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SEPARATION OF MONOCLONAL ANTIBODY VARIANTS

Dissertation

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Eingereicht von

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Zusammenfassung

Monoklonale Antikörper sind eine interessante und lukrative Klasse von Proteinen, die mittlerweile einen festen Bestandteil der biopharmazeutischen Industrie darstellen. Die Mikroheterogenität ist eine inhärente Eigenschaft dieser Klasse von therapeutischen Proteinen und entsteht durch unterschiedliche posttranslationale Modifikation und chemische Modifizierungen bei der Herstellung. Die Erforschung dieser Mikroheterogenität gestaltet sich äußerst schwierig bedingt durch die schiere Anzahl dieser Varianten in einer typischen monoklonalen Antikörper Formulierung. Ionenaustauschchromatographie ist die Standardmethode für die Auftrennung von Biomolekülen, basierend auf Unterschieden in der Oberflächenladungsverteilung. Es wurde eine neuartige pH-Gradientenelutionsmethode für Kationenaustausch-chromatographie entwickelt, welche eine verbesserte Auftrennung von Immunglobulin G Ladungsvarianten ermöglicht. Ursprünglich als analytische Methode konzipiert, konnte das Verfahren allerdings auch in größerem Maßstab, für präparative Anwendungen umgesetzt werden. Die Möglichkeit Immunglobulin G Isoformen im großen Maßstab aufzutrennen, erlaubt die tiefgreifende Untersuchung der Eigenschaften von Immunglobulin G Varianten und die Analyse der Effekte von Mikroheterogenität.

Abstract

Monoclonal antibodies are an interesting and lucrative class of protein, that has gained big importance in the biopharmaceutical industry. The microheterogeneity inherent in this class of therapeutic proteins stems from differential post-translational processing and chemical modifications during production. Due to the large amount of variants present in a typical monoclonal antibody formulation, the study of this microheterogeneity is challenging. Ion exchange chromatography is a trusted and powerful tool for the separation of biomolecules based on the surface charge distribution. A novel pH gradient elution method for cation-exchange chromatography was developed. This method shows an improved separation of immunoglobulin G charge variants, compared to other chromatographic methods. The method was initially developed as an analytical tool, but can be scaled up for preparative applications. Being able to separate immunoglobulin G isoforms in a large scale enables the in-depth study of the characteristics of immunoglobulin G variants and the analysis of the effects of microheterogeneity.

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I would like to express my deepest gratitude to my advisors, Alois Jungbauer and Muriel Bardor for their help and giving me this opportunity. Their continued support and scientific advice helped me achieve my goals.

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1 Introduction

1.1 Monoclonal antibodies

MAbs have become an incredibly useful and versatile recombinant therapeutic glycoprotein. Due to their ability to bind to a large variety of antigens and to interact with the human immune system, they have become an important molecule in the biopharmaceutical industry [1]. In humans five isotypes of antibodies have been identified (IgA, IgD, IgE, IgG, IgM), which are used by the adaptive immune system. The immunoglobulin G (IgG) isotype has been the most successful to be used as a biopharmaceutical [1]. IgG consists of two heavy chain and two light chain sub units, covalently linked with disulfide bonds. With a molecular mass of 150,000 Da it is a rather large protein [1]. The structure of an IgG molecule is shown in Figure 1.

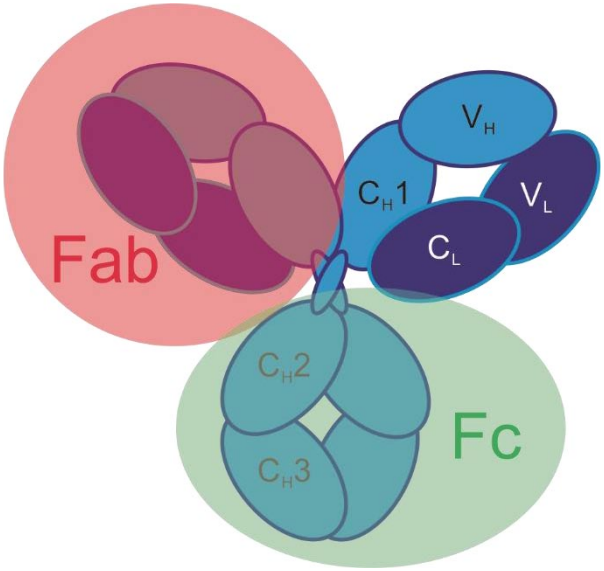

Figure 1: Schematic cartoon of an immunoglobulin G. Light blue denotes the heavy chain and dark blue the light chain. The heavy chain consists of four domains, three constant domains: C_H1, C_H2, C_H3, and a variable domain V_H. The light chain consists of two domains: a constant domain C_L and a variable domain V_L. The Fc (Fragment, crystallizable) region consists of the C_H2 and C_H3 domains of both heavy chains. It is marked by the green area. The Fab (fragment, antigen-binding) region consists of the C_H1 and the V_H domain of one heavy chain and the C_L and the V_L domain of one light chain. It is marked by the red area and there are two Fab regions per IgG.

IgG is an important part of the adaptive immune system and is produced by plasma cells. The binding of the antibody to its antigen can either neutralize the target by binding or help to identify foreign entities, which can then be targeted by the immune system. The antigen is targeted through the complementarity-determining region

(CDR) three of which are located on each V_H and V_L domain. Two major pathways exist, through which antibodies can elicit the destruction of targeted cells: the complement dependent cytotoxicity (CDC) and the antibody dependent cellular cytotoxicity (ADCC) pathways. After binding to an antigen the Fc region of an IgG can bind to C1q of the complement system and start the recruitment of the membrane attack complex or it can bind to Fcγ receptors, e.g. FcγRIII, which are located on natural killer cells and other cells of the immune system. The Fc region can also bind the neonatal Fc receptor (FcRn) of endothelial cells through which the IgG is internalized and released at a later point. This greatly increases the serum half-life of IgG.

In the biopharmaceutical industry, the product is still often defined by the process [2], which means that changes in the process lead to a new product. Under the quality by design (QbD) framework proposed by the US FDA, the influence of the process on the product should be sufficiently transparent, so that changes in the process become possible, without changing the product. In order to realize this idea, the critical quality attributes (CQA) have to be defined and correlated with the process parameters [3, 4]. This requires extensive knowledge on microheterogeneity of the therapeutic protein, for which high resolution analytical tools are required [4]. For mAb, this is a challenging task, due to the sheer number of variants present, due to the large size of the molecule. Being able to separate and identify variants, and gain insightful knowledge on CQA is not only of importance under the QbD framework, but is of immense advantage in the development of a biosimilar.

In this work, microheterogeneity is defined as slight differences in the structure of essentially identical proteins. This is due to the presence of: protein variants, which exhibit identical amino acid sequences, but different posttranslational or chemical modifications; glycoforms, which only exhibit differences in the saccharide portion; and isoforms, which show minor differences in the amino acid sequence due to point mutations. Some of these variants, glycoforms and isoforms can exhibit charge heterogeneity, in which case they are charge variants.

Microheterogeneity of antibodies is an active field of research. The sources of antibody microheterogeneity are manifold. The modifications are post-translational and can be classified as either enzymatic or non-enzymatic, but can also be created at a later point of the production process of a therapeutic mAb [5]. The formation of disulfide bonds, glycosylation, glycation, *C*-terminal lysine processing, N-terminal pyroglutamate formation from glutamine or glutamic acid, deamidation of asparagine and glutamine, oxidation and other modifications can all result in a heterogeneous population of antibodies, produced from the same sequence in the same cell line [5, 6]. This heterogeneity has first been observed by isoelectric focusing in 1970 by Awdeh, *et al.* [7] and has since been studied in detail [8-27].

While the sources of microheterogeneity are often known, the elucidation of its effects was hardly successful. Khawli *et al.* [17] set out to quantify the effects of charge heterogeneity on some selected effector functions and pharmacokinetics, but no general rules for how certain modifications affect antibodies have emerged since.

1.2 Microheterogeneity

A main source of microheterogeneity of IgG is a conserved *N*-glycosylation site at Asn-297 in the C_H2 domain of the heavy chain. This results in two *N*-glycosylation sites per IgG. Unlike many other glycoproteins, the glycan is facing inwards toward a pocket between the C_H2 and the C_H3 domain (Figure 2), which seems to limit the extent to which the cellular glycosylation apparatus can modify the *N*-glycan [28, 29]. Only bi-antennary structures can be found in IgG, the largest possible *N*-glycan is shown in Figure 3. The glycosylation is very important for the correct function of the protein in vivo and influences half-life and effector functions. A lot is known about the effects of glycosylation on the function of mAbs [30-42]. The key findings are that *N*-glycosylation is necessary for the FcRn pathway and different glycosylation patterns affect the binding to different receptors of the complement system and of cells of the immune system, e.g. bisecting *N*-acetyl glucosamine or lack of core fucose can increase ADCC, galactosylation can increase CDC [29, 42-44]. It seems to be the case that the sugars elicit slight changes of the conformation of the Fc region of the IgG, which in turn affects the binding to receptors [45]. Most *N*-glycans found on IgG carry

no charge, but addition of *N*-acetylneuraminic acid can change the isoelectric point (pI) of the whole glycoprotein.

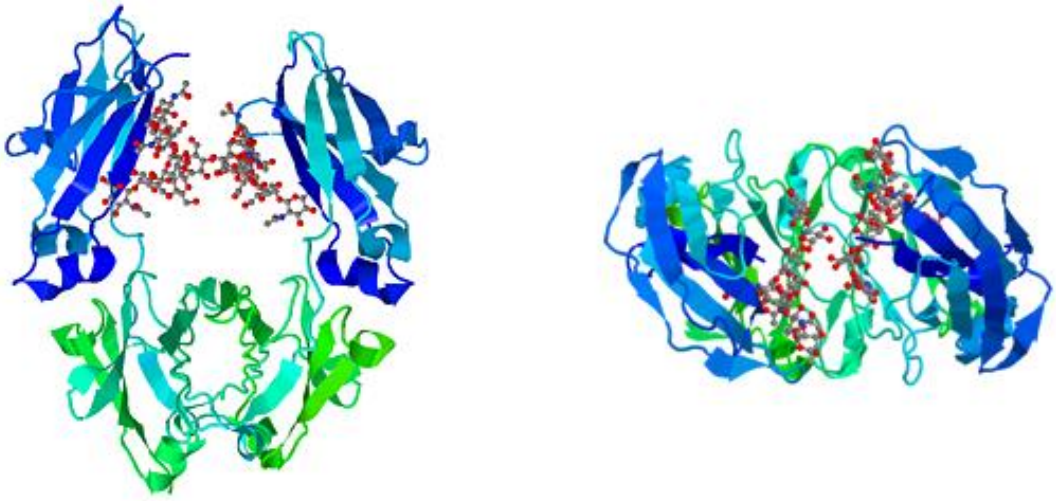

Figure 2: Crystal structure of Fc fragment, seen from two angles. The N-*glycan (stick and ball model) is facing towards the inside of the protein (rainbow ribbon model). From RCSB PDB: human IgG1 Fc fragment Heterodimer 4DZ8 [28].*

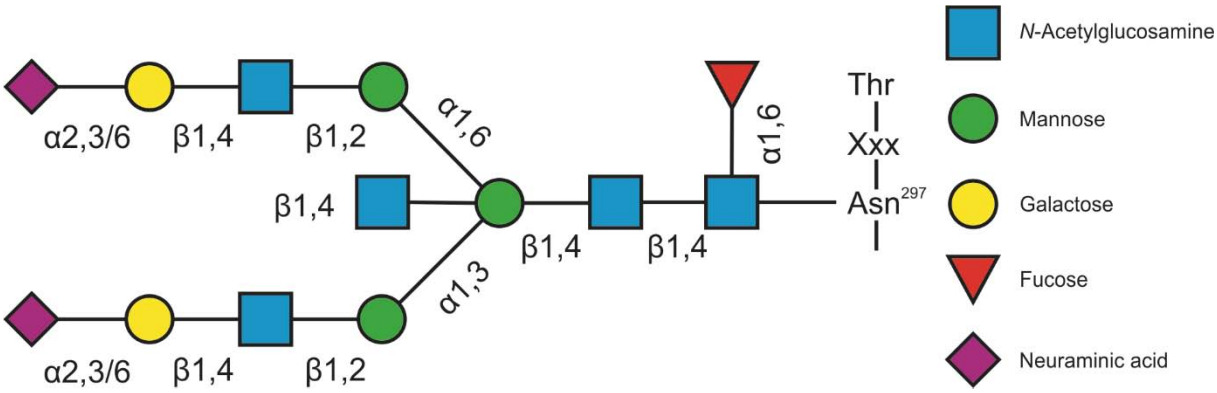

Figure 3: The largest N-*glycan found in human IgG.*

While glycosylation is generally restricted to the conserved Asn-297, there are reports of IgG with additional non-canonical *N*-glycosylation sites [31, 46]. These glycans can influence antigen binding if they are located close to the CDR region and can feature tetra-antennary glycosylation patterns. Foreign glycosylation can be the source of immunogenicity, which can occur when producing in mouse derived cell lines [4, 47, 48]. Some patients had severe anaphylactic reactions to cetuximab, produced in the murine NSO cell line. This antibody presented the gal epitope, i.e. galactose α(1-3) linked to galactose, an epitope not present in human glycoproteins, and some patients

primed to this epitope due to environmental exposure showed severe adverse reactions to an IgE response of their immune system. Murine cell lines, such as NSO and Sp2/0 can present immunogenic glycans such as the gal epitope and *N*-glycolylneuraminic acid [48]. Such aberrant glycoforms have to be avoided, to avoid severe adverse reactions. Antibodies produced in CHO or in glycoengineered plant cells feature human like glycoforms, which is important both for safety and efficacy of therapeutics [49-54].

Glycation is a non-enzymatic reaction between amines of the N-terminus or lysine and reducing sugars. This reaction occurs both *in vivo* and *in vitro* but seems to have a low impact on the function of antibodies [5, 55]. Due to the masking of amines after glycation, the pI of the antibody shifts towards the acidic.

Oxidation of methionine side chains can occur under oxidizing environments, at elevated temperatures or after prolonged storage. The oxidation of methionine to the sulfoxide increases the polarity of the side chain [5]. Even though methionine oxidation should not affect the pI of an antibody, Chumsae *et al.* have reported differential binding of oxidized IgG_1 in cation exchange chromatography, which is most likely due to conformational changes [56]. Stracke *et al.* have shown altered binding behavior of oxidized IgG to FcRn [57]. Oxidation of tryptophan has also been observed, but occurs rarely compared to the oxidation of methionine [58].

C-terminal lysine processing is the most common post-translational modification found in monoclonal IgG [5, 59]. The heavy chain gene codes for this lysine, but is removed either partially or completely by carboxypeptidases. The shift in pI is the only observable effect on the antibody, and there is evidence that C-terminal lysine processing even takes place in the serum after injection [60, 61].

N-terminal pyroglutamate formation is another common post-translational modification, which has similar negligible effects [5]. It changes the pI of the antibody by removing a primary amine, but otherwise seems to play no role for the function [59].

Incomplete removal of the signal peptide from the N-terminus has been reported by Meert *et al.* [18]. No measurable effects of the signal peptide have been observed so far [59].

Deamidation is another common modification found in mAb. The amino acid sequence asparagine – glycine is most susceptible and results in the formation of aspartate and iso-aspartate [5]. Due to its high frequency and the importance that asparagine can play both for antigen and receptor binding, deamidation can have deleterious effects on antibody function [62]. The reaction is influenced by many factors including pH, temperature and buffer composition, with elevated temperatures and alkaline pH greatly increasing the rate of deamidation [63, 64]. Deamidation of glutamine occurs at a much slower rate and as such is found far less frequently [6]. Deamidation always results in a change of the pI of the antibody.

Iso-aspartate can also be formed by the direct isomerization of aspartate, which leads to changes in the pI of the antibody and can induce conformational changes, due to the introduction of a methyl group to the peptide backbone [5]. As with deamidation, this can have detrimental effects on the function of the antibody [65]

Disulfide bonds can also be the source of isoforms, although this affects mostly the IgG2 subtype, which is utilized far less as a biopharmaceutical [16, 66-68].

Very harsh conditions such as extreme pH values or elevated temperatures can also induce peptide bond cleavage in the hinge region or between the C_H2-C_H3 domains [69]. During a standard downstream process for mAb purification a low pH step for virus inactivation is often used, which can lead to the creation of fragments. These fragments, with varying pI and size, have to be removed in subsequent polishing steps.

The intra and inter chain disulfide bonds in IgG are susceptible to forming non-reducible covalent bonds [69]. This cross-linking might be a common phenomenon in IgG, with very little effect on structure and function [5].

IgG is not a symmetrical protein, as a consequence the same modification sequence wise can have a different effect depending on which light or heavy chain is affected.

Aggregation is commonly observed in all types of antibodies, just as most other proteins [5]. Misfolded or partially unfolded proteins and temporarily unfolded areas of proteins can aggregate due to interactions of hydrophobic regions. Conditions such as elevated temperatures and extreme pH values can favor this rearrangement. High titer cell cultures can exhibit environments that induce aggregation, e.g. large amount of improperly folded proteins, high concentrations. Improvement of the storage buffer composition can reduce aggregation and other sources of heterogeneity [70]. Aggregates contribute to size heterogeneity and are routinely monitored using size exclusion chromatography.

All of these modifications can have some effect on antibodies, which lead to microheterogeneity being defined as a CQA under the QbD initiative of the US FDA [3].

1.3 Chromatography

Liquid chromatography is a standard method both in the downstream processing [71] and the analysis of antibodies [27]. The downstream processing of antibodies has become standardized, following a common pattern [71]. Protein A chromatography is most often used for the capture of the antibody from the clarified cell culture supernatant. Protein A from *Staphylococcus aureus* has the ability to bind the Fc region of antibodies, which makes it incredibly useful in affinity chromatography. The capture step is followed by up to three polishing steps, which are generally a combination of anion exchange, cation exchange or hydrophobic interaction chromatography (AEX, CEX and HIC respectively). While protein A chromatography uses evolved affinity for binding, ion exchange and hydrophobic interaction chromatography utilize electrostatic or hydrophobic interactions for selective binding.

Ion exchange chromatography (IEC) uses strong or weak ionic groups immobilized on the stationary phase to bind proteins [72]. IgG generally have a pI in the alkaline range, owing to the conserved Fc region, which differentiates it from most host cell proteins, which have a pI below 7. This makes IEC a favorable unit operation for the polishing, and sometimes the capture, of mAb. Two different modes often find use as polishing steps. AEX is often used in flow-through mode, in which the target protein

has the same charge as the stationary phase and as such do not bind to the stationary phase, whereas impurities carrying an opposite charge are bound. CEX is generally used in a bind-elute fashion, in which the target protein is bound on the stationary phase and subsequently eluted. In order to bind a CEX column the protein needs to be positively charged, which proteins are at a pH below their pI. The elution is carried out by increasing the modifier concentration, NaCl is the preferred modifier in ion exchange chromatography, since it is cheap and safe to use. The modifier concentration can be increased in a linear gradient or in one or more step gradients [73].

A major advantage of IEC are the mild operating conditions, compared to other chromatographic methods, i.e. reversed phase chromatography. In IEC, aqueous solutions with a pH around neutral and no organic modifiers are routinely employed. This feature makes it very attractive for the use in the biopharmaceutical industry, since proteins are prone to denature under harsh conditions, which compromises their biological activity. IEC also boasts high resolving power, high binding capacities, a large theoretical body of knowledge and a plethora of different stationary phases offered by many manufacturers [74]. As mentioned before, elution usually takes place by increasing the NaCl concentration, which is a safe and cheap chemical that interacts well with virtually all proteins. This is usually done in a linear gradient fashion, which provides certain advantages over isocratic elution. By increasing the concentration of salt during the chromatographic experiment, two things are achieved: the elution of stronger retained proteins is sped up and the method becomes more robust compared to isocratic elution. The increase in robustness stems from surveying a variety of salt concentrations, making the elution time and the separation less dependent on the actual salt concentration in either one of the buffers. Alternatively, pH can be used as the modifier in IEC, and just as salt, the change in pH modifies the strength of the interaction between solute and stationary phase.

