAbs using a ProPac WCX-10 column by pH gradient IEC. Each mAb was analyzed using the same pH-IEC method, and each mAb was injected sequentially. mAb pI values ranged from pI 7.3 to pI 9.4.

Similar to salt-gradient IEC methods, pH-IEC separations are developed by choosing a cation or anion exchanging column and varying the buffer system, pH of the mobile phases, and other operating parameters, such as temperature and flow rate. Figure 5 shows a typical development workflow for pH-IEC, and can serve as a guide for initial pH-IEC method development.

8.1 Column selection, buffers and operating parameters for pH gradient IEC

Like conventional IEC, the conditions chosen for pH-IEC separations, such as buffer, pH, column temperature, and sample load, are dependent on the type of column selected. To choose a column, the pI of the mAb and the expected charge species should be considered. Considerations for column selection may differ slightly for pH-IEC compared to conventional IEC. For example, because the column will be exposed to a pH gradient, the column must be able to perform adequately over a large pH range, i.e., the charged groups on the chromatography resin must maintain their charge over the operating pH range. Also, buffer strength can affect resolution, and pH-IEC mobile phases typically have lower buffer strengths than conventional salt-gradient IEC. Several pH-IEC buffer systems have been published for mAb separations; these buffer systems can be used as starting points for formulating buffers for pH-IEC methods (Farnan and Moreno, 2009; Rea et al., 2011; Rozhkova, 2009).

8.2 High-throughput multi-product separations using pH-IEC

To increase the throughput of the analytical methods, smaller particle sizes and shorter column lengths are being utilized to reduce run time. In Figure 10, the utilization of a 3 μm particle size column reduced analysis time 16-fold compared to a 10 μm particle size column. Analysis times are greatly reduced using smaller particle sizes because as the particle size decreases, there a significant gain in column efficiency, and the efficiency does not decrease at increased flow rates or linear velocities (Swartz, 2005). In addition, because different mAbs can be analyzed using the same pH-IEC method in the same sequence, these high-throughput methods are capable of analyzing hundreds of mAbs per day, which is not possible with conventional, product-specific salt-based IEC.
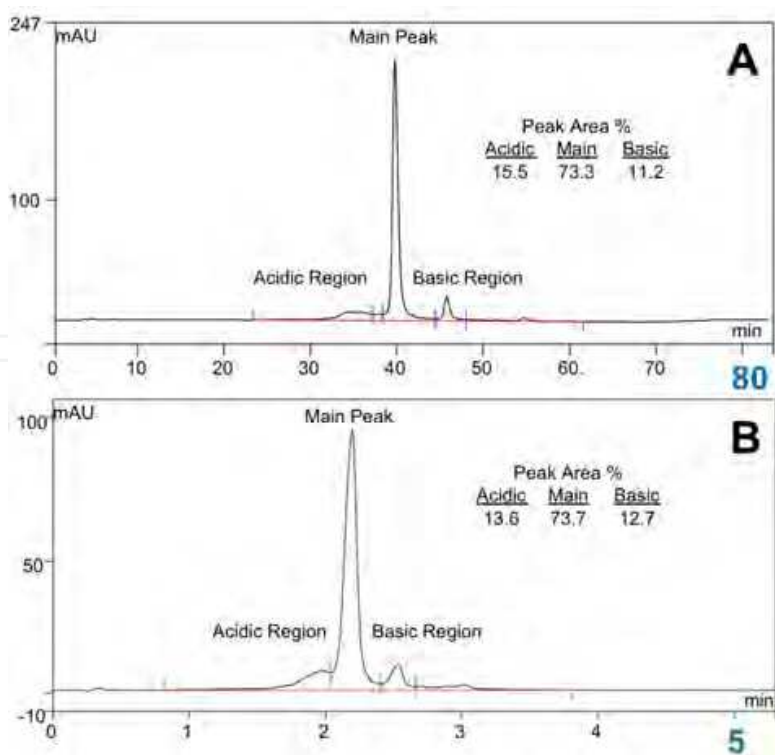

Fig. 10. Separation of a mAb using (A) a WCX column, 10 μm and (B) a SCX column, 3 μm, by pH gradient IEC. Each mAb was analyzed using the same buffers and gradient volume.

9. Hybrid/combination modes of IEC

Salt and pH may be combined to elute proteins from IEC columns. Combination or hybrid methods can be employed if either salt-based or pH-based methods prove inadequate for resolving species of interest, especially at extreme pHs. When pH increases above their pKa, amines, as used exclusively in the pH-IEC piperazine/imidazole/tris buffer system, become deprotonated and uncharged, resulting in decreased ionic strength. The bound proteins will also deprotonate and carry less charge. However, adequate amounts of positively charged ions are required to displace the bound proteins and to elute them off the cation exchange resin. Since the buffer salts alone can not provide enough positively charged ions at higher pH, additional salt is added to the pH-IEC elution buffers to maintain ionic strength. Figure 11 depicts measured conductivity as a function of elution time in pH-IEC with and without salt. In this case, adding salt to the elution buffer will compensate for the loss of ionic strength (represented by conductivity) due to deprotonation of buffer ions.

In Figure 12, separation of the charge species of three mAbs using pH-IEC with and without salt is compared. Without salt, mAb-1 with a pI of 9.4 did not elute, and mAb-3, with a low pI of 6.2, showed a very broad peak with significant tailing and no resolution of charge species. With the addition of salt, adequate separation of charge species is obtained for both high pI and low pI mAbs.
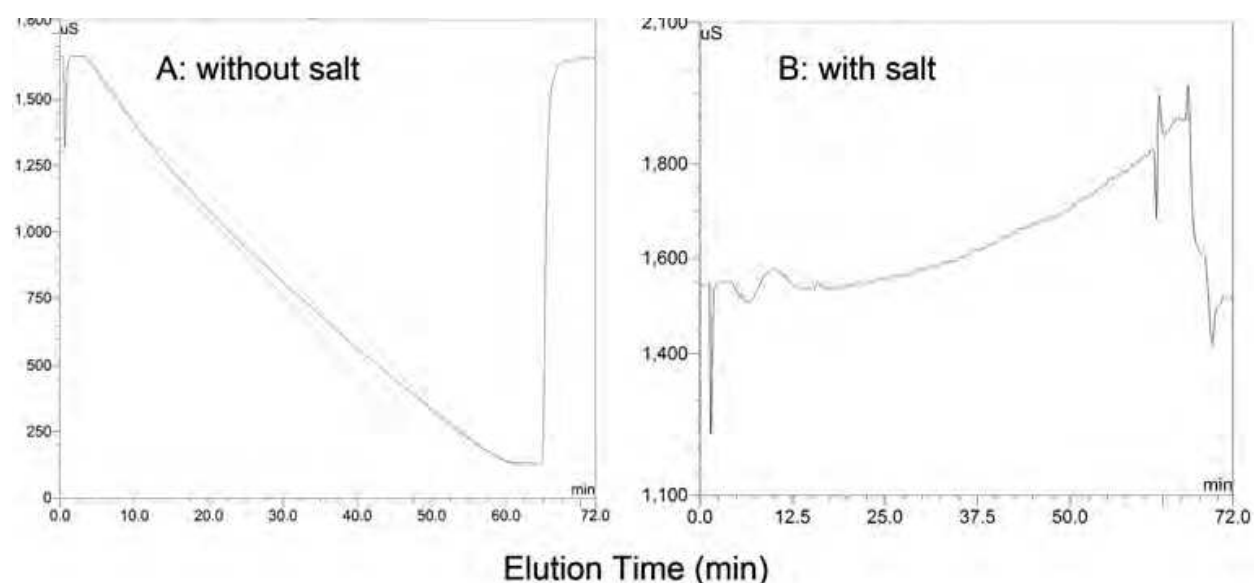

Fig. 11. Measured conductivity as a function of elution time in pH-IEC A) without, and B) with salt. Buffers are A) 11.6 mM piperazine, 1.5 mM imidazole, 2.4 mM Tris, B) 4 mM piperazine, 4 mM imidazole, 4 mM Tris, 16 mM NaCl.