1.4 Steric mass action model

The equilibrium between protein adsorbed on the stationary phase and protein in solution is highly dependent on the modifier concentration [72]. The steric mass-action

model [75] can be used to describe the interaction between protein and stationary phase according to the following equation,

$$\log k' = \log(\beta K_s \Lambda^{\nu}) - \nu \log(Na^{+}) \quad (1)$$

where k' is the retention factor, K_s is the equilibrium of the protein with the counter-ion, Λ is the ion-exchange capacity of the stationary phase, ν is the number interacting sites between protein and stationary phase and Na^+ is the Na ion concentration. Figure 4 shows how the capacity factor changes with the salt concentration. The steric mass-action model is based on the mass action law [76, 77] and further refined the descriptive nature of the model by adding a factor accounting for steric shielding of charges on the stationary phase by the bound protein.

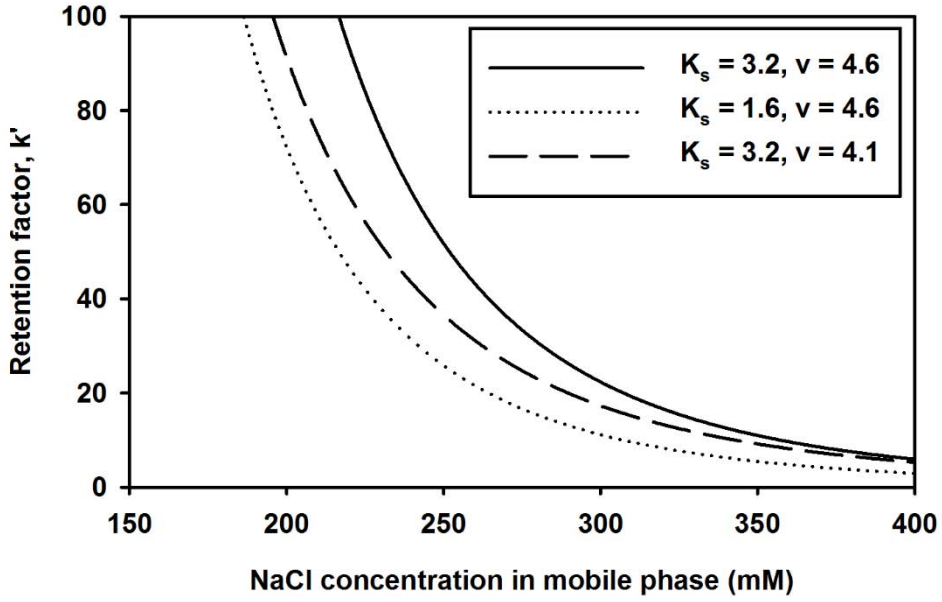

Figure 4: The relationship of the capacity factor and the mobile phase modifier concentration in ion exchange chromatography for three hypothetical antibody charge variants.

The sharp decrease of retention factor due to small increases in the modifier concentration allow for high resolution in IEC. It also follows from this, that isocratic elution of proteins is highly dependent on the exact composition of buffers, which is why linear gradient elution is a more robust method. By increasing the amount of modifier in a linear fashion, it is ensured that the elution window required for separation is met. In industrial processes, a compromise is made by using step gradients for elution, since the equipment for creating linear gradients is often not available.

1.5 Separation of charge variants

IEC is able to separate proteins and protein variants from each other, e.g. Harris [78] was able to separate variants of IgG_1 based on the presence of C-terminal lysine and tissue plasminogen activator based on the presence of C-terminal arginine. This separation was carried out using a salt gradient on a cation exchange column and was able to separate proteins based on the presence or absence of only one amino acid. Weitzhandler *et al.* [11] were able to separate cytochrome C from three different species, which are very similar in their primary structure, and their pI. The high resolving power of IEC can be exploited to separate variants of mAb based on their difference in charge distribution.

IEC salt gradient elution is therefore suitable for separation of charge variants of mAb. As shown earlier many modifications responsible for microheterogeneity in mAb microheterogeneity induce a difference in the pI of the antibody or in the charge distribution through conformational changes [5]. A method for the analytical or preparative separation of mAb charge variants is highly valuable in the study of the effects of charge heterogeneity on the function. Du *et al.* [79] pointed out the need for a preparative separation method in order to study the effect of charge heterogeneity. Teshima *et al.* [80] were successful in separating IgG charge variants in the single digit mg scale. They used AEX salt gradient elution at a neutral pH, a method that is not applicable for all mAb, due to their pI values in the range of 8-9. A protein generally binds to an AEX column at a pH above or close to its pI, which can be a problem for very basic proteins, as very high pH can denature proteins.

Another example for mAb charge variant separation is given by Melter *et al.* [81]. They employed a Dionex ProPacWCX-10 cation exchange column to separate C-terminal lysine variants of an IgG antibody using salt gradient elution. A lumped kinetic model was used to optimize the separation of charge variants at two different pH values. The stationary phase they chose is highly suitable for such difficult separations as it has a fast mass transfer compared to many other stationary phases. This is due to the fact that the ProPac packing material does not consist of porous beads, but instead is made up of non-porous particles on which a layer of polymer chains is grafted on [11]. This pellicular layer of polymer chains carry ion exchange

functional groups, which makes the paths for diffusive transport very short, compared to conventional porous media. The increase in separation power stems from the decreased peak spreading that is caused by mass transfer resistance [73]. This high rate of mass transport comes at the cost of surface area, which results in a much lower overall binding capacity. This problem is further amplified by the fact that Melter *et al.* are operating in the linear region of the adsorption isotherm, which means that such high resolution methods are only useful for analytical approaches with very low loadings [81, 82]. Guélat *et al.* were also successful in modelling the retention of mAb in cation exchange chromatography by using an adsorption equilibrium model [83]. They used the amino acid sequence and structural information about the stationary phase to predict the influence of ionic strength and pH on the retention time of mAb. The approach can be used for the separation of charge variants, but it suffers from the inaccuracy of the model used. The interaction between protein and chromatography surface as simplified as spheres and planes and the protein charge is calculated as a charge density on spheres, whereas the actual interaction depends on charge distribution. In order to improve such an approach, detailed structural information has to be known.

1.6 Displacement chromatography

Another mode of IEC is displacement chromatography, in which the proteins are eluted by a strong binding displacer. In general, the column is loaded with protein to a high degree, above 50% of the dynamic binding capacity, and subsequently a displacer is introduced in a step change at the column inlet. The displacer has a higher affinity to the stationary phase than the sample and the proteins are displaced into the mobile phase. At this point competitive binding between the proteins takes place while they travel down the column in an isotachic displacement train [75, 84]. The proteins in the displacement train will be separated by their affinity to the stationary phase, with the weakest binding protein eluting first and the strongest binding protein eluting just before the displacer. The proteins are separated into zones of pure proteins, bordered by zones containing mixtures.

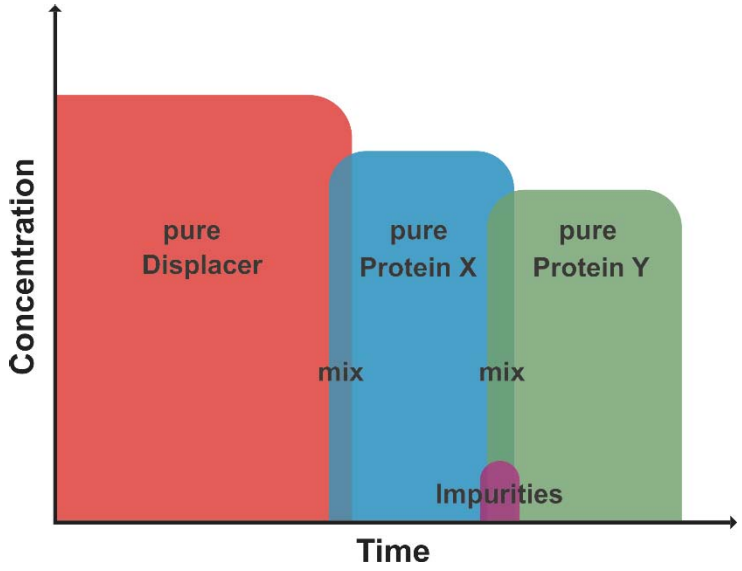

Figure 5: A schematic representation of an ideal displacement train. In this example protein, Y has a lower binding affinity than protein X, with the impurities having intermediate affinities. The weaker binding protein is eluted first, in a zone of pure protein, followed by a zone containing a mixture of both proteins and the impurities. Protein X elutes in a pure zone, followed by the displacer, again creating a mixed zone.

Zhang *et al.* have successfully used displacement chromatography to separate charge variants for one mAb at a hundreds of mg scale [85]. This method is highly useful for the study of mAb microheterogeneity, since they were able to separate ~100 mg of IgG charge variants in one experiment using a high performance anionic displacer, Sachem Expell SP1, for cation exchange chromatography. The CHO produced mAb they used in their work was of the IgG1 type, with a pI of 8.7, but otherwise undisclosed properties. Khawli *et al.* managed to purify even larger quantities of another CHO produced IgG1 antibody [17]. In their work, they managed to separate charge variants in the scale of 1 g, which is enough for a variety of analytical procedures, which allowed them to compare the pharmacokinetics of IgG charge variants.

Without a doubt, displacement chromatography is a very elegant method for the separation problem at hand, but the set up and optimization are not straightforward. Brooks and Cramer have suggested the use of the steric mass action (SMA) model to optimize separations in displacement chromatography [75]. This model builds on the stoichiometric displacement model, which is based on the mass-action law [77]. Simple isocratic or linear gradient elution experiments are enough to determine the protein parameters required for the SMA model. For the displacer, breakthrough experiments are required. With the knowledge of the protein, displacer and stationary

phase parameters the optimal displacement conditions can be determined. In order to optimize the displacement of mAb charge variants, the parameters of each or at least a number of charge variants have to be determined. This means that in order to use the SMA model to arrive at conditions for successful displacement chromatography, pure material of the charge variants is required. This poses a significant problem and hinders the implementation of displacement chromatography for the separation of mixtures, for which no pure substances are available. In summary, even though there are positive results in the literature, the separation does not seem to be consistent.

In our own experiments, we have observed limited separation between IgG charge variants. In the first experiments, the only separation achieved was between IgG and impurities as shown in Figure 6, and confirmed through Western blot. In this experiment, a micropellicular column with 5µm SCX particles and an aspect ratio of around 70 was used. The large aspect ratio allows enough time for a displacement train to develop. The mobile phase conditions were standard conditions taken from the supplier and from Zhang *et al.* [85].

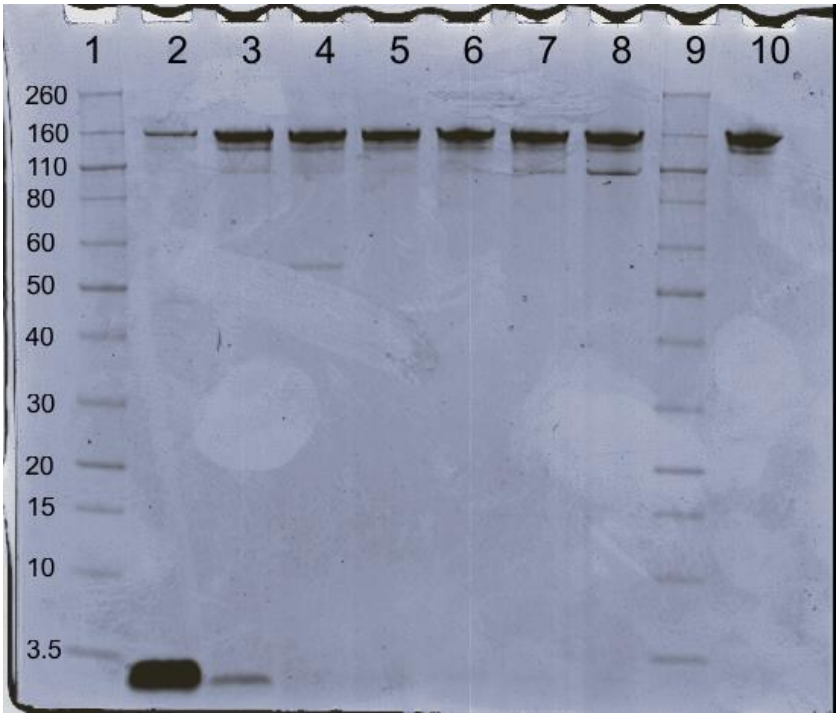

Figure 6: Non-reducing SDS-PAGE of displacement fractions. Stationary phase: Sepax SCX NP 5µm, 2.1 x 150 mm. Mobile phase: 30 mM MES, 10 mM NaCl, pH 6.1. Displacer: 5 mM Expell SP1. Sample IgG 84 at 50% of DBC. Lanes 1 and 9 are marker. Lanes 2-7 are displacement fractions in order of elution. Lane 8 is the original sample, IgG 84. Lane 10 is trastuzumab biosimilar. An early eluting impurity was detected in the first two fractions.

We hypothesized that the binding capacity of the core-shell particles we used was not sufficient to ensure the development of a displacement train, since the only separation we saw was between the impurity and the antibody. An IEF showed no differences in charge variant distribution between the fractions. We then decided to use a stationary phase with improved binding capacity, and chose the GE Mono S stationary phase. Figure 7 shows the separation possible with this stationary phase. The antibody used in these experiments, IgG 84, is an in house product of the Bioprocessing Technology Institute.

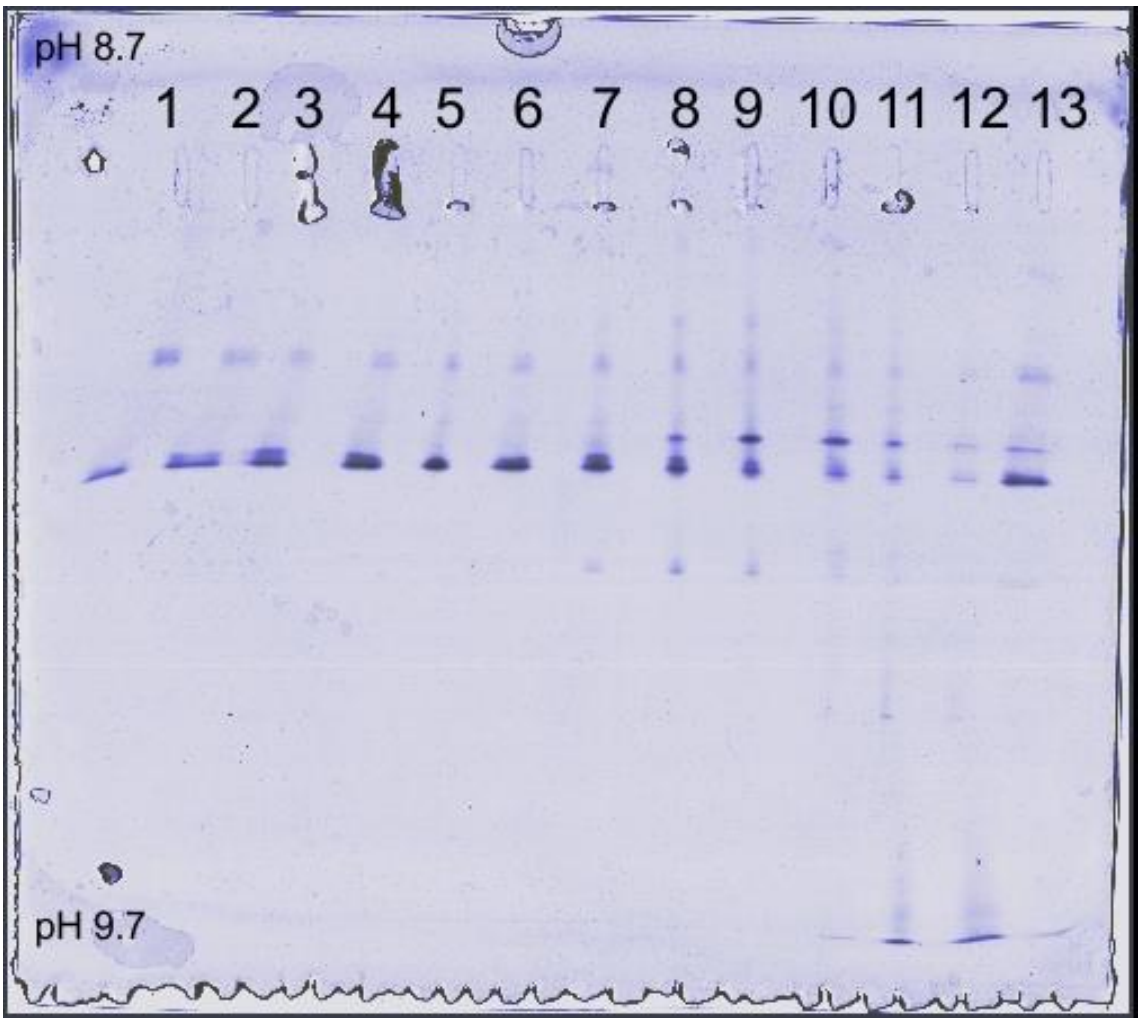

Figure 7: IEF of displacement fractions. Stationary phase: GE Mono S, 4.6 x 100 mm. Mobile phase: 40 mM MES, 10 mM NaCl, pH 5.5. Displacer: 5 mM Expell SP1. Sample IgG 84 at 55% of DBC. Lanes 1-12 are displacement fractions in order of elution. Lane 13 is the original sample, IgG 84. An increase in acidic variants in the later eluting fractions is apparent.

It was attempted to recreate the separation achieved with a different antibody, APN311, a chimeric IgG1 antibody provided by Apeiron Biologics. Interestingly the

initial experiments performed under similar experimental conditions provided no separation of charge variants, as shown in Figure 8.

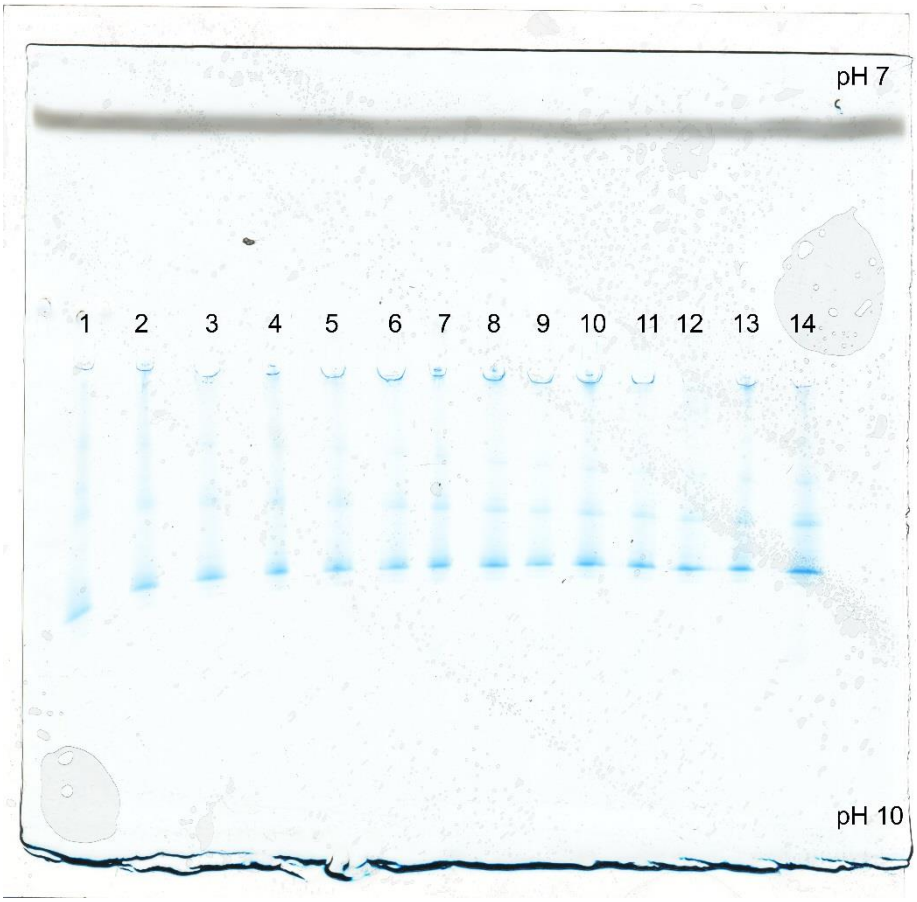

Figure 8: IEF of displacement fractions. Stationary phase: GE Mono S, 5 x 200 mm. Mobile phase: 30 mM MES, pH 6.1. Displacer: 5 mM Expell SP1. Sample APN311 at 50% of DBC. Lanes 1-13 are displacement fractions in order of elution. Lane 14 is the original sample, APN311. No separation of charge variants could be observed.

The optimization of the displacement separation is detailed in Martina Berndtsson's Master's thesis [86]. Isocratic elution experiments were performed at different pH values to find the SMA parameters, as shown in Table 1, and used to create an operating regime plot.

Table 1: The SMA parameters, characteristic charge and equilibrium constant, of the APN311 determined by isocratic elution experiments. Taken from [86].

pH	Characteristic charge, ν	Equilibrium constant, K
5.0	3.2	20
5.5	6.7	178
6.1	5.7	9.2
6.5	4.6	3.2
7.0	3.7	1.1

The operating regime plot was used to find improved conditions for displacement, but unfortunately, even those conditions did not show any separation of charge variants, as shown in Figure 9.

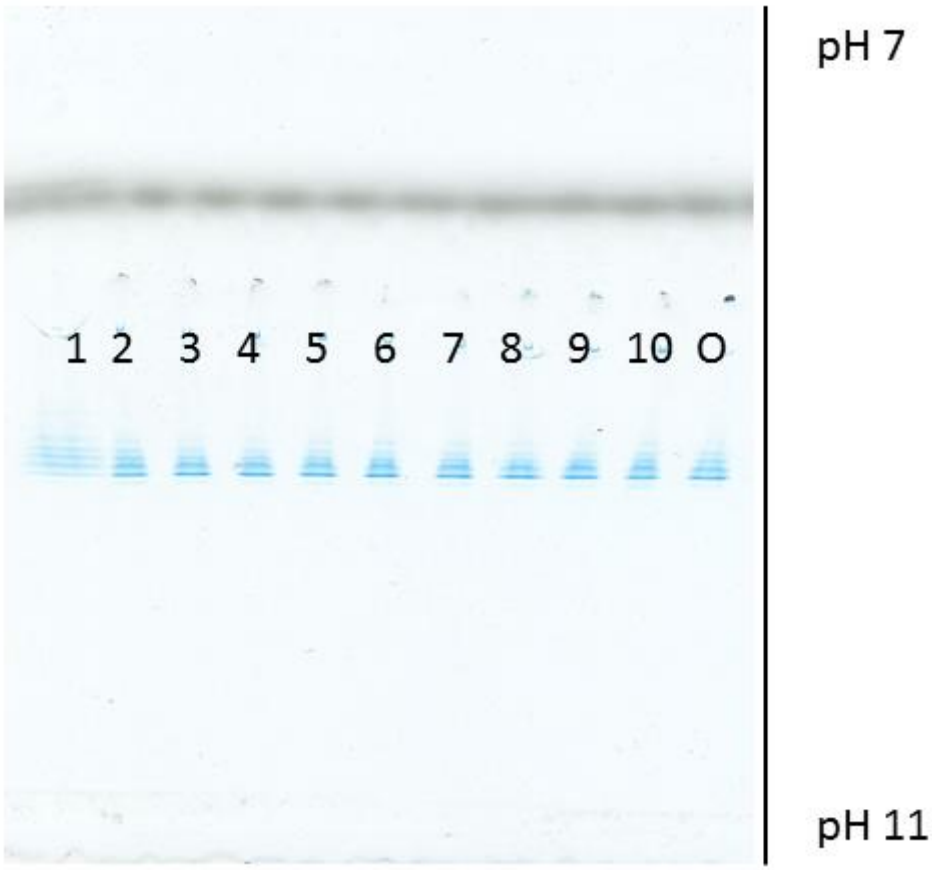

Figure 9: IEF of displacement fractions. Stationary phase: GE Mono S, 5 x 200 mm. Mobile phase: 30 mM MOPSO, 15 mM NaCl, pH 7.0. Displacer: 5 mM Expell SP1. Sample APN311 at 55% of DBC. Lanes 1-10 are displacement fractions in order of elution. Lane O is the original sample, APN311. No separation of charge variants could be observed.

While displacement chromatography can provide excellent separation as shown by others [17, 85], we found the performance to not be consistent and furthermore, dependent on the antibody used. In order to overcome the limitations of displacement chromatography we turned to linear gradients with a different modifier than salt.

1.7 pH gradients

Sluyterman was the first to describe the use of pH gradients for the elution of proteins on ion-exchange stationary phases [87-90]. The basic principle was to apply the separation known from isoelectric focusing (IEF) in a liquid chromatography setting. Sluyterman identified two basic strategies for creating a pH gradient in an ion-

exchange column, an external and an internal gradient. An external pH gradient is achieved by simply mixing two buffers in a gradient mixer and applying that gradient to the column inlet. An internal gradient on the other hand requires the interaction of the mobile phase with the buffering capacity of the stationary phase.

Table 2: An overview of publications dealing with pH gradient ion-exchange chromatography.

Author	Year	Methodology	Reference
Sluyterman	1978	Ampholyte driven internal gradients	[87, 88]
Sluyterman	1981	Induced gradients on weak ion-exchange resins	[89, 90]
Kaltenbrunner	1993	External gradients created with borate/mannitol	[91]
Bates	1998	Model based induced gradients	[92, 93]
Kang	2000	Model based induced gradients	[94-96]
Ahamed	2007	Long pH range external gradients for AEX	[97, 98]
Pabst	2007	Model based induced gradients	[99-101]
Tsonev	2008	Long pH range external gradients for IEX	[102, 103]
Ng	2009	Scale up of induced gradients for acidic proteins	[104]
Rozhkova	2009	Narrow range external gradients for mAb	[105]
Kröner	2013	Systematic generation of external pH gradients	[106, 107]
Zhang	2013	Non-linear external gradients for mAb	[108]
Vetter	2014	Mixed bed induced gradients	[109, 110]

A simple way of creating an induced pH gradient on an ion-exchange column is the use of ampholytes as the elution mobile phase [88]. Ampholytes are chemicals which buffer in a pH range close to their pI [111]. After applying the ampholyte mixture at the column inlet, the ampholytes are separated by their pI and due to their inherent buffering capacity at their pI, they will form a pH gradient in the column. This is analogous to the creation of a pH gradient in carrier ampholyte IEF. This method works as long as the column is equilibrated at a pH at one end of the pH spectrum of the ampholyte mixture and the pH of the mobile phase is adjusted to the other end of the spectrum. This method can provide surprisingly linear pH gradients, but the quality of the gradient is dependent on the composition of the ampholyte mixture. Since ampholytes are generated in a "chaotic synthesis" [111], it should not be surprising to

learn that there are large batch to batch variations in ampholytes. The amount of good ampholytes created in this chaotic synthesis also decreases with increasing pH, i.e. for ampholytes designed for the alkaline range, a smaller portion of the molecules found in the mixture will actually be good buffers around their pI. Moreover, ampholytes react with proteins with unknown consequences. This and other problems surrounding ampholyte pH gradients, also called chromatofocusing, make them a less than ideal method [99].

A more elegant approach to inducing a pH gradient in ion-exchange chromatography is to utilize the inherent buffering capacity of the stationary phase [88]. Weak ion-exchangers possess buffering capacities in pH ranges that are attractive for pH gradient elution, i.e. not too basic or too acidic. For a weak cation-exchanger, which is functionalized with carboxylic groups, this buffering capacity will generally be between pH 4 and 6. The functional groups will be protonated when the stationary phase is equilibrated with a mobile phase with a pH below the pK_a of the functional groups. By prompting a step change in the pH of the mobile phase, i.e. by switching to a buffer with a pH above the functional group pK_a, the stationary phase will be titrated [100]. By adapting the buffering capacity of the mobile phase to the buffering capacity of the stationary phase, the induced pH gradient can be modified [99]. In order to be useful for the separation of proteins, the stationary phase needs additional strong ion-exchange groups that stay in the ionic form, instead of being protonated, in order for them to bind proteins. This means that it is not possible to use most commercially available ion-exchange media, either a strong ion-exchange stationary phase has to be modified, e.g. by alkaline hydrolysis of the resin's backbone, or commercially available strong and weak ion-exchange media can be mixed [109]. This provides both the weak ion-exchange groups needed for the creation of the gradient and the strong ion-exchange groups required for protein binding.

As Sluyterman has observed in 1978 [87, 88], the Donnan potential plays an important role for the separation of biomolecules using pH gradients. Hindered diffusion of some ionic species due to electrostatic repulsion between them and immobilized ionic groups, causes an unequal distribution of ions in the mobile and the stationary phase in liquid ion-exchange chromatography. This relationship is mathematically described by

the Donnan potential. The resolution achievable in pH gradient elution is directly proportional to the Donnan potential, which can be increased by decreasing the ionic strength of the mobile phase, or increasing the buffering capacity of the stationary phase [26, 88].

The resolution is determined by peak separation and peak width. The peak width in pH units can be written as,

$$\Delta \mathrm{pH}^2 \approx \left[D * \left(d\mathrm{pH}/d\mathrm{V}\right)\right] / \left[\varphi * \left(d\mathrm{pH}/d\mathrm{V}\right)\right] \quad (2)$$

where D is the protein diffusion coefficient, *d*pH/*d*V is the pH gradient slope and φ is the dimensionless Donnan potential [26]. Therefore, a stationary phase with a high ionic capacity and a mobile phase with a low ionic strength is beneficial for high resolution in pH gradient elution of proteins in ion-exchange chromatography.

One advantage of induced pH gradients over salt gradients for the elution of proteins is the high peak compression that is inherent in induced pH gradient elution. Peak compression is generally observed when steep gradients are used in salt gradient elution [73]. The compression effect stems from the difference in chromatographic velocity that an analyte experiences at the front of the peak versus the back of the peak. At steep gradients, there will be a considerable difference in salt concentration through the length of a column, and an analyte peak will not be exposed to one discrete salt concentration, but instead will experience an axial salt gradient. Since the chromatographic velocity is a function of the modifier concentration, it follows that there exists a velocity gradient through the analyte peak as well. This results in the front of the peak being slowed down, relative to the center, whereas the tailing end of the peak is travelling at a faster chromatographic velocity than the peak average. This axial modifier and the resulting chromatographic velocity gradient, creates the peak compression effect.

In pH gradient elution, the change in pH acts as the modifier, and akin to salt gradient elution there also exists an axial modifier gradient [74]. Due to the peculiar fashion in which the pH gradient is induced, the temporal pH gradient that can be measured at the column outlet, also exists as an axial gradient in the column [87]. As pointed out earlier, instead of changing the modifier concentration by combining two buffers in a

gradient mixer, a pH gradient is induced by switching to a different buffer altogether, that then proceeds to titrate the stationary phases buffering groups. This results in an immediate pH change at the column inlet, and a delayed response at the column outlet. The consequence of this behavior is a steep axial gradient even when a shallow temporal gradient is used. This results in a focusing of the analyte peaks, driven by the same mechanism that creates peak compression in steep salt gradient elution.

Giorgio Carta's group were successful in using induced pH gradients for the separation of proteins in CEX [99] and AEX [101]. For these separations, they used modified chromatography media with additional functional groups that contained the buffering capacity required for the induced pH gradients. While they were even able to separate mAb charge variants, with this approach, the fact that it requires special stationary phases is a drawback. Recently they introduced a new method of providing the functional groups required for buffering and for binding, by using columns packed with two types of media; a weak ion-exchange resin with small pores for creating the gradients and a strong ion-exchange resin with larger pores to add the binding capacity [109, 110]. While induced pH gradients offer an inherent focusing effect, their main drawback is the non-linearity of the resulting gradient, which is most often concave [104]. This results in uneven gradient slopes over the gradient range, which means that not it is not equally suitable for separation problems. Moreover, the shape of the created gradient depends on the stationary phase used and as such cannot be modified easily.

In order to avoid the restrictions and issues surrounding induced, or internal, pH gradients a different approach has to be employed. External gradients, as mentioned earlier, are not dependent on the buffering capacity of the stationary phase, but also do not offer the inherent focusing effect associated with the steep axial gradients of internal gradients.

Schmidt *et al.* succeeded in modelling the elution behavior of proteins under salt and pH gradient elution, by using the stoichiometric displacement model combined with Yamamoto's linear gradient elution model [74, 112]. Lysozyme and IgG were used as model proteins and Interesting differences were found in the binding behavior. For

Lysozyme, about 50% of the total charges of the protein are interacting with the stationary phase, which is plausible considering the small size and the globular shape of the protein. For IgG only about 15% of the net charge is involved in binding, mostly due to the large mass and the smaller contact surface area. They further investigated the elution behavior of IgG charge variants in salt and pH gradient elution. Differences in the distribution coefficients between the charge variants were large at higher pH values, possibly explaining the increased resolution observed in pH gradient elution [74].

Considering that, pH gradients are more suitable for the elucidation of antibody microheterogeneity and internal pH gradients having various limitations that limit their usefulness, what other pH gradient methods are available? Already in 1993 external pH gradient elution was performed in this lab [91]. Kaltenbrunner *et al.* used a chemical reaction to release H_3O^+ from the reaction of mannitol with borate to create a linear pH gradient. While this creates a pH gradient of good linearity and can be used in an analytical and preparative scale, relying on this chemical reaction limits the use to cation exchange chromatography. Borate may also react with the glycans present on the mAb, forming stable complexes with the protein of interest, which is problematic if the protein is to be recovered and analyzed further.

Other attempts were made to create controlled pH gradients with simple buffer compounds. Kang and Frey used mixtures of simple, low molecular weight buffers, such as citric acid and phosphates to create linear pH gradients spanning several pH units [96]. The resulting pH gradients are comparable in linearity to those created by using ampholytes as the mobile phase, making them a worthwhile replacement for chromatofocusing.

In 2008 Tsonev and Hirsh introduced a new method of pH gradient elution for both modes of ion-exchange chromatography [102]. The pH gradients span 10 pH units, from pH 2 to 12, enabling the elution of a wide variety of proteins. The method relies on using a mobile phase consistent of anionic and cationic buffering compounds with pK_a values evenly spread across the pH range of the gradient. In total only five buffering compounds are used and the resulting pH gradients are not linear. In order to

overcome this issue, an algorithm is used to correct for the non-linearity in the gradient, by adjusting the actual gradient that the pumps of the liquid chromatography system are administering. This results in very complicated methods, instead of a linear increase of mobile phase ratios. The issue is further complicated by using both anionic and cationic buffering compounds on both anion and cation exchangers. The obvious drawback is further deviation from linearity by interaction of the buffering compounds and the stationary phase functional groups, which results in partial retention of the mobile phase. This results in changes of the buffer concentrations and therefore changes in the buffering capacity. Unsurprisingly, even though an intricate gradient delivery method has to be programmed, the algorithm employed cannot also correct for the deviations in buffering capacity caused by the interaction of the mobile phase with the chromatographic media. While Tsonev and Hirsh's approach was a big step away from traditional chromatofocusing by using defined buffers, the method itself was still lackluster due to the non-linear gradients.

Dell Farnan was involved in a number of publications dealing with pH gradient elution of mAb charge variants [15, 19, 108]. By using a mixture of three cationic buffer substances, namely piperazine, imidazole and Tris, with pK_a values of 9.8, 7.1 and 8.1 respectively, it is possible to maintain a relatively stable buffering capacity over a pH range from ~6 to 9.5 [15]. By varying the relative amounts of buffer compounds used, it is possible to influence the shape of the resulting pH gradient. The method was developed for the routine analysis of charge heterogeneity of a group of mAb, as a replacement of salt gradient elution. The biggest advantage is the not need of optimizing the method for each antibody, as in the case of salt gradient elution were stationary phase, mobile phase pH and additives and gradient conditions have to be optimized for each product [15]. Other researchers have presented similar pH gradient methods with varying pH ranges [14, 97, 105].

No thorough investigation of the fundamental reasons for the non-linearity of the previously discussed external pH gradients has been performed until 2013, when Kröner and Hubbuch published a theoretical framework that offers an in depth treatment of the requirements for linearity [106]. Firstly, they correctly identified one of the main problems of many previously presented methods, namely utilizing

buffering compounds carrying the opposite charge of the stationary phase in their ionic state. This obviously leads to interaction between the mobile and the stationary phase, resulting in retention of some buffering compounds depending on their charge state, distorting the desired concentration profile.

Secondly, and most importantly they recognized that the linearity of an external pH gradient depends solely on the buffer capacity of the mobile phase over the gradient pH range, if the buffering compounds are unretained [106]. They identified an abundance of commercially available cationic, anionic and zwitterionic buffering compounds with pK_a values from 2 to 11 for the use in either cation- or anion-exchange chromatography. By using an algorithm that assumes additive buffering capacity of all ions in a solution, they were able to predict two buffer systems for both modes of ion-exchange chromatography. A pH 4.0 to 11.0 gradient using anionic or zwitterionic buffers for the use in cation-exchange chromatography and a pH 10.5 to 3.5 gradient using basic buffers for the use in anionic-exchange chromatography. The resulting pH gradients were exceptionally linear, quantified by a coefficient of correlation $R^2 > 0.99$ and could be used for the elution of proteins in ion-exchange chromatography.

Chromatographic ion-exchange methods, based on either pH gradient or salt gradient elution or displacement are able to separate mAb charge variants. While displacement is a highly efficient chromatographic mode, the practical implementation of the method can be tricky. Elution chromatography is straightforward and the literature in the recent years has confirmed that pH gradients are superior for the elution of mAb charge variants. Various methods exist for the creation of pH gradients, each with its own set of advantages and disadvantages. Cation exchange pH gradient methods can be used on an analytical scale for the determination of the chromatographic pI, based on the surface charge distribution, and for the quantification of charge heterogeneity and on a preparative scale for the separation and purification of mAb charge variants for the study mAb microheterogeneity.

1.8 Alternative methods

For the analysis of antibody charge heterogeneity, a few gel-based methods are also available. They can be useful as orthogonal methods, but can only be operated at an analytical scale, making them ineffective for the preparation of charge variants for further analysis [27, 113]. IEF can be performed using two different methods to create a pH gradient in the polyacrylamide gel, either using ampholytes or immobilizing a pH gradient using acrylamide with buffering side chains [111, 114, 115]. The immobilized pH gradient method is far superior, providing stable gradients over a large range of pH values, but both methods are generally used for proteins with a pI below seven.

Capillary zone electrophoresis can be coupled to mass spectrometry detectors and has been used somewhat successfully for the analysis of mAb variants, but cannot be considered a method of choice [16, 20, 24, 116].

Capillary IEF is a powerful analytical method that is analogous to gel based IEF. Excellent resolution and repeatability makes this method very useful for the standard monitoring of mAb charge heterogeneity. Different approaches for detection are available were the analyte is either eluted and detected in a one or two step process or a transparent capillary can be used in so called imaging capillary IEF [18, 22, 117].

A main difference in the determination of pI values by electrophoretic methods and chromatographic methods is based on the separation mode. While the analyte travels to a pH region in which its net charge is zero, which corresponds to the true pI, a chromatographic method will only be able to measure the surface charge of an analyte. Large deviations between pI values have been observed for many proteins [106], so a pI measured chromatographically should be labeled as such.

While most of these electrophoretic methods offer high-resolution separations, they cannot be scaled up to preparative scale, a large drawback compared to a chromatographic method.

2 Objective

The objective of this doctoral thesis was the development of a high-resolution method for the separation of mAb variants. The method should not only be usable at analytical scales, but should be scalable to preparative and even industrial scale, to allow not only the generation of material for further analysis but to improve the possibilities in the downstream processing of mAb. Conceptually it should be based on ion-exchange chromatography, due to its low cost and ease of implementation. The method should be able to resolve isoforms based on the various chemical modifications found on mAbs, i.e. glycosylation, deamidation and others. The method should be evaluated with different IgG, produced in Chinese hamster ovary cells. For sample analysis, an electrophoretic method for the identification of charge variants should be developed that is viable for proteins with an alkaline pI.

3 Conclusion

In a review article, the influence of one source of mAb microheterogeneity is reviewed, namely glycosylation. The effect of differential glycosylation on three biopharmaceuticals, among them IgG, is outlined. Furthermore, various state of the art strategies for glycan analysis are presented and discussed. It should be apparent that glycosylation is a big influence on the efficacy of a therapeutical mAb, and therefore should be controlled and monitored. Glycosylation being a big part of the microheterogeneity observed in monoclonal antibodies, it was important to review the literature of the current state of glycobiology of biopharmaceuticals.