10. Equipment configurations to accelerate development

Ionic strength gradient ion-exchange methods are typically product-specific, with each method requiring a unique pair of mobile phases and experimental conditions. As discussed above, a significant number of mobile phase pH values and gradient profiles need to be evaluated. Changing mobile phase pH values normally requires user intervention to supply new mobile phase pairs; the time needed to manually change the system can slow down

development. In order to more efficiently develop analytical IEC methods, alternative equipment configurations have been utilized to accelerate the selection of operational parameters, including quaternary buffer systems and customized solvent selection valves on the pump inlets to allow selection from an array of available solvents. Such modifications to the equipment and workflows can allow consecutive performance of significantly more experiments without requiring user intervention.
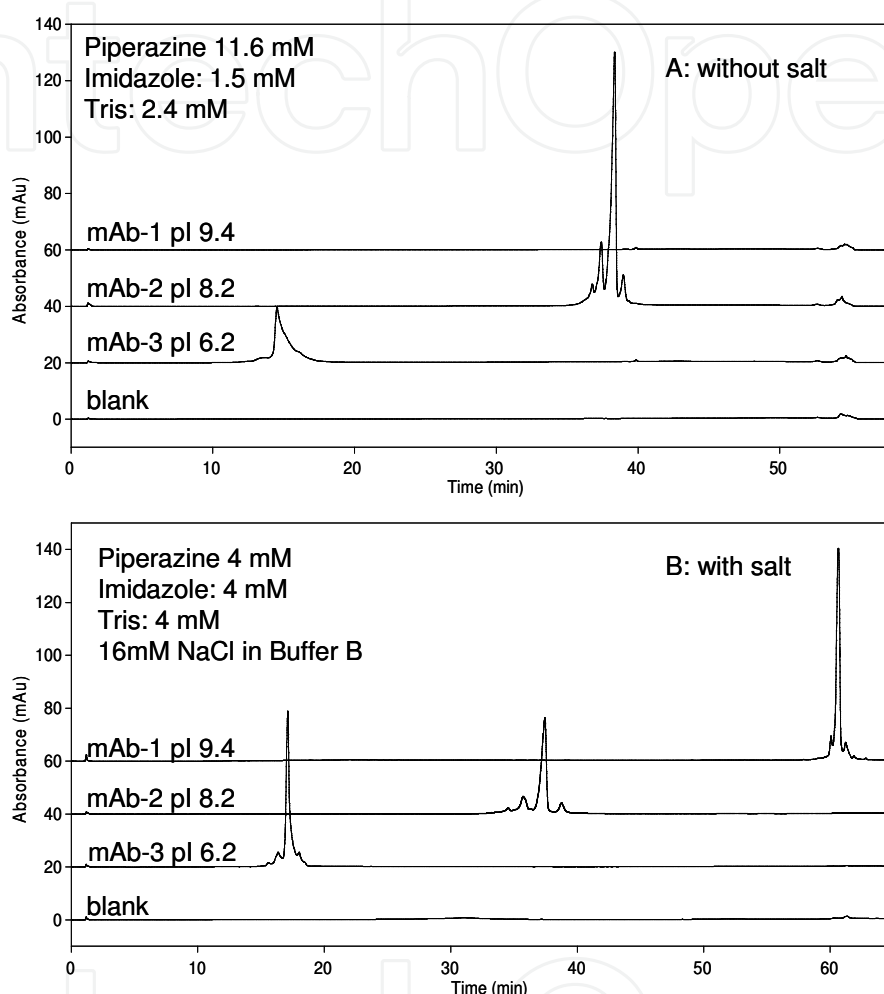

Fig. 12. Separation of the charge species of three mAbs using pH gradient with and without salt in a ProPac WCX-10 column. A) pH 5 to 9.5 in 45 minutes, gradient 0.1 pH unit/min; B) pH 5 - 10.8 in 58 minutes, gradient 0.1 pH unit/min.

A quaternary buffer system can be utilized to develop a method and reduce the amount of user intervention by using a pair of buffer solvents (solvent lines A and B) to allow the pump to admix to achieve the desired mobile phase pH, and using two other solvents (solvent lines C and D) to generate the ionic strength gradient for elution. The quaternary system can apply different combinations of salt and pH by automatically programming the percentages of the four solvents to be mixed and applied to the column. Thus, programs can be generated to screen a variety of salt and pH conditions in a single sequence using only four buffers.

Another approach, which is particularly important for binary pump systems, is to add a multi-port solvent selection valve to the system prior to the pump. Although the customized

valve system requires the production of many buffers, the multiple valve configurations can allow users to further customize buffer components and concentrations (as opposed to only salt gradient and pH) compared to the quaternary system. Such a system allows up to a dozen more buffer combinations to be evaluated without intervention.