A pH gradient cation exchange chromatography method for the separation of IgG charge variants was developed. The work was focused on the IgG isotype, since it is the clearly the dominant antibody variant in the pharmaceutical industry. Cation exchange chromatography media were chosen as the stationary phase due to their ubiquity in mAb downstream processes and analytical applications. A linear pH gradient was chosen as the driving force for elution, as it offers a higher selectivity for IgG charge variants than salt gradients. The methods for creating pH gradients in ion-exchange chromatography found in the literature offered only unsatisfying gradients. A new method to creating external pH gradients for ion-exchange chromatography was developed, based on keeping the buffering capacity constant. This approach was subsequently confirmed by Kröner and Hubbuch's theoretical framework [106]. In detail, it was observed that other groups have tried using buffer compounds with pK_a values covering the intended pH gradient range, but their concentrations were generally just kept the same for all compounds. This results in increased buffering capacity close to the pK_a of the buffers, and valleys of buffering capacity between two buffer compounds. This deviation in buffering capacity creates concave and convex pH gradients when the mobile phases are mixed in a gradient mixer, the gradient becomes steeper at a lower buffering capacity and shallower when the buffering capacity increases. In order to counter act this behavior, it was hypothesized that using a simple equation to calculate the total buffering capacity of the solution at discrete pH

values along the gradient based on the assumption that buffering capacities behave in an additive fashion, as shown in the equation below,

$$\beta_t = \sum_{i=1}^{n} \beta_i \quad (3)$$

where β_t is the total buffering capacity and β_i the buffering capacity of each buffering compound. This additive effect of buffering capacities should hold true at low concentrations.

The buffering capacity of each buffering substance i, can in turn be calculated with the following equation,

$$\beta_i = \frac{d\mathrm{B}^+}{d\mathrm{pH}} = \ln(10) \times \frac{C_i K_{Ai} [H^+]}{(K_{Ai} + [H^+])^2} \quad (4)$$

where $d\mathrm{B}^+$ is the infinitesimal amount of base added, dpH is the resulting change in pH, C_i is the concentration of buffering compound i, and K_{Ai} is the dissociation constant of compound i. After accounting for all buffers, the inherent buffering capacity of water has to be taken into consideration, which is important at very low or high pH.

In publication I, these concepts are presented and it is experimentally confirmed that a buffer system with a constant buffering capacity, creates a more linear pH gradient. It is also shown that, for the separation of IgG charge variants, a more linear pH gradient gives a higher resolution separation. The performance of pH gradient elution is also compared to conventional NaCl gradient elution, using the same stationary phase. In this direct comparison, the pH gradient method provided almost double the peak capacity of the conventional method. This improvement comes only at the cost of using a more complex, and therefore, more expensive mobile phase. Figure 10 and Figure 11 show the difference in resolution achieved between linear salt and pH gradients. Is difference in resolution is mostly due to the higher selectivity at higher pH values and due to the high Donnan potential.

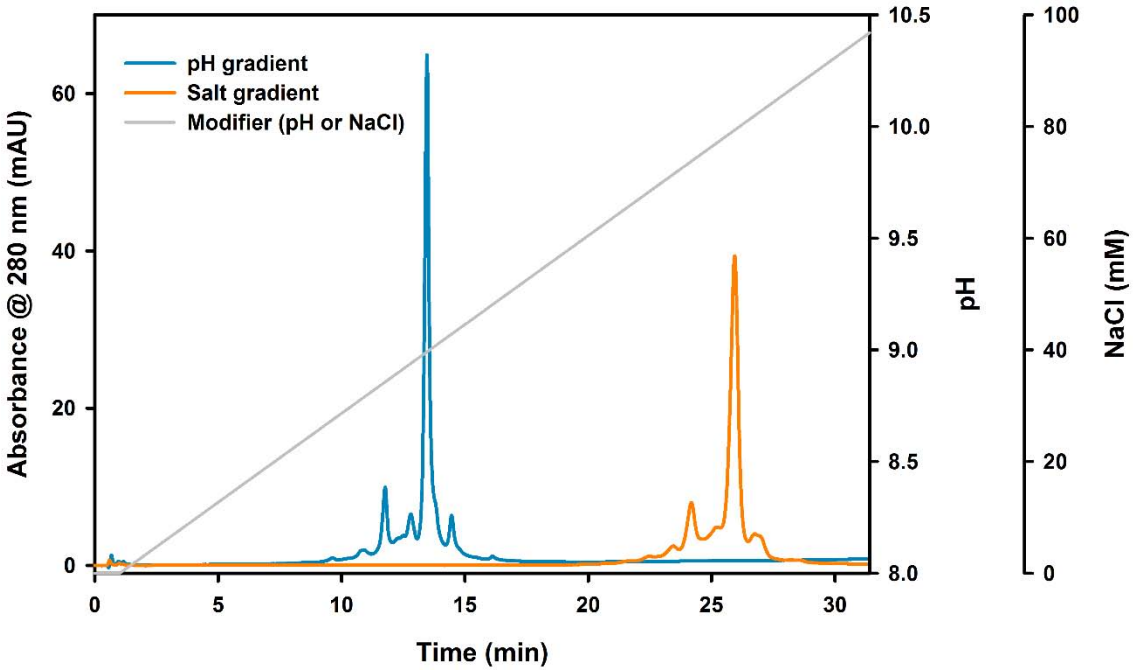

Figure 10: Analytical scale linear salt and pH gradient CEX with a trastuzumab biosimilar antibody. These chromatograms are from the experiments presented in publication I. The pH and NaCl values are estimated as no pH or conductivity sensors were available.

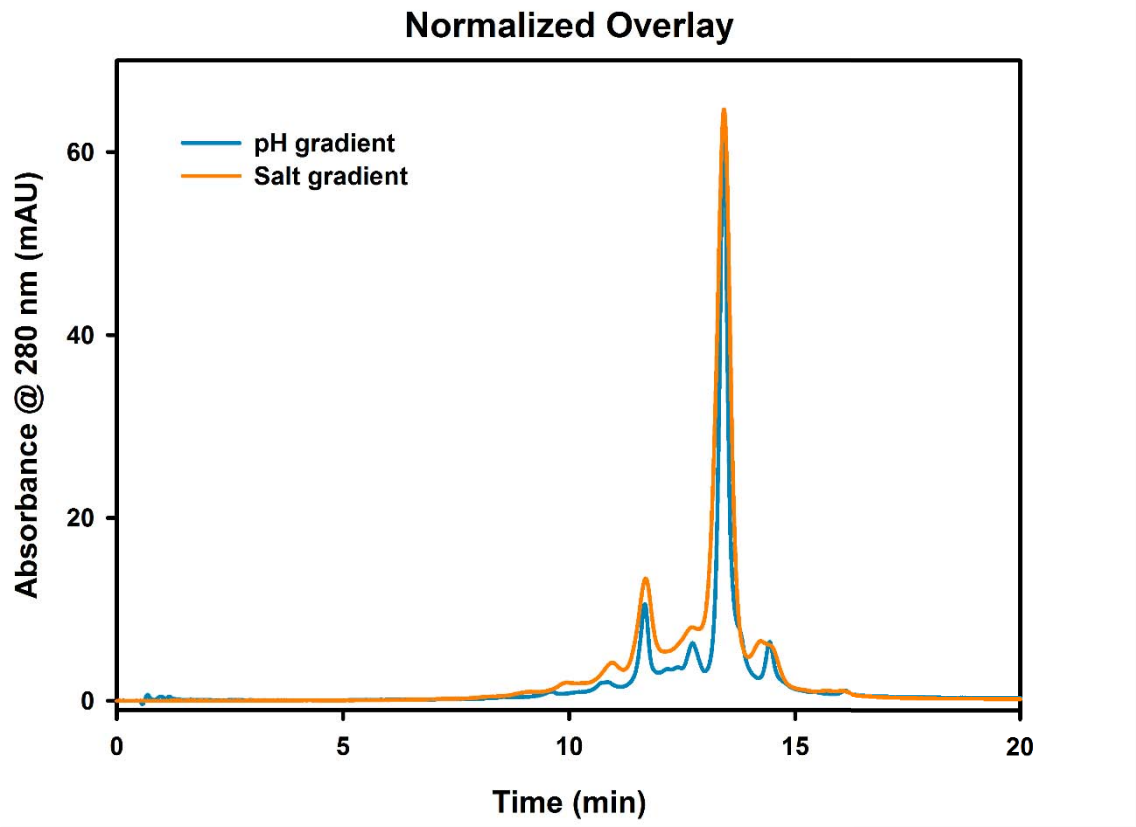

Figure 11: Analytical scale linear salt and pH gradient CEX with a trastuzumab biosimilar antibody. The chromatograms were modified in order to allow an overlay, to compare the difference in resolution. These chromatograms are from the experiments presented in publication I.

In publication II, the same pH gradient method is validated for the routine use as a monitoring method for charge heterogeneity. In order to validate an analytical method, a defined standard material is required. Different routes of obtaining IgG charge variant standard material were explored. The three most fruitful were all based on cation-exchange chromatography, and utilized either salt or pH gradient elution or displacement chromatography.

It was observed that salt gradient elution has a very narrow operating window of pH and starting and final NaCl concentration, in which sufficient resolution between charge variants was possible. Unfortunately, the binding capacity under these operating conditions was very low. Therefore, Salt gradient elution was not a feasible method for the generation of sufficiently pure standard material.

Displacement chromatography was attempted with various IgG and under various conditions, but even the best separation observed was inferior to preparative pH gradient elution. Consequentially most of the experiments performed remain unpublished. The reasons for this disappointing performance, compared to some results reported in the literature [17, 85, 118], were never elucidated, even though a systematic approach based on the SMA model was pursued [86]. Due to the disappointing results, no charge variant standard material could be generated with displacement chromatography.

Eventually, the pH gradient method was scaled up to semi-preparative scale, in order separate IgG charge isoforms in the hundreds of mg scale, to generate the required amounts of standard material. The scale up was performed by keeping the residence time constant. In practice, the residence time had to be increased in the semi-preparative approach, due to pressure limitations. The resulting chromatograms were similar, if the normalized gradient slope was identical in terms of column volumes. Owing to the lack of an orthogonal method to create the standards, the distinctiveness of the acidic and the main charge variant standards had to be confirmed using another method.

In detail, the dimensionless gradient slope, γ, expressed in modifier/column volume was calculated. In the analytical scale, a γ of 0.25 pH/CV was used, i.e. per column

volume of mobile phase the pH was increased by 0.25. This γ was kept constant when scaling up from the 4 x 250 mm column to the 9 x 250 mm column, and when scaling up from the 9 x 250 mm column to the 22 x 250 mm column. This ensured that the resolution observed in the quantitative scale would be achieved in the two larger scales. Since the residence time had to be increased when scaling up, due to system limitations, the overall resolution was increased slightly.

IEF in IPG acrylamide gels was adapted for the analysis of proteins with isoelectric points in the alkaline range. The use of ampholyte based gels was fruitless, owing to the many problems reported for gradients above neutral [111], but immobilized pH gradients could successfully be modified as shown by Görg *et al.* [119] for 2D electrophoresis. This IEF method was then used as a quasi-orthogonal analytical method for the confirmation of the quality of the standard material.

The validation of the pH gradient method with the standard material showed excellent linearity for samples with a relative content of between 1 and 50% of charge variants. The other method parameters were also in agreement with standards for bio-analytical methods [120, 121].

The wider scope of the project is aimed at the elucidation of the origin and the effects of the microheterogeneity of IgG. In order to achieve this goal glycosylation analysis, peptide mapping, in-vitro receptor binding assays as well as cellular activity assays were performed. The, as of yet, unpublished glycan data shows that some separation of IgG glycoforms was achieved with the pH gradient method described in this thesis. It was possible to obtain fractions containing almost no charged glycan species, i.e. sialylated glycans. The peptide map showed differences in deamidation state between variant fractions. Receptor binding and activity assays showed varying results across fractions, suggesting that the antibody isoforms, which could be separated with the pH gradient method, have different biological activity. This is a promising start for proving a long-standing biological doctrine false, namely that antibody modifications leading to microheterogeneity do not affect the in vivo function of the drug product.

Under the quality by design (QbD) framework introduced by the US food and drug administration, analytical tools for the elucidation of complex product characteristics,

such as antibody microheterogeneity, are of immense importance in the development and production of a biopharmaceutical. The pH gradient method presented in this work can be used for the study of a drug product's microheterogeneity, in order to quantify the biological activity of the different variants. This in turn helps identifying a design space of acceptable isoforms, leading to safer and more effective biopharmaceuticals. The method can also be important in comparability studies, proving consistency between production batches [2].

In summary, it can be said that the objectives of this thesis were met:

1 A high-resolution method for the separation of mAb variants based on ion-exchange chromatography was developed.
2 The method was scaled up to preparative scale, and 180 mg of monoclonal antibody variants could be separated in one run.
3 Enough charge variant material could be separated to allow the use of various analytical methods, including biological assays.
4 While reproducible separation of charge variants was accomplished, a conclusion on the exact influence of all modifications and their influence on retention time is not available yet.
5 The method was evaluated using various CHO produced antibodies.
6 The method was validated for the quantification of monoclonal antibody charge variants, which allows the use in analytical labs and for process monitoring.

Contributions to the publications:

In publication I, Nico Lingg devised the method, conducted the chromatographic experiments and drafted the manuscript.

In publication II, Nico Lingg planned the validation scheme, conducted the chromatographic experiments and drafted the manuscript.

In the additional publication I, Nico Lingg drafted the introduction, the chapters about IgG, HPLC profiling of released glycans, LC-MS approach for site-specific glycosylation and the conclusion.

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5 Publications

I. Lingg, N., Tan, E., Hintersteiner, B., Bardor, M., Jungbauer, A., *Highly linear pH gradients for analyzing monoclonal antibody charge heterogeneity in the alkaline range*, Journal of Chromatography A 1319 (2013) 65-71.

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6 Additional publications

I. Review article: Lingg, N., Zhang, P., Song, Z., Bardor, M., *The sweet tooth of biopharmaceuticals: importance of recombinant protein glycosylation analysis*, Biotechnology Journal 7 (2012) 1462-1472.

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Highly linear pH gradients for analyzing monoclonal antibody charge heterogeneity in the alkaline range

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ABSTRACT

Recombinant antibodies with high isoelectric point are frequent since most of them are constructed from the same framework. Classically, cation exchange chromatography is used as a standard method for the determination of antibody charge heterogeneity. In contrast, in this study highly linear pH gradients were achieved by keeping the buffering capacity over the length of the gradient constant. The buffering compounds were selected to be unretained on the column and their respective concentration was adjusted in the start and end buffer of the pH gradient to achieve constant buffering capacity. This helps conserve linearity and stability of the gradient. The method allows quantification of charge variant distribution and the determination of chromatographic isoelectric point. To demonstrate the effectiveness of this novel method, a ProPac WCX-10 column was used to separate isoforms of trastuzumab biosimilar antibodies. Effects of pH gradient linearity and of varying the analytical amount of sample on the separation are shown.

1. Introduction

Cation exchange (CEX) chromatography is a standard method for the determination of charge heterogeneity of recombinant antibodies with high isoelectric point [1]. NaCl gradient elution is the preferred method at the moment, but requires optimization for each macromolecule analyzed [2]. Linear pH gradient elution has been shown to be a reliable alternative [2–4]. However, to create a linear pH gradient the following conditions must be met: the buffering capacity of the buffers used has to be constant over the pH range used and the buffer substances should not interact with the stationary phase. Kröner and Hubbuch [5] have applied these principles to obtain highly linear pH gradients over a wide pH range for proteomic applications. Such principles can be applied to create highly linear gradients for the elution of immunoglobulin G (IgG) on cation exchange columns.

Monoclonal antibodies (mAbs) are a constantly growing class of biopharmaceuticals, owing to their ability to bind antigens with high specificity and to elicit receptor functions [6,7]. MAbs, which are most often IgG, are already approved for the treatment of a variety of diseases, such as various cancers [8] and multiple sclerosis [9], showcasing their ability to be adapted for a plethora of uses.

A series of recombinant IgG molecules are based on the same framework, which has an isoelectric point (pI) around 9. Thus a lot of medically and commercially relevant antibodies present an isoelectric point in the alkaline range. Moreover, besides purity [10] charge heterogeneity is often considered as one critical quality attribute, due to several reasons, such as serum half-life, effector functions, solubility and stability, which may all be connected to the surface charge of the molecule [11–13].

Many of the modifications affecting IgG could influence the pI of the protein, e.g. sialylation of the glycan, oxidation of methionine residues, N-terminal lysine processing, and isomerization of aspartic acid [14–17]. Some of these modifications also influence the effector functions and serum half-life of IgG and as such have to be closely monitored to ensure safety and efficacy of the final product [18].

CEX, as mentioned before, is a robust method for the characterization of the charge heterogeneity of IgG, exploiting small binding differences of the charge variants to the stationary phase under salt gradient elution. However, when using a salt gradient for elution, the pH of the equilibration buffer strongly influences binding, generally necessitating optimization of the pH and the NaCl concentration of the equilibration and elution buffers for each mAb product [2]. A different mode of ion exchange gradient chromatography utilizes pH gradients to affect the surface charge of the

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Table 1
pK_a values of the selected buffer substances at room temperature.

Substance	pK_a
MOPSO	6.90
HEPES	7.48
Bicine	8.26
CAPSO	9.60
CAPS	10.40

protein to modify the binding to the stationary phase. Using this strategy, many different recombinant antibodies have been analyzed using the same unmodified method and buffers, as shown by Farnan et al. [2]. Previous pH gradient methods, such as those used by Zhang et al. [4], Farnan and Moreno [2], Rea et al. [19] and Tsonev and Hirsh [20], used buffer systems that were either interacting with the stationary phase or with large variations in buffering capacity resulting in non-linear pH gradients. As pointed out earlier, this deviation from pH gradient linearity results in lowered resolution. In contrast, the current work presents highly linear pH gradients for the analysis of mAb.

2. Material and methods

2.1. Buffer systems for pH gradients

The buffer compounds used for the generation of the pH gradients were3-morpholino-2-hydroxypropanesulfonic acid (MOPSO), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), *N*,*N*-bis(2-hydroxyethyl)glycine (bicine), 3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid (CAPSO) and 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS), all Sigma Aldrich (St. Louis, MO, USA). Sodium chloride (Merck, Darmstadt, Germany) was used to adjust the conductivity of the buffers to the same level. Sodium hydroxide was obtained from Merck (Darmstadt, Germany).

Buffer systems were designed for two different pH ranges, covering either pH 8.0 to pH 10.5 or pH 7.0 to pH 10.5. Acidic or zwitterionic buffer substances were selected with pK_a values evenly distributed across the desired pH range. For the pH 8.0 to 10.5 gradient, the following buffers were selected: HEPES, bicine, CAPSO, CAPS and for the pH 7.0 to 10.5 gradient, HEPES was replaced by MOPSO. The pK_a values of the buffers used can be found in Table 1.

The buffer composition was optimized by keeping the buffer capacity constant over the whole buffered range. The constancy of the buffer capacity was quantified by calculating the difference between the minimum and the maximum buffer capacity over the covered pH range. By varying the concentration of buffers in both buffer A and B, instead of using one stock buffer, the buffering capacity was further improved. The average buffer capacity was set at 5 mmol/l to keep the ionic strength low. The conductivity of buffer A was adjusted to the conductivity of buffer B with a 1 M solution of NaCl.

Five buffer systems, listed in Table 2, were tested on an ÄKTA Explorer 100 system with a Dionex ProPac WCX-10 column (Thermo Fisher, Sunnyvale, CA, USA). This column consists of a pellicular cation-exchange layer grafted to a non-porous support particle, resulting in high mass transfer rates [11] and is considered as the gold standard for mAb charge heterogeneity analysis [18]. The length of the gradient was 10 column volumes. Each buffer system was used to run the experiment five times. The linearity was quantified by calculating the coefficient of correlation. The details of the buffer systems used can be found in Table 2.

2.2. Gradient elution of IgG

An Agilent 1100 HPLC system was used with a Dionex ProPac WCX-10, 4 × 250, column to elute IgG under linear pH and salt gradients. The sample IgG used was a Trastuzumab biosimilar antibody produced in CHO cells and purified using protein A chromatography [21]. The samples were buffer exchanged into the running buffer A and diluted to a concentration of 1 mg/ml for all experiments. A volume of 25 μl was injected for all experiments, unless stated otherwise.

The chromatograms of the pH gradient were blank corrected, by subtracting a blank run, due to the difference in absorbance of the buffers at high and low pH. The IgG was eluted in 10 CV linear gradients and the absorbance was measured at 280 nm. The chromatograms were then integrated to calculate the relative distributions of the main peak, the acidic variants (eluting before the main peak) and the basic variants (eluting after the main peak). The peaks were also fitted using the exponentially modified Gaussian function [22] to calculate first moments and second central moments.

2.3. Isoelectric focusing

Immobilized pH gradient (IPG)-polyacrylamide-gels (size: 125 × 260 × 1 mm) were cast on a GELbond-PAG-film backing (GE Healthcare), polymerized, washed and dried following a procedure previously described by Westermaier et al. [23]. The desired pH gradient from 8.0 to 10.0 was obtained by graphic interpolation from a recipe previously published by Görg et al. [24].

Directly before use, the gel was cut into 3 mm strips, which were rehydrated for 2 h in a solution containing 6 M Urea, 2% Tergitol, 2% Pharmalyte 3–10, 10% Glycerol and 16% Isopropanol.

The rehydrated strips were put onto the IPGphor (GE Healthcare) using the manifold. The electrodes were positioned on the acidic and basic ends of the strips with wetted paper wicks between gel and electrode to ensure good contact. The samples were applied by cup loading under a covering layer of paraffin and electrophoresis was performed overnight (at 150 V for 1 h, followed by 300 V for 1 h, a gradient step to 3000 V for 30 min, 3000 V for 18 h, and 3500 V for 5 min). After electrophoresis, the strips were stained with Coomassie G250.

Table 2
Composition of the five buffer systems used.

pH 8.0 to 10.5 buffers		HEPES	Bicine	CAPSO	CAPS
System 1	Buffer A [mM]	5.5	4.2	9.5	0.8
	Buffer B [mM]	0.0	10.5	2.5	7.0
System 2	Buffer A & B [mM]	5.0	5.0	5.0	5.0
System 3	Buffer A [mM]	20.0	0.0	0.0	0.0
	Buffer B [mM]	0.0	0.0	0.0	20.0
pH 7.0 to 10.5 buffers		MOPSO	Bicine	CAPSO	CAPS
System 4	Buffer A [mM]	7.1	5.3	14.9	0.7
	Buffer B [mM]	14.6	4.9	1.4	7.1
System 5	Buffer A & B [mM]	7.0	7.0	7.0	7.0

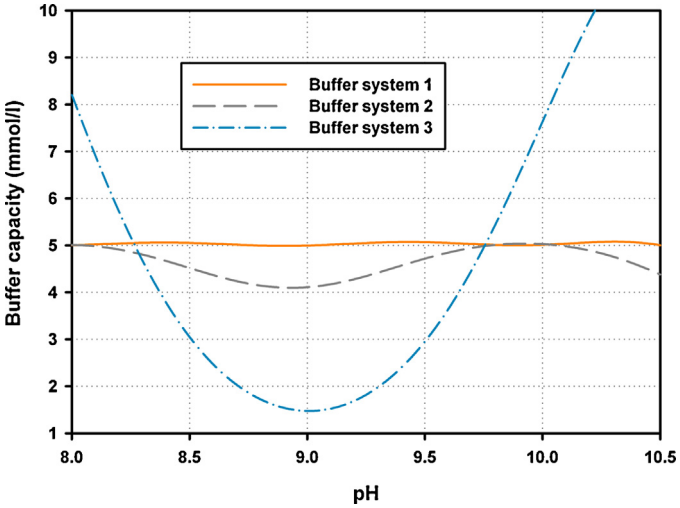

Fig. 1. Buffer capacity of buffer system 1 to 3. By varying the concentrations in buffer A and B of buffer system 1, the buffer capacity can be kept constant in the working range from pH 8 to 10.5.

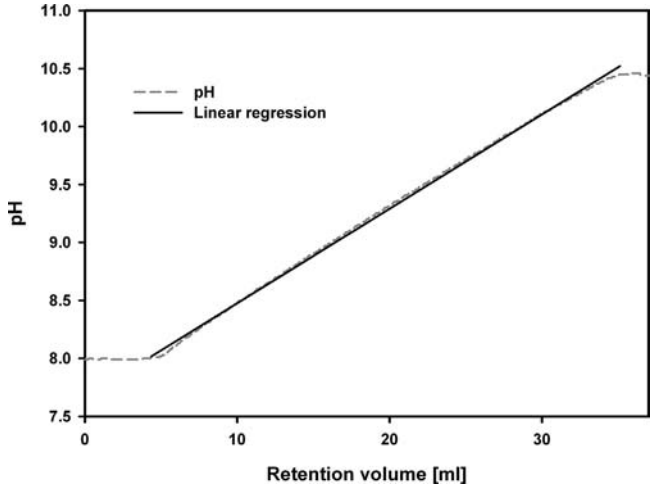

Fig. 2. Comparison of experimental pH gradient and the linear regression. The experimentally determined pH gradient, of buffer system 1, is shown as a gray dashed line and the linear regression used to calculate the coefficient of correlation is shown as a solid trace.

3. Results

3.1. Linearity of pH gradient

Eq. (1) was used in order to generate a linear gradient with a constant buffer capacity over the entire pH.

$$\beta = \frac{dB^+}{d\mathrm{pH}} = \ln(10) \times \frac{[H^+] + C_A K_A [H^+]}{K_A + [H^+]^2 + [OH^-]} \quad (1)$$

where β is the buffer capacity, dB^+ is the infinitesimal amount of added base, dpH is the resulting change in the pH, C_A is the concentration of buffering weak acid and K_A is the dissociation constant of the weak acid. We assumed thermodynamically ideal conditions and concentrations were used instead of activities, because of the dilute solutions. The buffer capacity of the optimized buffer system (buffer system 1), and the non-optimized buffer systems 2 and 3 are shown in Fig. 1. For the optimized system, the capacity fluctuated less than 0.1 mM for a 5 mM buffer over the entire pH range, while the buffer capacity of buffer system 2 and 3 were less stable. Buffer systems 1 to 5 were then used to create a linear pH gradient on a weak cation exchange stationary phase. Systems 1 to 3 cover pH 8.0 to 10.5 and systems 4 and 5 cover pH 7.0 to 10.5. Systems 1 and 4 were optimized by changing the amount of buffering component in buffer A and B. Systems 2 and 5 were optimized by using buffer components with pK$_a$ values covering the pH range. System 3 uses one buffer component for the start pH and another for the end pH. A clear increase in linearity is observed for each optimization of the buffering capacity.

The resulting pH gradient from buffer system 1 is given as an example in Fig. 2. The largest deviations from linearity can be seen at the beginning and the end of the gradient. The coefficient of correlation is equal to 0.9809 if a linear regression is performed on only the first 10% of the gradient, and 0.9901 for the last 10%. The part of the gradient in between those chosen beginning and end points, i.e. the remaining 80%, show a much higher coefficient of correlation of 0.9995. These initial deviations from linearity can be attributable to non-ideal pumps and gradient mixers [25]. Table 3

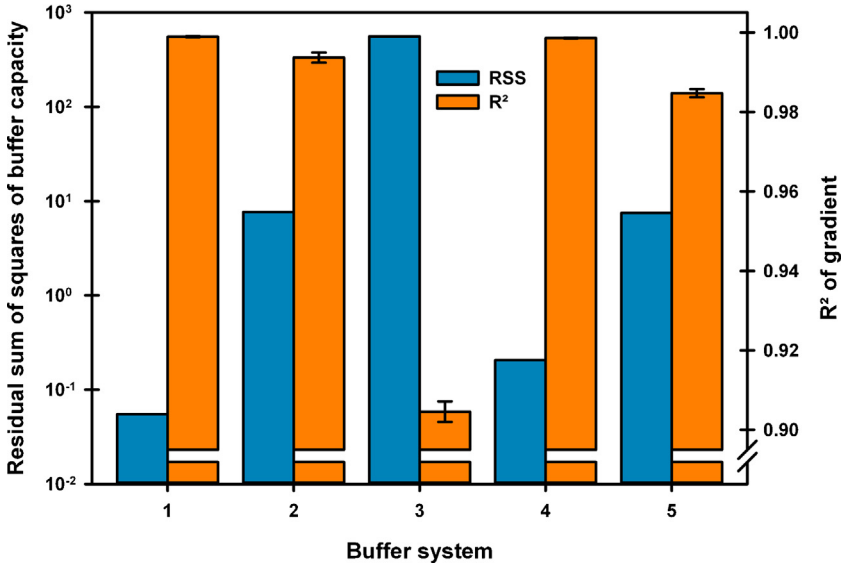

Fig. 3. Maximum relative deviation in buffering capacity. The dark grey bars represent the maximum relative deviation in buffering capacity of the buffer system and the light grey bars represent the linearity of the pH gradient. The dependence of the gradient linearity on the buffer capacity stability can be seen.

Table 3
Summary of the maximum relative deviation in buffering capacity Δ β, the residual sum of squares, the coefficient of correlation (R) and the standard deviation (s.d.) of R^2 for the five buffer systems.

	$\Delta\beta$ (%)	RSS	R^2	s.d.
pH 8.0 to 10.5 buffers				
System 1	98.2	0.055	0.99894	0.00014
System 2	81.3	7.7	0.99368	0.00130
System 3	12.6	560	0.90455	0.00257
pH 7.0 to 10.5 buffers				
System 4	96.5	0.21	0.99860	0.00009
System 5	68.3	7.5	0.98473	0.00104

and Fig. 3 show a clear relationship between buffering capacity and linearity of the pH gradient.

From these results shown in Table 3 and Fig. 3, it is clear that the stability of the buffering capacity determines the linearity of the pH gradient. For buffer systems 2 and 5, the same buffer compounds were used as in buffer system 1 and 4 but their concentrations were not adjusted. Because the pK_a values of those buffer compounds cover the whole pH range of the gradient and are at least 0.7 pH units apart, the resulting buffer capacity is relatively stable. This is an easy way to create mostly linear gradients, especially for narrow gradients up to three pH units.

To obtain highly linear pH gradients, the deviation from linearity of the buffering capacity can be kept minimal by varying the buffer concentrations in both buffers A and B as illustrated for example in buffer systems 1 and 4.

3.2. Linearity of pH gradient and chromatographic performance

A Trastuzumab biosimilar antibody was used to demonstrate the effect of linearity of pH gradient on chromatographic specificities such as resolution and peak capacity. The buffer systems described in Section 3.1 were used for pH gradient elution of 25 μg of recombinant antibody. A NaCl gradient was also performed in parallel for comparison. Each experiment was performed in replicates of 5. The resulting chromatograms were fitted by exponentially modified Gaussian function [22] and the peak capacity and the resolution between adjacent peaks were calculated. Due to IgG consisting of a large number of charge variants, the chromatogram contains many peaks. In order to perform the analysis of the data, only the 4 representative peaks, which could be identified easily in all 6 experiments, were evaluated. The chosen peaks are labeled from 1 to 4 in Fig. 4, which shows one of the chromatograms obtained with buffer system 1. Isoelectric focusing performed with the same protein gave similar results in regards to isoelectric point and number of major peaks.

The resolution and the peak capacity both increased with the linearity of the pH gradient. Fig. 5 and Table 4 show the peak capacity and the resolution obtained by elution with all5 buffer systems and with a salt gradient used as a reference in parallel. A large difference between the salt gradient and the highly linear pH gradients can be seen. The non-linear pH gradient obtained with buffer system 3, shows peak capacity and resolution values comparable to the ones from the salt gradient.

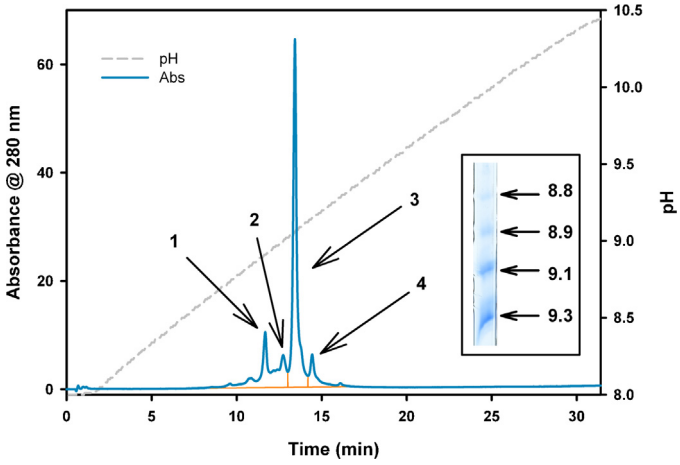

Fig. 4. Representative chromatogram from the experiments outlined in Section 3.2. An amount of 25 μg Trastuzumab biosimilar antibody were injected to a WCX-10 column and eluted using a linear gradient over 10 column volumes with buffer system 1. The black solid trace shows the chromatogram obtained by measuring the absorbance at 280 nm. The gray dashed trace gives the pH gradient, which had to be measured independently, due to the lack of online pH monitoring on the HPLC system. It can be seen that the IgG is a mixture of many isoforms, all eluting between pH 8.6 and 9.3. To analyze and compare all seven experiments, only four representative peaks, marked with arrows, were chosen for analysis. The calculated chromatographic pI values for the labeled peaks are 8.85, 8.94, 8.99 and 9.07, respectively. These values correspond to the pI values determined by IPG IEF, shown in the inset. The boundaries for integration are shown under the peaks.

Table 5
Determination of the isoform composition with different buffer systems. In these experiments, a salt gradient has been used for comparison in parallel to the five buffer sytems studied.

Buffer	Acidic isoforms (%)	CV (%)	Main isoform (%)	CV (%)	Basic isoform (%)	CV (%)
1	30	4	61	3	9	5
2	33	1	59	1	8	4
3	35	2	55	1	9	7
4	31	2	59	2	10	7
5	36	1	55	1	10	7
Salt	33	3	55	2	12	3

The chromatograms were also integrated fully, by perpendicular drop method [26], and the peaks assigned to acidic, main or basic isoforms as previously reported for mAb [18]. Fig. 5(C) and Table 5 show the distribution determined by all six buffer systems used, which are all in agreement about the composition (>50% main peak, ~35% acidic variants and ~10% basic variants). A trend towards higher main peak content can be seen for buffer systems 1, 2 and 4, which is due to the better resolution and the better separation from the acidic peaks.

Table 4
Summary of the results of the pH gradient elution experiments. The peak capacity and the resolution between all 4 representative peaks are given with the coefficient of variation (CV) from 5 experiments. In these experiments, a salt gradient has been used for comparison in parallel to the 5 buffer sytems used in this study.

Buffer	Peak capacity	CV (%)	Resolution	Peak 1 & 2	CV (%)	Peak 2 & 3	CV (%)	Peak 3 & 4	CV (%)
1	4.9	0.5		1.86	0.5	1.21	0.3	1.79	0.6
2	4.3	5.1		1.63	6.6	1.12	3.9	1.54	5.3
3	2.9	6.3		1.12	12.3	0.85	2.9	0.90	2.3
4	4.5	0.4		1.86	0.8	1.09	0.2	1.57	0.6
5	3.9	0.6		1.55	1.2	0.94	0.8	1.37	0.2
Salt	2.9	0.6		1.06	1.0	0.85	0.4	1.03	0.6

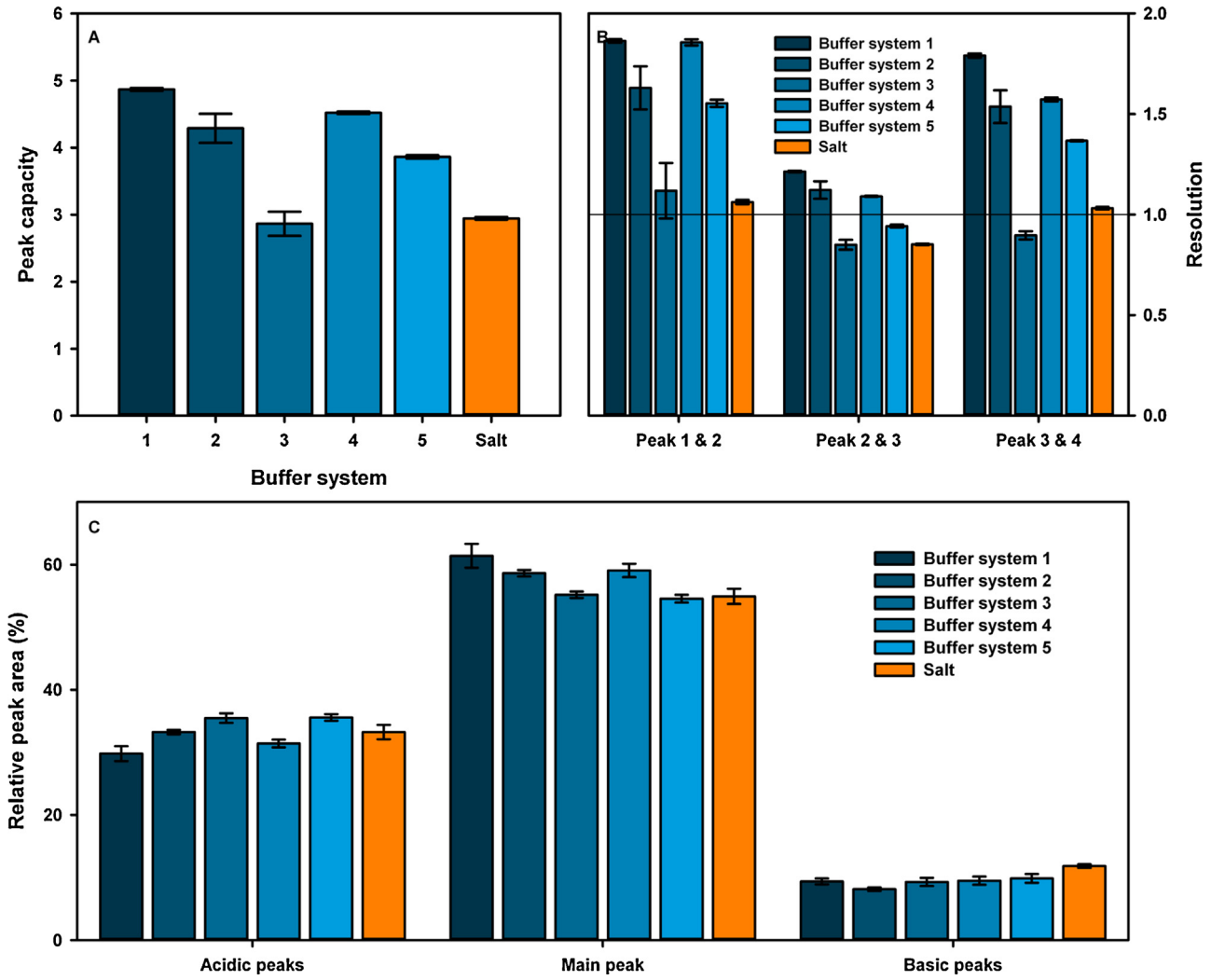

Fig. 5. The dependence of the peak capacity (A) and the chromatographic resolution (B) on the linearity of the pH gradient. Buffer systems with a gradient linearity of $R^2 > 0.98$ (buffer 1, 2, 4, 5) show a peak capacity between 4 and 5, while the salt gradient and the buffer system 3 show a peak capacity lower than 3. The resolution between the 4 peaks behaves similarly. Panel (C) shows the relative distribution of charge variants determined by integration of the chromatogram.

Table 6
Influence of the injection amount on the peak capacity and the resolution of the pH gradient elution.

Injection amount [μg]	Peak capacity	CV (%)	Resolution:	Peak 1 & 2	CV (%)	Peak 2 & 3	CV (%)	Peak 3 & 4	CV (%)
10	3.9	3.0		1.5	1.4	1.0	0.8	1.5	0.9
25	4.9	2.3		1.9	0.9	1.2	0.4	1.8	1.0
50	4.8	0.6		1.8	0.5	1.2	0.2	1.8	0.1

3.3. Influence of sample amount

To test the robustness of an optimized buffer system, the effect of sample amount on the chromatographic key values was determined by injecting 10, 25 or 50 μg of IgG and eluting it with the buffer system 1. The column supplier recommends an injection amount of 25 μg. Very similar peak capacity, resolution and isoform distribution for the 25 and 50 μg injections were observed (Fig. 6, Tables 6 and 7). The 10 μg injection shows lowered values for peak capacity and resolution as well as higher standard deviations in isoform distribution. Nevertheless, the isoform distribution varies little with the injection amount.