Because pH-IEC is performed by using a pH gradient and not a salt gradient, simply reversing the pH gradient allows for the chromatography mode to be switched between CEX and AEX. This reversal of gradient can be automatically performed through the chromatography software. Thus, multiple CEX and AEX columns can be screened by using only two buffers at different pH's and using a column switching valve to screen different column types (Figure 13). During development, it is helpful to have online pH and conductivity meters to ensure that the pH gradient is roughly linear and that the conductivity does not interfere with the separation efficiency.

Fig. 13. HPLC column compartment equipped with a 6-port column switching valve, for screening of up to six different columns for pH-IEC without the need to change buffers or columns.

11. Method robustness and validation

The robustness of an analytical procedure is a measure of its capability to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. For IEC, robustness can be evaluated by varying parameters such as injection volume, buffer pH, flow rate, and column temperature. In addition to robustness, intermediate precision can be demonstrated by evaluating inter-laboratory variations, such as different days of analysis and different analysts. Furthermore, the ability to use different instrument and column manufacturers for a particular method greatly reduces the business risk of the method; if a column supplier cannot meet demand or if an instrument manufacturer ceases production of a particular instrument model, method transfer to other instruments and columns can occur without loss of performance.

11.1 Obtaining robust performance

Obtaining robust performance of an IEC method often goes beyond the design of the method itself, and involves good equipment hygiene, elimination of metal corrosion (e.g. formation of iron oxide) and contamination (e.g. presence of metal ions such as Fe^{3+} ions),

and mitigates the differences between instrument types. Problematic metal contamination typically results from corrosion of the fluid-contacting metal parts and can be avoided by using PEEK or titanium materials in the fluid paths. Good practices on obtaining robust method performance are discussed in the following sections.

11.1.1 Equipment hygiene

Maintaining good equipment hygiene is important in order to achieve robust performance. The following are good practices to ensure instrument hygiene:

1. Filter mobile phases that are amenable to microbial growth with 0.2 mm filters prior to use; replace solvent reservoir filters (sinkers) each time mobile phase bottles are replenished;
2. Flush and store HPLC system in 10% isopropanol in water when not in use, to prevent growth of microbes;
3. Leave the system running at low flow rates to prevent salt build up and clogging;
4. Keep all lines flushing as opposed to just a single channel;
5. Flush auto-sampler components as needed with 10% isopropanol in water;
6. Follow manufacturer's instructions regarding proper maintenance of HPLC instrumentation.

11.1.2 Metal contamination

Metals can negatively affect the ion-exchange chromatography of proteins. Protein chelation with metals are a secondary retention mechanism to the primary electrostatic interaction of ion-exchange chromatography. This secondary interaction results in peak tailing. These interactions can either occur with metal contaminating the column or with corroded surfaces within the HPLC. In addition to affecting separation, corrosion can result in physical damage to system, such pump seal failure and compromised performance of the detector cells. Halide containing eluents readily corrode HPLC systems manufactured from stainless steel, as stainless steel has the propensity to form rust (Collins et al., 2000a). Sodium acetate or sodium sulfate can be used as an eluting salt instead of halides; however, sulfate is divalent, thus concentrations in the eluting mobile phase would be different compared to using a halide, as halides are monovalent.

Metal contamination may be reversed by flushing with chelating agents such as oxalic acid dihydrate (Rao & Pohl, 2011). Also, stainless steel systems may require periodic passivation for reliable usage (Collins et al., 2000b). In light of the drawbacks of using a stainless steel HPLC system, more manufacturers are including biocompatible equipment (e.g. Titanium or PEEK) for analyzing mAbs and other protein products.

11.1.3 Transferring methods between instrument types

Transferring methods between instruments from different manufacturers can pose challenges due to the differences between instruments. As mentioned previously, equipment composition (e.g. stainless steel vs. titanium) is one of the factors to be considered when transferring a method between instrument types, in addition to gradient delay, mixing volumes, pump capabilities, and column compartment temperature ranges. Gradient delay and mixing volumes can differ between instruments, but they are generally

only a significant concern for very fast gradient separations. In addition, shallow IEC gradients can challenge the performance of an HPLC; however, most gradients are >70 mM salt and can be proportioned over 30-40% of the pump range, well within the capabilities of modern pumps. Often a gradient hold for 5 minutes at the initial salt concentration is included, just in case a method is particularly sensitive when being transferred from one equipment type to another. In such cases the hold time can be adjusted to compensate for differences in the gradient delay volume between the instruments.