4. Discussion

Kröner et al. have shown that by keeping the buffering capacity constant over the length of the gradient, the resulting pH gradient will be linear [5]. The value that was being used for optimization

Table 7
Influence of the injection amount on the determination of the relative isoform distribution.

Injection amount [μg]	Acidic isoforms (%)	CV (%)	Main isoform (%)	CV (%)	Basic isoform (%)	CV (%)
10	30	7	59	5	11	13
25	30	4	61	3	9	5
50	32	0	60	0	8	6

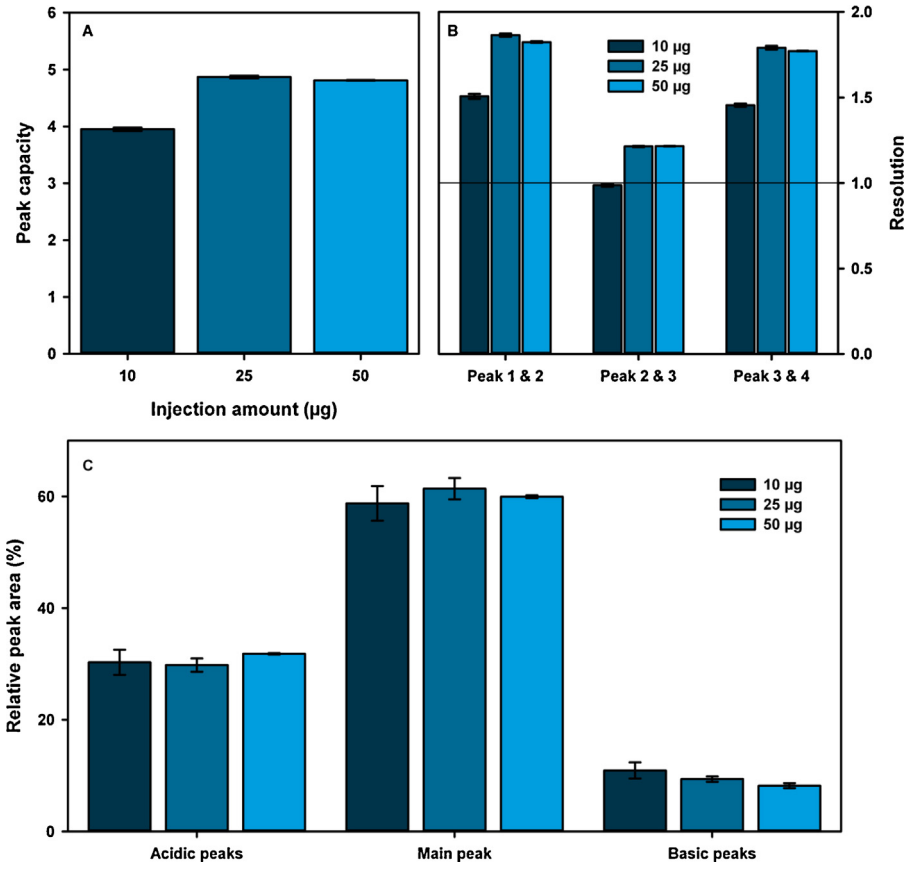

Fig. 6. Influence of the injection amount on the peak capacity (A) and the resolution (B) of the separation. A decline in separation performance can be seen for injections below the recommended 25 µg. Panel (C) shows the influence of the injection amount on the isoform distribution determination. Minor differences can be seen in the acidic peaks between all three injection values. The main and basic peak values for the 10 µg injection do not agree with the 25 and 50 µg injections.

was the ratio of minimum to maximum buffer capacity over the pH range. This variable turned out to be a more suitable metric for optimization in the relatively narrow pH gradients covered here, compared to the metrics used by Kröner et al. [5] or the residual sum of squares used by Giaffreda et al. [27] for the optimization of the immobilized IEF pH gradient recipes.

Kaltenbrunner et al. [3] have used pH gradient elution for the characterization of mAb in 1993, but their method relied on the chemical reaction of mannitol with borate. This limits the customization possibilities of such a gradient. Zhang et al. [4], Farnan and Moreno [2], Rea et al. [19] and Tsonev and Hirsh [20] are all using cationic buffering species on a cation exchange stationary phase. This means that the buffering species themselves are interacting with the functional groups, resulting in partial retention and therefore deviation of the pH gradient from the optimum. In the case of the works of Tsonev and Hirsh [20], they are using algorithms to correct for the deviations. With the method presented in this work, such corrections are not necessary. Kang et al. [28] and Ng et al. [29] used induced pH gradients for heterogeneity analysis of antibodies, but the method is limited to proteins with a neutral or acidic pI. Nordborg et al. [30] used buffering compounds with pK_a values across the pH range to create linear pH gradients, but did not optimize the buffering capacity of their buffers. Rozhkova [31] used phosphate as the only buffering compound in very low concentrations, leading to unstable buffering capacity in the pH range used and losing the ability to adjust the pH range of the pH gradient.

Kröner and Hubbuch [5] chose buffering components that carry the same charge as the immobilized ligands and therefore do not interact with the stationary phase. Their pH gradients are very wide range for proteomics applications and are based on one stock buffer that gets titrated to the pH values spanning the pH gradient. By using a narrower gradient in the present work, we were able to vary the buffer composition in both buffers, which further resulted in a more even distribution of the buffering capacity.

As shown in Fig. 5, the measured isoform distribution changes depending on the method used. The trend towards larger relative amounts of main peak in methods with higher peak capacity stems simply from the better resolution and the more accurate integration of the chromatograms. These differences have to be considered for technology transfer or the characterization of biosimilars, but should not be seen as a disadvantage of the method presented here.

The determination of isoforms is independent of the injected amount of sample, as shown in Fig. 6. The quantification of minor isoforms, next to more abundant isoforms is possible, which speaks for the robustness of the whole method.

Isoelectric focusing (IEF) and capillary electrophoresis (CE) methods are popular methods for the evaluation of charge heterogeneity of mAbs [1,32]. Capillary IEF (cIEF) is a high resolution method for the determination of the pI and the intrinsic protein net charge. Thus, this method can be considered orthogonal to linear pH gradient elution cation exchange chromatography which determines the protein surface charge only. CIEF does have a very high resolution and requires low sample amounts. However, problems are encountered with cIEF like the use of ampholytes, which can lead to reduced robustness of the method due to batch to batch variations; the ampholyte mix and the electrode stabilizers have to be optimized for the pH range [33]. CIEF in immobilized gradients have also been reported [34] and would alleviate the problems associated with the use of ampholytes. Nevertheless, IEF and CE

methods require their own instruments and know-how, while linear pH gradient CEX can be easily adopted as a method in a lab where other chromatographic methods are already in place.

5. Conclusions

In this study, we proposed a novel method for the characterization of mAb charge variants. The new method requires optimized buffer systems for the creation of a highly linear pH gradient. The buffer systems described here are able to quantify the general distribution of acidic, basic and the main charge variants in IgG samples. For accurate quantification of each charge variant, further complementary analysis of the peaks with other method, e.g. mass spectrometry, is required to confirm that each peak corresponds to only one specific charge variant.

Furthermore, it was shown that the linearity of the pH gradient is correlated with the constancy of the buffer capacity over the pH range. The linearity of the pH gradient is correlated with the resolution and peak capacity of the pH gradient elution. The peak capacity improves from 2.9 to 4.9 when eluting with a highly linear pH gradient instead of a linear salt gradient.

The advantage of pH gradient methods over conventional salt gradient elution methods is their broad applicability for a variety of IgG, without the need for optimization. This is the main difference over salt gradient elution, in which the initial and final salt concentrations have to be adjusted for each IgG. The buffers presented in this work can cover pH ranges from 7 to 10.5 and so should be useful to elute most IgG.

The separation problem at hand, i.e. the separation of IgG charge variants, is a difficult one. IgG consists of many charge variants, sometimes with only minor differences in surface charge distribution. Even with high performance stationary phases and improved elution methods, base line separation of all isoforms is not possible at the moment. The method presented in this work offers another step towards improved characterization of monoclonal IgG.

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Highly linear pH gradients for analyzing monoclonal antibody charge heterogeneity in the alkaline range: Validation of the method parameters

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Validation

ABSTRACT

Cation exchange chromatography has been routinely used for the quantification of monoclonal antibody (mAb) charge heterogeneity. A previously developed method utilizing pH gradients for the elution instead of salt gradients was validated according to current guidelines proposed by the ICH. The linearity, stability, accuracy, precision and the lower limit of quantification have been determined, using pure charge variant standards. The method is valid for the quantification of mAb samples with a charge heterogeneity between 1% and 50%. Three different approaches to obtaining pure standard material for the validation of bio-analytical methods for the quantification of charge heterogeneity of IgG are presented. These methods are based on salt gradient elution, pH gradient elution and displacement in cation exchange chromatography.

1. Introduction

Monoclonal antibodies (mAb) are a highly researched class of proteins which represent a very profitable part of the biopharmaceutical industry [1]. Indeed, their ability to target a wide range of antigens, e.g. other proteins, carbohydrates, and the strong effector functions they can elicit, e.g. antibody dependent cellular cytotoxicity, complement dependent cytotoxicity, make them an ideal molecule to be used as a therapeutic [2]. Immunoglobulin G (IgG) is the most commonly used isotype. It is a rather large protein with ~150 kDa and consists of four subunits. In order for the protein to function properly it requires proper folding, correct disulfide bonds and correct *N*-glycosylation for effector functions [3,4]. Other post-translational modifications, e.g. methionine oxidation, C-terminal lysine processing, deamidation of asparagine and isomerization of aspartic acid influence the behavior of mAb, as such they can be considered a critical quality attribute (CQA) [5,6]. The emergence of novel purification strategies demands sophisticated analytical methods to ensure comparability of the product [7–11], and such analytical methods should be validated for their intended purpose [12,13].

In order to facilitate this goal, we decided to validate our previously presented pH gradient method for the analysis of antibody charge heterogeneity based on cation exchange chromatography (CEX) [14]. This method is intended to be used for the determination of acidic and basic charge variants, relative to the main charge variant found in a monoclonal antibody batch. In order to validate the method parameters, we purified mAb charge variants, to be used as a standard. The preparative separation of charge variants requires methods with a high resolving power. In this work, we describe different approaches to obtain pure standard material using CEX. The charge variants will be referred to as the main charge variant (MCV) and the acidic charge variants (ACV). The MCV is the main peak eluting in the chromatogram, and is usually not unmodified antibody, it is rather the antibody with the most common modifications [15]. The ACV are simply all charge variants eluting before the MCV in CEX. Basic charge variants could not be purified in sufficient amounts to allow usage as standard material, but the method should be valid for all charge variants. Isoelectric focusing

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Table 1
Summary of the salt gradient elution experiments performed on a semi-preparative ProPac WCX-10 column. [M] is the modifier (NaCl) concentration, with the indices *i* and *f* meaning initial and final respectively.

Buffer	[NaCl] buffer A (mM)	[NaCl] buffer B (mM)	pH	[M]*i* (mM)	[M]*f* (mM)	Gradient slope (CV)
30 mM MES	0	1000	6.0	50	350	20
10 mM phosphate	50	100	7.0	50	100	5
10 mM phosphate	50	100	7.0	70	77	5
10 mM phosphate	50	100	7.0	63	70	5
10 mM phosphate	50	100	7.0	63	65.5	5
10 mM phosphate	50	100	7.0	60	65.5	5

Table 2
Summary of the displacement chromatography experiments performed on a Mono S column.

Buffer	pH	Displacer (mM)	Load (mg/ml)	Load (% DBC_{10})
40 mM MES, 15 mM NaCl	5.5	5	35	50
30 mM MES, 100 mM NaCl	6.1	5	14.9	60
30 mM MOPSO, 15 mM NaCl	7.0	5	33.6	60

(IEF) was used as an orthogonal method to check the purity of the standards.

Our first approach for the preparative scale separation of charge variants was standard CEX elution chromatography, using NaCl as the mobile phase modifier. This method has been used successfully for decades [16] and requires only a simple setup. The next approach was displacement CEX [17], which has been shown to be a method capable of high resolution separations of mAb charge variants [18]. With the advent of high performance displacers, this approach was very promising. Our last approach focused on the scale up of our own pH gradient elution CEX method into preparative scale, which we have previously shown to have a higher resolution than standard NaCl gradient elution CEX [14].

In order for this method to be used as a standard for the quantification of mAb charge heterogeneity, we validated the key performance parameters of the method, by following the recommendations for the validation of bioanalytical chromatographic methods presented by Hartmann et al. [12], while using the terminology from Peters et al. in this work [19]. In detail the following parameters have been determined: selectivity, calibration model (linearity), stability, accuracy (bias), precision (repeatability, intermediate precision) and the lower limit of quantification (LLOQ). This was done in a similar fashion to the work published by Tscheliessnig and Jungbauer [20] and Rozhkova [21]. The upper limit of quantification (ULOQ) was not determined, instead a charge heterogeneity of 50% was taken as the upper limit.

Our final goal was to provide evidence that our previously presented method is indeed valid for the quantification of mAb charge variants.

Other methods, such as imaging capillary isoelectric focusing (iCIEF), can also be used for quantification of mAb charge variant content and are well established in the biopharmaceutical industry [22,23]. Such methods have shown great inter-laboratory robustness and are reliable methods [24].

2. Material and methods

2.1. Materials and chemicals

All chemicals were of analytical grade, unless stated otherwise. 3-Morpholino-2-hydroxypropanesulfonic acid (MOPSO), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), *N*,*N*-bis(2-hydroxyethyl)glycine (bicine), 3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid (CAPSO) and 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS), 2-(*N*-Morpholino)ethanesulfonic acid (MES), NaH_2PO_4, Tergitol (70% in water) and isopropanol were purchased from Sigma-Aldrich (St. Louis, MO, USA). NaCl, Na_2HPO_4, NaOH, Urea and glycerol was purchased from Merck (Darmstadt, Germany). Pharmalyte 3–10 was purchased from (GE Healthcare, Uppsala, Sweden). Histidine, research grade (>98.5% purity) was from Serva (Heidelberg, Germany). Tween 20 was obtained from Bio-Rad (Hercules, CA, USA). Expell™ SP1 (Sachem Inc., Austin, TX, USA) was used as the displacer in displacement chromatography experiments.

Table 3
The buffer system used throughout this work for the creation of linear pH gradients.

	HEPES	Bicine	CAPSO	CAPS	NaCl	pH
Buffer A [mM]	5.5	4.2	9.5	0.8	6.3	8.0
Buffer B [mM]	0.0	10.5	2.5	7.0	0.0	10.5

2.2. Preparative chromatography

The mAb used for the validation was a chimeric IgG 1, provided by Apeiron Biologics (Vienna, Austria). An ÄKTA Explorer 100 (GE Healthcare) was used for all preparative chromatography steps. The outlet was monitored at 280 nm.

2.2.1. Salt gradient elution

A Dionex ProPac WCX-10 column, 9 × 250 mm (Thermo Fisher, Sunnyvale, CA, USA) was used. The flow rate was 2.5 ml/min (236 cm/h). The buffers used for the salt gradient experiments are described in Table 1. The method used consisted of a 1 CV equilibration step at 0% B, 10 ml injections via sample loop, a 1 CV wash step at 0% B, a 1 CV gradient to the initial elution conductivity and the elution gradient with varying gradient length, found in Table 1.

2.2.2. Displacement chromatography

For displacement chromatography, a Mono S column, 4 × 200 mm (GE Healthcare, Uppsala, Sweden) was used. The flow rate was 0.25 ml/min (76 cm/h). Before each experiment, the dynamic binding capacity (DBC) at 10% breakthrough was determined for each mobile phase. The buffers and conditions used for displacement chromatography can be found in Table 2. The displacement experiments were performed according to Zhang et al. [18] and McAtee and Hornbuckle [17].

2.2.3. pH gradient elution

For salt gradient and pH gradient preparative experiments, a Dionex ProPac WCX-10 column, 9 × 250 mm (Thermo Fisher, Sunnyvale, CA, USA) was used. The flow rate for the ProPac column was 2.5 ml/min (236 cm/h). The buffers used for pH gradient experiments are found in Table 3. The various gradients used are detailed in Table 4. The collected acidic and main fractions from each

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Table 4
Outline of the pH gradient elution method used for the purification of the standard material.

Experiment	Wash (CV)	Initial pH	Gradient (CV)	Final pH	Gradient (CV)	Final pH
1st round	1	8.0	14	9.8		
2nd round, acidic	1	8.0	2	8.9	10	9.4
2nd round, main	1	8.9	10	9.4		

run were concentrated and buffer exchanged with Amicon Ultra-15 Centrifugal Filter Units, 50 kDa (Merck Millipore, Darmstadt, Germany). The diafiltration buffer was 20 mM histidine, 0.1% Tween 20, pH 6.0. The gradient for the second purification round of the ACV standard consisted of two segments, a steeper initial gradient and a shallower gradient to separate the ACV from the main charge variant. The concentrated and buffer exchanged mAb was adjusted to a concentration of 2 mg/ml and used as the MCV and ACV standards.

2.3. Isoelectric focusing

Immobilized pH gradient (IPG)-polyacrylamide-gels (size: 125 × 260 × 1 mm) were cast on a GELbond-PAG-film backing (GE Healthcare), polymerized at 50 °C for 2 h, washed and dried following a procedure previously described by Westermaier [25]. The desired pH gradient from 7.0 to 11.0 was obtained by graphic interpolation from a recipe previously published by Görg et al. [26].

Directly before use, the gel was cut in 3 mm strips, which were rehydrated for 2 h in a solution containing 6 M urea, 2% tergitol, 2% pharmalyte 3–10, 10% glycerol and 16% isopropanol.

The rehydrated strips were put onto the IPGphor (GE Healthcare) using the manifold. The electrodes were positioned on the acidic and basic ends of the strips with wetted paper wicks between gel and electrode to ensure good contact. The samples, 15 μg of mAb, were applied by cup loading under a covering layer of paraffin and electrophoresis was performed overnight (at 150 V for 1 h, followed by 300 V for 3 h, 3500 V for 18 h). After electrophoresis, the strips were stained with Coomassie blue G250.

2.4. Analytical chromatography

An Agilent 1220 Infinity LC System (Santa Clara, CA, USA) equipped with a SIM sample-cooler (Scientific Instruments Manufacturer GmbH, Oberhausen, Germany) was used for all validation experiments. The column used was a Dionex ProPac WCX-10, 4 × 250 (Thermo Fisher). The column oven and the auto-sampler temperatures were set to 30 °C and 10 °C, respectively. These temperatures were chosen due to instrument limitations.

The samples for the calibration model, the accuracy, precision and the stability experiments were prepared by mixing appropriate volumes of ACV and MCV standard. The outlet was monitored at 280 nm. The flow rate was 1 ml/min (477.5 cm/h). The buffers were the same as those used for the preparative experiments in Table 3. A gradient from 35% to 55% buffer B in 8 CV was used. The chromatograms were then integrated with the Chemstation software suite (Agilent).

3. Results and discussion

3.1. Creation of charge variant standard

In order to validate the method parameters, pure IgG charge variant standards were necessary. For such preparations, different CEX approaches were conceived and tested. The first approach was cation exchange chromatography combined with salt gradient elution.

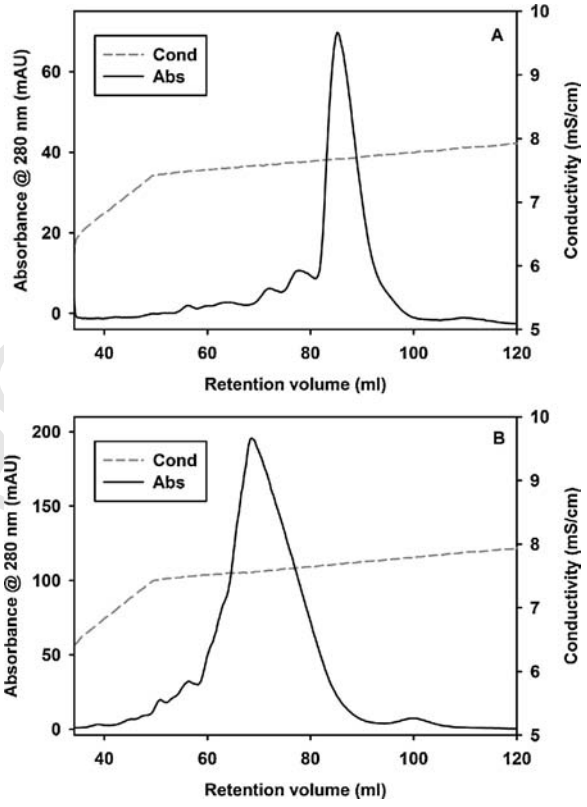

Fig. 1. Chromatogram of the semi-preparative separation of charge variants using salt gradient elution. Panel (A) shows a load of 3 mg (~190 μg/ml column) and panel (B) shows a load of 15 mg (~950 μg/ml column). A drastic decrease in resolution is apparent at higher loading. Stationary phase: Dionex ProPac WCX-10 column, 9 × 250 mm. Flow rate: 2.5 ml/min (236 cm/h). Mobile phase: 10 mM phosphate, pH 7.0, buffer A with 50 mM NaCl, buffer B with 100 mM NaCl. Gradient from 60 to 65.5 mM NaCl in 5 CV.

3.1.1. Salt gradient elution

Both the Mono S and the ProPac WCX-10 columns were tested, but the latter column was used for further optimization due to the higher resolution, owing to the faster mass transfer.

The method was optimized by testing elution behavior at different pH values and adjusting the salt gradient, the conditions tested for the optimization can be found in Table 1. The elution was optimized by running increasingly narrow gradients around the elution peaks of interest, thereby increasing the resolution. Optimization experiments were performed with 190 μg/ml injections, with a total volume of 10 ml, but subsequent scale up to 1.9 mg/ml was unsuccessful. Fig. 1 shows the optimized salt gradient with 190 μg/ml (Fig. 1A) and 950 μg/ml (Fig. 1B) injections. A drastic decrease in resolution was observed even under relatively low loading amounts (<1 mg/ml stationary phase). Due to the low binding capacity observed at pH 7, we did not attempt separation at a higher pH. Since scale up of salt gradient elution failed, we pursued displacement chromatography as a method for creating the standard.

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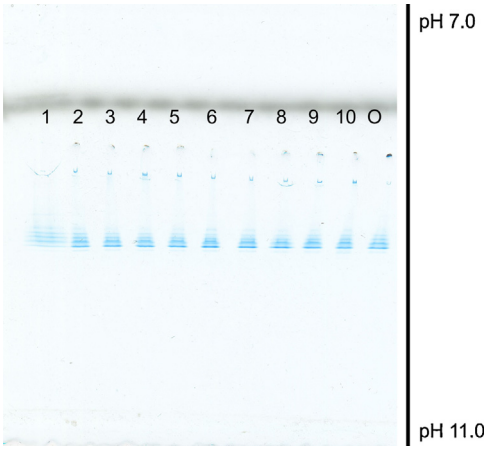

Fig. 2. IPG IEF of the displacement fractions from a displacement experiment on a Mono S column, 5 × 200 mm. Flow rate: 0.25 ml/min (76 cm/h). Mobile phase: 30 mM MOPSO, 15 mM NaCl, pH 7.0. Displacer: 5 mM Expell SP1. Lanes 1–10 contain displacement fractions with higher numbers denoting later eluting fractions. O is the initial sample. No difference in any fraction could be observed. No marker was used, since only the isoelectric point (pI) relative to the initial sample was relevant.

3.1.2. Displacement chromatography

Displacement chromatography was tested at three different conditions, as described in Table 2. Expell SP1 was chosen as the displacer, as it has proven to be useful in IgG separations [17,18,27]. No separation of charge variants was detectable after analysis with IEF. A representative result from the pH 7.0 displacement run is shown in Fig. 2. Due to the disappointing results in all three displacement experiments, we decided to scale up our pH gradient elution method to a semi-preparative scale.

3.1.3. pH gradient elution

Finally preparative separation of the charge variants using pH gradient elution was used, lacking an orthogonal method for creating the standard. The initial mAb sample was purified as shown in Table 4 and acidic and main peak fractions were collected, as shown in Fig. 3(A). After concentration and buffer exchange of the eluate with ultra-/diafiltration, the standards were used in a second round of purification using a shallower gradient to remove the remaining unwanted charge variants. All three chromatograms can be seen in Fig. 3, including the fractionation that was used to obtain the pure standard material. It should be noted that the linearity of the pH gradients stays almost unaffected, even with higher loads of antibody. The gradients used can be found in Table 4.

The standards obtained this way will be referred to as main charge variant (MCV) and acidic charge variants (ACV). Because of non-orthogonality of the method used for creating the standard, IEF was used to confirm that the standard material indeed consisted of purified and separated charge variants. Fig. 4 shows the original mAb sample and both of the standards used in this work. No main band can be detected in the ACV sample, and only a faint band with a lower isoelectric point (pI) is visible in the MCV sample. It was concluded that these two samples will be of sufficient purity to validate the analytical method.

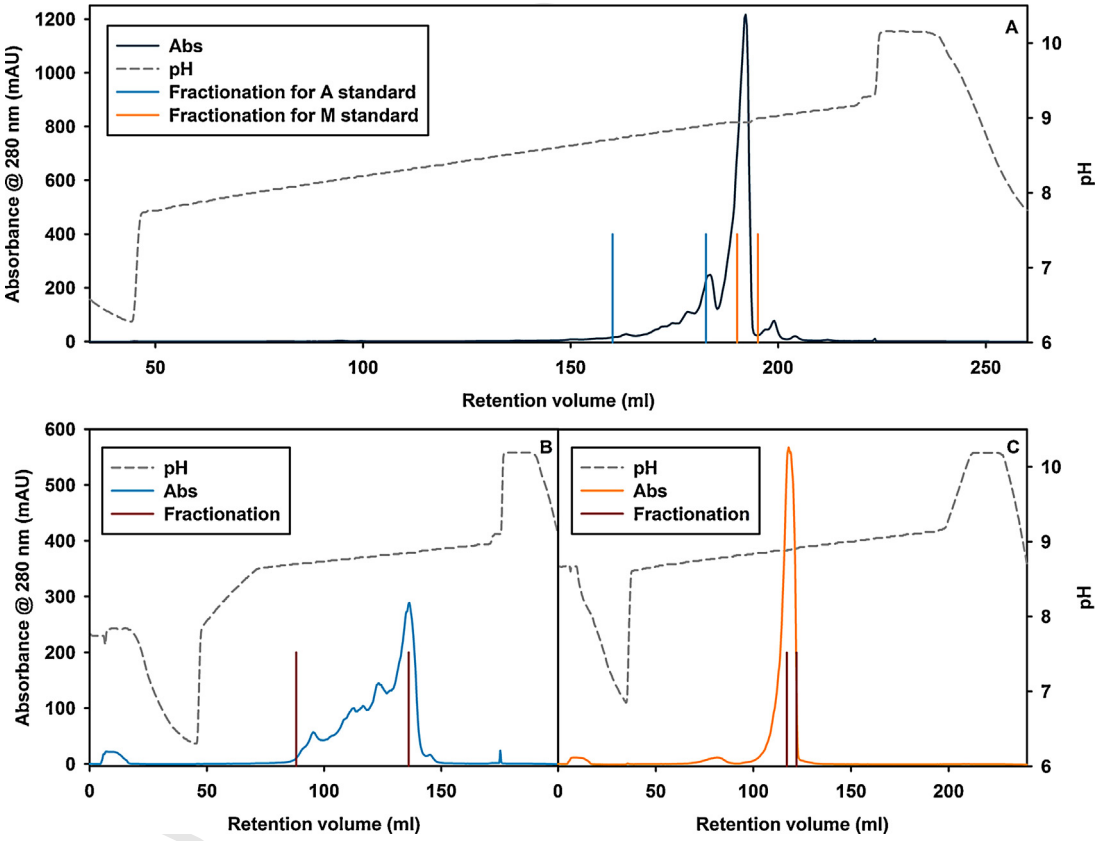

Fig. 3. Chromatograms of the preparative pH gradient elution separation of charge variants on a ProPac WCX-10, 9 × 250 mm column. Flow rate: 2.5 ml/min (236 cm/h). Mobile phase A: 5.5 mM HEPES, 4.2 mM Bicine, 9.5 mM CAPSO, 0.8 mM CAPS, 6.3 mM NaCl, pH 8.0, mobile phase B: 10.5 mM Bicine, 2.5 mM CAPSO, 7.0 mM CAPS, pH 10.5. Panel (A) shows the initial purification and fractionation for the acidic (blue) and the main (orange) standard. Graqdient from pH 8.0 to 9.8 in 14 CV. After concentration and buffer exchange those fractions were used in a second round of purification to further increase the purity. Panels (B) and (C) show the purification and fractionation of the second round for acidic and main standard, respectively. For the acidic standard a 2 CV gradient from pH 8.0 to 8.9 was followed by a 10 CV gradient to pH 9.4. For the main standard a 10 CV gradient from pH 8.9 to 9.4 was used. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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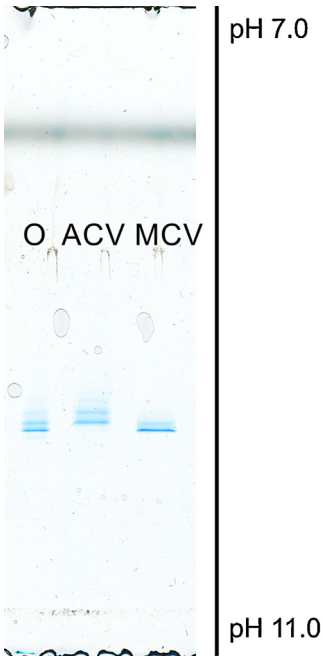

Fig. 4. IEF gel of the initial mAb (O), the acidic charge variants (ACV) and the main charge variant (MCV). A faint band is visible with a pI lower than the main band in the MCV standard. No main band can be detected in the ACV standard.

Table 5
Calibration results.

ACV content (%)	Response (AU*s)	RSD (%)
0	97.2	7.7
1	280.2	6.7
3	632.2	1.4
5	945.3	0.3
10	1719.7	0.6
20	3406.9	0.6
40	6355.5	4.4
50	8079.8	1.0

3.2. Testing of the standard material

The purified MCV and ACV standards were analyzed using the analytical pH gradient elution method to check if the standards are of sufficient purity to be used for the validation and compared with the original mAb sample. A gradient from 35% to 55% buffer B (pH ~8.9–9.4, see Table 3 for composition) in 8 CV was used. This specific gradient was able to separate the charge variants, as shown in Fig. 5, while allowing to reduce the run time. Therefore, it was used for the whole validation. Both standards are of over 99% purity and elute at different times, as shown in Fig. 5C. It was concluded from these experiments that MCV and ACV will be suitable standard material for the subsequent validation experiments.

3.3. Calibration model

In order to confirm the linearity of the calibration model and to find the linear range of the method, spiking experiments were performed. MCV was spiked with ACV, to obtain relative abundances from 1% to 50% acidic species. The calibration model was tested by fitting a linear model to the data obtained with eight concentration levels evenly spaced across the calibration range with three replicates [19]. The results can be found in Table 5 and Fig. 6. The results of the linear regression gave a slope of 157.9 and an intercept of 143.0 with a correlation of regression of 0.9996. The residuals were equally (Fig. 7) distributed and the RSD was lower than 10%

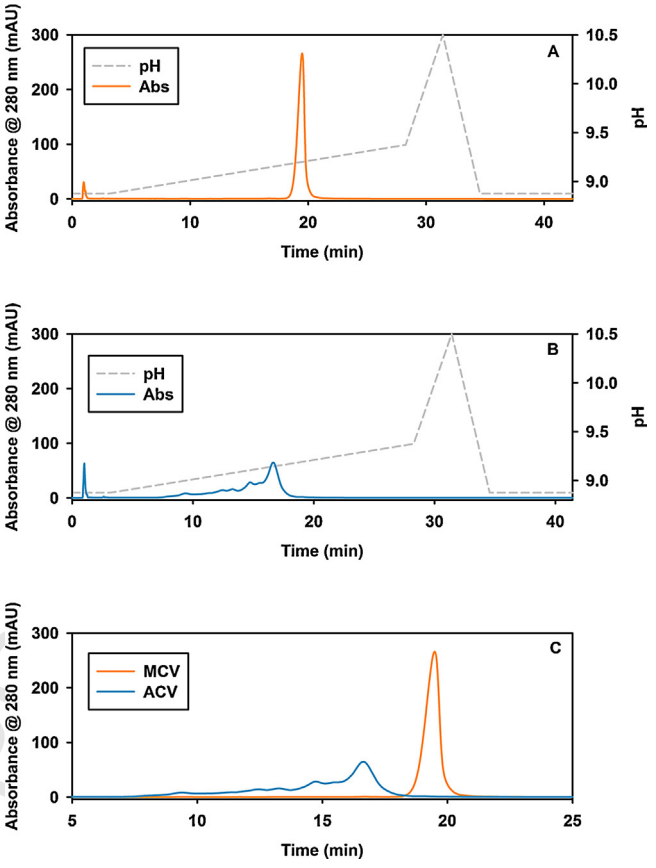

Fig. 5. Evaluation experiments for the suitability of the standard. The MCV (orange) and ACV (blue) standards were tested using the pH gradient elution method. Stationary phase: Dionex ProPac WCX-10, 4 × 250 mm. Flow rate: 1 ml/min (477.5 cm/h). Mobile phase A: 5.5 mM HEPES, 4.2 mM Bicine, 9.5 mM CAPSO, 0.8 mM CAPS, 6.3 mM NaCl, pH 8.0, mobile phase B: 10.5 mM Bicine, 2.5 mM CAPSO, 7.0 mM CAPS, pH 10.5. A gradient from 35% to 55% B in 8 CV was used. Panel (A) and (B) show the chromatograms of the MCV and ACV standard, respectively. The gray dashed line represents the pH at the column inlet, not a measured signal. Panel (C) shows the overlay of both chromatograms from 5 to 25 min. The samples are of high purity and are suited for the use as a charge variant standard. The small initial peak is a solvent peak, consisting of the histidine and Tween 20 of the mAb buffer. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

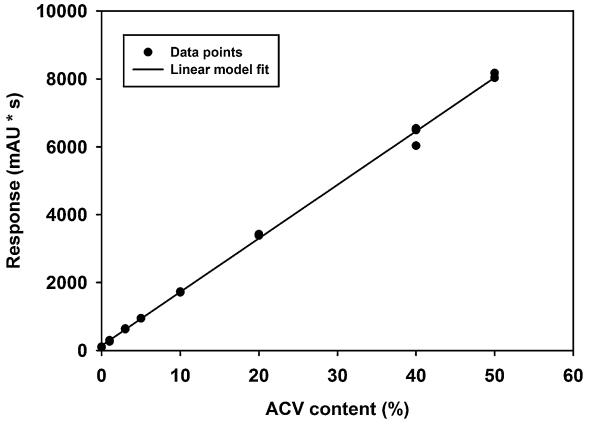

Fig. 6. The calibration curve for the acidic charge variant (ACV) content range of this study.

Table 6
Results of the accuracy and precision measurement.

Relative amount of spiked ACV (%)	Average relative charge variant content measured (%)	Bias (%)	Standard deviation	Relative standard deviation (%)
1	0.82	−17.6	0.07	8.9
25	25.37	1.5	0.38	1.5
50	49.96	−0.1	0.32	0.6

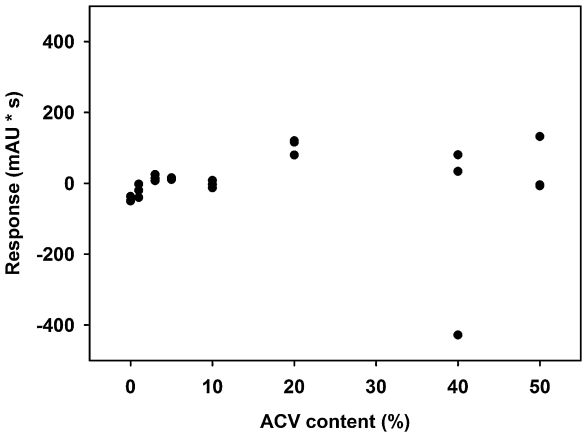

Fig. 7. Residuals of the calibration curve.

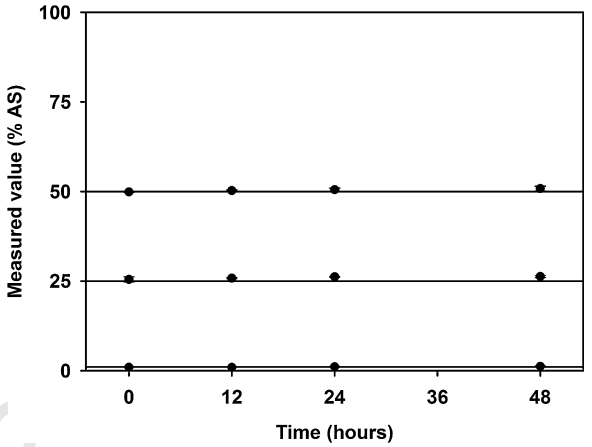

Fig. 8. Results from the stability experiments over 48 h. Samples with 1%, 25% and 50% ACV were measured in triplicates at 0, 12, 24 and 48 h.

over the whole range. One value with an unusually large residual was observed, but Grubb's outlier test ($\alpha = 5\%$) determined that this was not an outlier [12]. ANOVA was used to confirm the validity of a linear calibration model, with the following results: linearity F test ($\alpha = 1\%$), $F^* = 16640.41 > F = 7.95$, which leads to the acceptance of linear model; lack of fit F test ($\alpha = 1\%$), $F^* = 1.23 < F = 4.20$, which leads to the acceptance of the null hypothesis of good model fit.

3.4. Accuracy and precision

The accuracy and bias were determined by the statistical evaluation of duplicate experiments performed on eight days at three different concentrations, as recommended by Peters et al. and Hartmann et al. [12,19]. The concentration levels selected were the LLOQ, the ULOQ and the median, i.e. 1%, 50% and 25% ACV, respectively. The precision was determined as the overall RSD, with acceptance criteria being 20% RSD for the lower limit of quantification and 15% for the other two spiking levels. The accuracy was determined as the deviation of the grand means from the respective reference values, with acceptance criteria being ±20% bias for the lower limit of quantification and ±15% for the other two spiking levels [19,28,29]. The results from measuring at the LLOQ, the median and the ULOQ can be seen in Table 6. Both the bias and the RSD are within the acceptance criteria. The bias at the LLOQ is rather high, with −17.6%, suggesting that quantification of charge variants below 1% in relative abundance will not be practical. This also served as confirmation of our estimation of the LLOQ for this method.

3.5. Selectivity

We assume that this method will be used for the determination of charge heterogeneity of purified monoclonal antibodies only, and as such the components that might be expected to be present in the samples analyzed are small molecules, such as buffering compounds, amino acids, sugars and detergents. As such, selectivity, "the ability to measure the analyte in the presence of components which might be expected" [19], has been shown previously [14].

3.6. Stability

The stability of mAb samples inside the autosampler, at 10 °C, and the stability of the pH gradient buffers was tested over a time frame of 48 h. Samples were measured in triplicates at 0, 12, 24 and 48 h. The distribution of the measured values over time can be seen in Fig. 8. Those results demonstrated that the samples and the method are stable for 48 h and the method can therefore be used over night or over the weekend.

4. Conclusions

We presented the development of a highly linear pH gradient elution method in our previous work [14]. In the present complementary work, we have validated key method parameters, such as the accuracy and precision. The method uses commercially available stationary and mobile phase components, which allows others to implement this method easily. Other methods such as IEF provide a higher resolution than our method, but can only be considered as semi-quantitative method, cannot be automated as easily and cannot be scaled up to semi-preparative scale. The method for the quantification of mAb charge variants is valid for the quantification of purified mAb samples with a charge variant content as low as 1%. The linearity of the calibration range was confirmed through statistical testing and also showed an excellent coefficient of correlation of 0.9996. 1% charge variants was confirmed as the LLOQ, with an accuracy (bias) of −17.6% and a precision of 8.9%. For samples measured at the median or the ULOQ, the method had excellent accuracy and precision of below 2%. We have previously presented methods for the creation of gradients from pH 7.0 to pH 10.5. The big advantage of our method is that the linearity of the gradient is still high even when a narrower gradient is being used to increase the resolution. This means that our method can be easily customized for the analysis of different mAb, just by varying the initial and the final buffer concentration. In this study, we have used an antibody with a chromatographic pI of 9.2 and have adjusted our method gradient from 35% to 55% buffer B accordingly, which corresponded to a pH gradient from 8.9 to 9.4.