Temperatures inside the column are dependent on oven design and plumbing configuration. Having a pre-column heat exchanger in line or out of line could make a several degree difference in the temperature at which the column chemistry occurs. This is particularly concerning for buffers with which the pH can change rapidly with temperature. Figure 14 shows a comparison of column compartment temperature settings for two different instruments from different manufacturers. To make the correlation, thermocouples were fitted into T-pieces in the fluid path inside the column oven, but just prior to the column, and temperatures were measured for a range of column compartment set points and mobile phase flow rates. These measurements were used to estimate the temperature of mobile phase going through the column for each set point. By equating the measured fluid temperatures for each flow rate, the correlation of column compartment temperatures were plotted. It is noted in this correlation that there was also a significant effect of the mobile phase flow rate on the correlation.

Different detectors can sometimes yield differences in baseline slope. This can occur when moving from a single/double wavelength detector with a reference beam to a photodiode array (PDA) detector. The selection of an appropriate reference wavelength and bandwidth on the PDA can overcome detector variance.
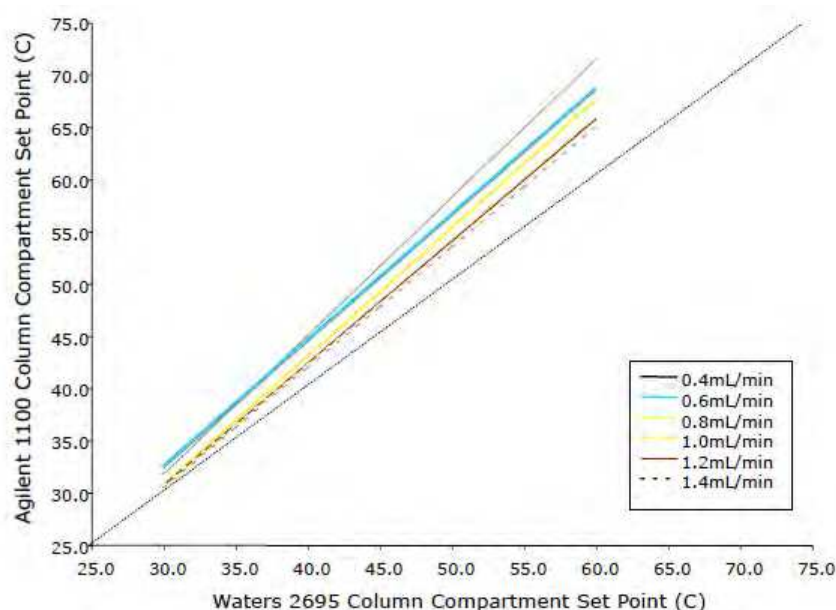

Fig. 14. Comparison of column compartment temperature settings required to achieve the same columns compartment temperature for two different HPLC models at different flow rates. Results are shown for a 4 x 250 mm Dionex ProPac column. The Agilent 1100 HPLC was configured using only the left hand side heat exchanger. The Waters 2695 HPLC was configured with the solvent pre-heater in-line.

11.2 Method validation

Before an analytical method can be incorporated into a characterization platform or a quality control system, it must first be demonstrated that the method is suitable for its intended purpose. Guidelines for validation of analytical methods have been published in the United States Pharmacopeia, by the International Conference on Harmonization (ICH), US Food and Drug Administration (FDA), and in published reviews (Bakshi & Singh, 2002). Methods must be evaluated considering regulatory requirements and validation procedures. In other words, the "validatability" of these methods must be assessed before implementation. Validation tests include precision, accuracy, and linearity. Intermediate precision is tested by using multiple instruments, multiple analysts, and multiple column lots. Methods must be validated and documented according to regulatory requirements prior to implementation into a control system for lot release of drug substance and drug product. Robustness studies can also be performed in conjunction with method validation. It has been our experience that the most significant effects on method robustness are: mobile phase pH value, column temperature, metal contamination and column age.

A system suitability range can be obtained from robustness studies. This range is often based on the standard deviation of the mean for a particular measured component, such as main peak relative area. The system suitability range indicates the precision of the method. pH-IEC may demonstrate an improvement in precision over conventional salt-based IEC (Rea et al., 2011). The 6σ ranges in Figure 15, which predicts a 99% method success rate, demonstrate the improved precision of the pH-gradient IEC method over conventional IEC, which can have a 6σ range of up to 8% main peak relative area (Figure 15).
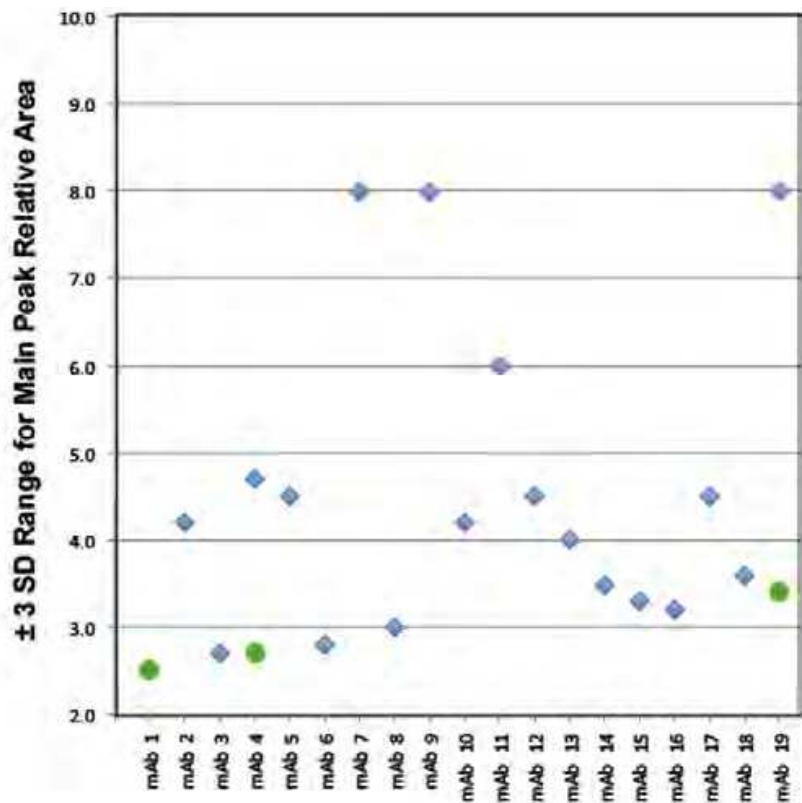

Fig. 15. Six sigma range (±3SD) for main peak relative area for salt gradient IEC (diamonds) and pH gradient IEC (circles) for a variety of mAbs.

12. Automation in sample preparation and data handling

In addition to high-throughput and multi-product analytical methods, the use of robotics for sample preparation automation may further reduce sample analysis time and cost. There are several companies that provide liquid handling automation instruments, including LEAP Technologies and TECAN. The LEAP Technologies CTC PAL liquid handling system is capable of on-the-fly sample preparation, such as protein dilution and digestion. On-the-fly sample preparations are viable if the sample preparation takes less time than the analytical method. For sample preparations that take longer than the analytical run time, batch sample preparation can be performed using robotic liquid handling systems such as the TECAN Freedom EVO, which can handle multi-well plates for increased sample throughput. Robotic liquid samplers can increase reproducibility, efficiency and safety compared to manual handling of samples.

The final steps to most characterization workflows include data analysis and report generation. Several software packages are available that are designed to reduce the time necessary to complete post-data acquisition tasks. For liquid chromatography applications, commercially available chromatography data software, such as Dionex's Chromeleon Chromatography Management Software and Waters Corporation's Empower Chromatography Data Software, include features such as automated peak integration and one-click report generation. In addition, laboratories are increasingly implementing electronic laboratory notebooks, which has advantages over traditional laboratory notebooks, including ease of data sharing and collaboration, streamlined review and witnessing processes, standardized documentation, and long-term data preservation.

13. Conclusion

Monoclonal antibodies are valuable therapeutic products that are approved for a variety of indications. In this chapter, mAb development, production, purification, formulation, characterization and regulatory requirements were discussed, followed by a more detailed discussion on charge species analysis using IEC. Method development strategies, method robustness, validation and automation, as well as applications of salt-gradient and pH-gradient IEC methodologies for the analysis of mAbs were also covered. This chapter is intended to be a reference text for scientists such that a concise strategy can be implemented for developing robust IEC methods for the characterization of therapeutic mAbs, resulting in shorter method development times and enabling faster analysis of mAb products to support biopharmaceutical pipelines.

14. Acknowledgment

15. References

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