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Compared to iCIEF, our method shows comparable precision [23], but no thorough validation could be found in which accuracy of iCIEF was determined. We conclude that our method is comparable to other standard methods. The biggest difficulty in performing this study was the production of the standard material. Our attempts at using standard salt gradient elution cation exchange chromatography proved unfruitful. We were unable to find conditions under which we had a sufficient resolution with an adequate protein load. The approach presented by Rozhkova [21], i.e. cation exchange chromatography combined with salt gradient elution, only worked for very small loading amounts for our mAb and additionally did not achieve the resolution we observed in pH gradient elution experiments. Unfortunately Rozhkova does not present analytical data of her standard material as well as more detailed information about loading amounts. Accordingly it is difficult to compare the two methods.

Our next approach, displacement chromatography [17], did not yield a separation of charge variants, under any conditions. This is in stark contrast to the results that Zhang et al. [18] have presented previously. Eventually, we used a preparative pH gradient elution method to prepare our standards, and confirmed their identity as charge variants by IEF. This approach proved to be highly effective in separating large amounts. It has previously been shown that induced pH gradients can be used for the preparative separation of mAb charge variants [30–32], and we are happy to report that our external pH gradients are able to achieve the same feat. Solubility problems due to elution close to the pI were not observed, even at the preparative scale used in this work, making this a high resolution method for the preparative separation of protein charge variants.

Acknowledgments

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Review

The sweet tooth of biopharmaceuticals: Importance of recombinant protein glycosylation analysis

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Biopharmaceuticals currently represent the fastest growing sector of the pharmaceutical industry, mainly driven by a rapid expansion in the manufacture of recombinant protein-based drugs. Glycosylation is the most prominent post-translational modification occurring on these protein drugs. It constitutes one of the critical quality attributes that requires thorough analysis for optimal efficacy and safety. This review examines the functional importance of glycosylation of recombinant protein drugs, illustrated using three examples of protein biopharmaceuticals: IgG antibodies, erythropoietin and glucocerebrosidase. Current analytical methods are reviewed as solutions for qualitative and quantitative measurements of glycosylation to monitor quality target product profiles of recombinant glycoprotein drugs. Finally, we propose a framework for designing the quality target product profile of recombinant glycoproteins and planning workflow for glycosylation analysis with the selection of available analytical methods and tools.

Keywords: Analytical biotechnology · Carbohydrates · Chromatography · Mass spectrometry · Recombinant proteins

1 Introduction

Biopharmaceuticals appeared 30 years ago with the approval of Humulin®, the first recombinantly produced insulin [1]. Today, the biopharmaceutical market is a highly competitive and growing sector of the pharmaceutical industry representing 99 billion US$ of sales in 2009 [2]. Many patents for blockbuster therapeutic proteins are about to expire and the question of biosimilar and biogeneric regulation has come to the fore [3]. Owing to the fact that the majority of those biopharmaceuticals are glycoproteins [4], the elucidation of their glycosylation patterns, a key determinant of functionality and efficacy, is of utmost importance.

Glycosylation is the most common modification found in secreted proteins, but also the most structurally diverse [4]. Indeed, unlike DNA, RNA or protein, glycan synthesis is not based on templates, but consists instead of an assembly of monosaccharides that are enzymatically linked in various ways [5]. Additionally, proteins can present different glycoforms that vary both in the type and number of glycans and their attachment sites. Because correct glycosylation is usually required for the optimal function of glycoprotein pharmaceuticals, they are currently produced in eukaryotic systems [6]. Efforts are underway to

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Abbreviations: ADCC, antibody-dependent cellular cytotoxicity; **CDC**, complement-dependent cytotoxicity; **CID**, collision-induced dissociation; **CQA**, critical quality attribute; **DMB**, 1,2-diamino-4,5-methylenedioxybenzene; **EPO**, erythropoietin; **ESI**, electrospray ionization; **ETD**, electron-transfer dissociation; **Fab**, fragment antigen-binding; **Fc**, fragment crystallizable; **Fuc**, fucose; **Gal**, galactose; **GalNAc**, N-acetylgalactosamine; **GCase**, glucocerebrosidase; **GlcNAc**, N-acetylglucosamine; **HILIC**, hydrophilic-interaction liquid chromatography; **HPAEC-PAD**, high pH anion exchange chromatography with pulsed amperometric detection; **HPLC**, high performance liquid chromatography; **HTM**, high throughput method; **IEF**, isoelectric focusing; **LC-MS**, liquid chromatography-mass spectrometry; **mAb**, monoclonal antibody; **MALDI-TOF**, matrix-assisted laser desorption ionization coupled with a time-of-flight analyzer; **Man**, mannose; **MS**, mass spectrometry; **MS^n**, multi-stage mass spectrometry; **Neu5Ac**, N-acetylneuraminic acid; **Neu5Gc**, N-glycolylneuraminic acid; **NP**, normal phase; **QbD**, quality by design; **QTPP**, quality target product profile; **RP**, reversed phase

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engineer prokaryotic cell lines to produce proteins with mammalian like glycosylation which could replace eukaryotic cell lines in the future [7]. The glycan structure present on proteins can have profound effects on their stability and function and mediates the efficacy of many biopharmaceuticals [4, 8].

Even after over 20 years of research characterizing biopharmaceutical glycosylation, many challenges remain. In this review, we will first describe the important role that glycosylation plays biopharmaceuticals. Secondly, current analytical methods for the characterization of biopharmaceutical glycosylation will be presented.

2 Functional importance of glycosylation

Glycosylation is ubiquitous on cell-surface and secreted proteins. In eukaryotic cells, glycans are produced and maturated in the endoplasmic reticulum and Golgi apparatus. An overview of the most common N-glycan structure found on human proteins is shown in Fig. 1. Mucin-type O-glycosylation will be introduced in the last part of this section and common human core O-glycan structures are illustrated in Fig. 2. Since extensive descriptions of general properties of glycosylation already exist [5, 9], this review will illustrate the importance of glycosylation through specific examples.

As glycosylation is a complex network of metabolic reactions in eukaryotic cells, the resulting end products (glycans) are a mixture of different structures. Heterogeneity of glycosylation is contributed by two main categories, microheterogeneity and site occupancy. In the context of biopharmaceuticals, glycosylation critically modulates the therapeutic efficacy and safety of the drugs. Most of these drugs are produced in cultured mammalian cells which effectively glycosylate proteins. A myriad of process conditions have been reported to influence the outcome of glycosylation [10]. Therefore, changes in process conditions may lead to inconsistent glycosylation profiles of the recombinant protein during process scale-up and between production batches. Recently, the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) proposed several guidelines detailing the specifications of biopharmaceuticals through characterization of site-specific glycan structures (ICH Q6B) [11], as well as comparability of such structure profiles during process scale-up and changes in manufacturing process (ICH Q5E) [12]. Hence, glycosylation should be considered as a key critical quality attribute (CQA) which should be carefully controlled and monitored throughout the development and production processes [13].

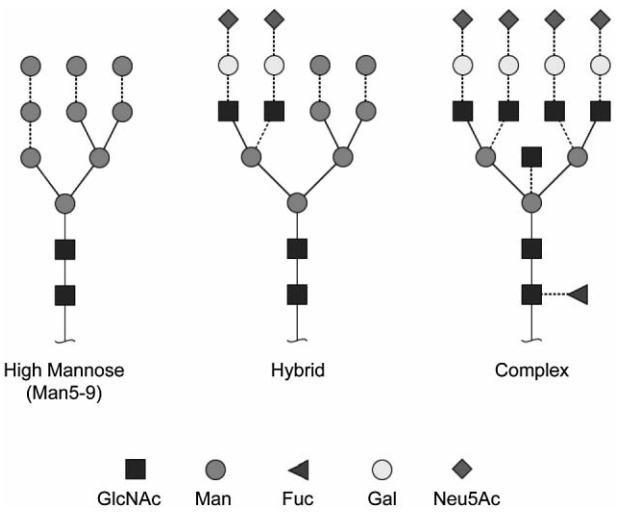

Figure 1. Overview of N-glycan structures found on human proteins. High mannose glycans can have between five and nine mannose residues as signified by the dotted lines. Hybrid type glycans carry only mannose residues on the Manα1-6 arm and one or two antennae, with varying degrees of extension on the Manα1-3 arm. Complex type glycans have between two to four antennae with varying degrees of extension. The antennae can terminate in GlcNAc, Gal or Neu5Ac in humans. In most other mammals, Neu5Ac can be replaced by another sialic acid called Neu5Gc. Bisecting GlcNAc can be present on the initial Man and Fuc substitution can be found on the proximal GlcNAc from the core of the N-glycan.

2.1 N-linked glycosylation

Next, we provide brief highlights of three biopharmaceuticals focusing on the functional importance of N-glycosylation in several efficacy aspects, including in vivo bioactivity, half-life and drug targeting. It is hoped that such review will help prioritize favorable quality target product profiles (QTPP). This will be followed by a short review of O-glycosylation and then a report of current analytical methods for the monitoring of such CQAs.

2.1.1 IgG

Since 1986, more than 30 monoclonal antibodies (mAbs) and mAb derived products have been approved in different countries for therapeutic purposes [6, 14]. The ability to bind any type of extracellular target with high affinity and specificity makes mAbs very versatile, which is part of the reason why they are the fastest growing category of therapeutic drugs [15]. At the time of writing, mAbs are approved for many different indications, with the majority being for the treatment of oncological and autoimmune diseases [15]. The immunoglobulin G (IgG) mAb subclass has been particularly targeted for biopharmaceutical development.

A human IgG consists of two Fab (fragment antigen-binding) domains and one Fc (fragment crystallizable) domain connected by a hinge region. While the Fab is responsible for antigen binding, providing the high affinity and specificity that makes them valuable as pharmaceuticals, the Fc region is responsible for effector functions. This is achieved by binding to different receptors, namely the binding of components of the complement pathway

like C1q, the different types and isoforms of Fcγ receptors (FcγRI to FcγRIII) and the neonatal receptor FcRn that, among other functions, regulates serum levels. The binding to FcγR and C1q depends highly on the glycosylation status of IgG [16]. Each heavy chain carries an evolutionarily conserved glycosylation site, totaling at least two glycan moieties for an intact IgG molecule. Due to the genetic variability of antibodies, especially in the Fab region, additional N- and O-glycosylation sites can be present, sometimes influencing antigen binding. For example, in the case of trastuzumab, an additional N-glycosylation site in the Fab region was found to be present, which was removed later on by mutation in the final humanized version of the drug for consistency [17].

The N-glycan of the Fc region is not located on the surface of the protein, but instead occupies a "pocket" inside the C_H2 domain. The oligosaccharides present in this region influence the structure of this C_H2 domain through non-covalent interactions and these structural changes can have an impact on receptor binding [18]. The N-glycan most commonly found on human IgG is of the complex biantennary type terminates either with N-acetylglucosamine (GlcNAc) or with one or two galactose (Gal), with low levels of N-acetylneuraminic acid (Neu5Ac), core fucose and bisecting GlcNAc also being present (Fig. 1).

The importance and effect of particular glycoforms on effector functions has been elucidated in several studies [6, 17, 19]. Indeed, IgG1 glycoforms without core fucose show increased antibody-dependent cellular cytotoxicity (ADCC) due to higher binding affinity to FcγRIIIa [6]. Ex vivo studies showed that this increased ADCC is observed only with natural killer cells as effector cells, while the ADCC mediated by polymorphonuclear neutrophils seems to be adversely affected by lower fucose levels [20]. Core fucosylation was shown not to affect complement dependent cytotoxicity (CDC) [21]. CDC on the other hand seems to be increased by the presence of terminal galactose [6]. The effect of terminal galactosylation on Fcγ receptor binding, and thus ADCC, is not fully understood and conflicting results exist [22, 23]. Bisecting GlcNAc, which can be found on the human antibody N-glycan but has not been found in other mammals, seems to have a positive effect on ADCC [24].

Increased levels of Neu5Ac on the N-glycan decreases ADCC, due to reduced binding affinity to FcγRIIIa [25]. Higher Neu5Ac content enhances anti-inflammatory activity [26], but Anthony and Ravetch [19] showed that this is only true for α2,6 linked sialic acid, not for α2,3 linked Neu5Ac. This anti-inflammatory activity is thought to be mediated by Fc binding to FcγRIIb, an inhibitory receptor on macrophages.

Effects of mAb glycosylation on efficacy require extensive analytical methods for the characterization and monitoring. Moreover, the inclusion of immunogenic sugars like Neu5Gc or α1,3gal epitope needs to be carefully evaluated since these glycoepitopes can generate adverse reactions [27].

2.1.2 *Erythropoietin*

Erythropoietin (EPO) is a glycoprotein hormone used for the treatment of anemia [28]. The mature EPO molecule consists of 165 amino acid residues and is decorated with three N-linked glycans and one O-linked glycan, which together constitute almost 40% of its molecular mass. The attached carbohydrates were found to critically affect the secretion and solubility of EPO [29]. It also has profound effect on the in vivo bioactivity of EPO, which in part is due to the clearance of undersialylated glycoforms of EPO by liver asialoglycoprotein receptor [30]. In fact, early studies showed that sialylated EPO had a half-life of about 3 hours while that of the desialylated EPO was only about 2 min [30]. Therefore, sialic acid content of EPO is a critical quality attribute (CQA) that determines its efficacy as a therapeutic drug. EPO as a biopharmaceutical product is produced recombinantly in CHO and BHK cells which are capable of producing "human-like" glycans [28], and more recently in a gene-activated human fibrosarcoma cell line, HT-1090 [31]. Because of the heterogeneity of glycosylation in mammalian cells, it is important to analyze the glycan structures and pay special attention to the sialylation of EPO to ensure safe, optimal, and consistent drug performance. Indeed, early structural analysis of sialylated N-glycan fractions of CHO-K1 and BHK-21-produced EPO revealed a few interesting features such as the presence of Gal-GlcNAc (LacNAc) repeating units in products from both cell lines, a paucity of Neu5Gc in CHO-derived recombinant EPO [32, 33], as well as the presence of Man-6-phosphate in BHK-derived recombinant EPO [34]. In a more recent report, Shahrokh et al. [33] compared the glycosylation of EPO produced in a gene-activated human cell line (Dynepo) with that of CHO-derived EPO (Eprix, NeoRecormon) and the glycoengineered EPO analog (Aranesp) and found significant differences in N-glycan structures. For example, EPO produced in the human cell line (Dynepo) was found to exclusively contain sLe^x structures on its N-glycans and possessed highest proportion of tetraantennary N-glycans and the lowest amount of poly-LacNAc repeating units. Aranesp was found to contain O-acetylated sialic acid residues, whereas such modification is absent in Dynepo [33]. Apart from the significant differences observed in innovator EPO products, a recent report highlighted the dramatic differences in glycosylation between innovator EPO and its biosimilars produced in Asia [35]. Significant differences in sialylation were detected, which may lead to inconsistent clinical performances of EPO biosimilars.

Taken together, this demonstrates that glycosylation of EPO can be significantly influenced by the host cell line, production process, or protein engineering. This example stresses the importance of glycosylation as a key

COA, which critically determines the safety and efficacy of EPO. As such, glycosylation patterns must be carefully characterized using a comprehensive array of analytical methods.

2.1.3 *Glucocerebrosidase*

Glycosylation of protein-based biopharmaceuticals can also play a critical role in a unique aspect of drug efficacy, which is targeting. The impact of glycosylation on drug targeting is well exemplified by the glucocerebrosidase. Glucocerebrosidase (GCase), or acid β-glucosidase, is a lysosomal enzyme that cleaves glucosylceramide into glucose and ceramide. It contains five N-glycosylation sites, the first four of which are occupied [36]. Loss-of-function mutations in the gene encoding GCase result in accumulation of glucosylceramide in the lysosomes of macrophages, leading to a lysosomal storage disorder termed Gaucher's disease. Treatment of Gaucher's disease relies on enzyme replacement therapy (ERT) in which exogenous GCase is administered intravenously into patients [37]. Effective targeting and internalization into macrophages critically depend on the terminal mannose residues on the N-glycans of GCase, which are recognized by macrophage cell surface mannose receptors. The first therapeutic preparation of GCase was purified from human placenta [37]. To ensure therapeutic efficacy, it was sequentially deglycosylated to reveal the core mannose (Man3) by a combination of exoglycosidase treatment steps [37]. Although the source of GCase was later changed to recombinant expression system using CHO cells (imiglucerase, or Cerezyme) due to the growing demand worldwide, the same post-purification enzymatic treatment steps are still necessary to ensure drug efficacy [37]. Alternative expression systems that are capable of producing mannose-terminated glycans have been proposed as safe and cost effective production methods [38, 39]. One example is the expression of recombinant GCase, subsequently targeted to storage vacuoles, in carrot cells [39]. As a result, structural analysis of this recombinant GCase demonstrates predominantly pauci- and oligomannose-type N-glycans ranging from Man3 to Man5, leading to a bioactivity similar to that of Cerezyme [39]. Recently, this recombinant GCase (taliglucerase alfa, brand name Elelyso), developed by Protalix, became the first FDA-approved biopharmaceutical protein produced in plants. Success of taliglucerase alfa paves the way for the therapeutic applications of more plant-produced glycoproteins.

In addition to the need for glycan structural characterization, the presence of an unoccupied N-glycosylation site on GCase requires complementary analysis to ascertain the site occupancy and site-specific distribution of glycans. As glycosylation can potentially modulate the conformation of a protein, GCase biosimilars must demonstrate a lack of N-glycosylation at the fifth N-glycosylation sequon to ensure comparable conformation and activity with reference to the original recombinant product, Cerezyme [38]. Recent advancements in site-specific distribution analysis by liquid chromatography-mass spectrometry (LC-MS) will be introduced in the second part of this review.

2.2 O-linked glycosylation

Mucin-type O-glycosylation is another type of glycosylation initiated in the *cis*-Golgi with the attachment of a GalNAc residue to a Ser or Thr residue in a protein. In contrast to N-glycosylation, the consensus sequence for such attachment is still unknown, despite evidence suggesting the preference of regions rich in Ser/Thr, Pro and Ala for the attachment of O-glycans [40]. The GalNAc residue O-linked to Ser/Thr is also known as the Tn antigen (Fig. 2). Modification of the Tn antigen by a sialic acid results in a sialyl-Tn antigen (STn), a "dead end" structure, whereas modification of Tn antigen by a Gal residue results in a core 1 structure, also known as a T antigen. Because Tn, STn and T antigen structures are exclusively expressed on the surface of some cancer cells, they are known as tumor-associated O-glycans [41]. Modification of Tn antigen by a GlcNAc residue leads to a core 3 structure. Addition of GlcNAc to core 1 and core 3 structures results in core 2 and core 4 structures respectively (Fig. 2). All these core structures can be further processed, leading to extended core 1–4 structures [40].

The current understanding of O-glycosylation is poor in the context of biopharmaceuticals. One of the few examples highlighting the importance of O-glycosylation is

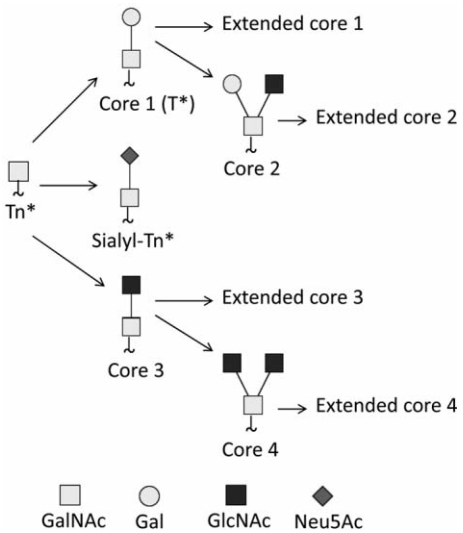

Figure 2. Common core O-glycan structures found on human proteins. Biosynthesis of mucin-type O-glycan is initiated with the attachment of a GalNAc residue (Tn antigen) to Ser/Thr in a protein. Modification of the Tn antigen gives rise to a collection of structures with common core structures (core 1–4). Tn, Sialyl-Tn and T antigens are "tumor-associated antigens" and are indicated by asterisks (*).

that the presence of O-glycan on granulocyte-colony stimulating factor (G-CSF) covers the underlying peptide sequence, thus protecting against immune response elicited by this peptide [42]. Conversely, recombinant G-CSF produced in *E. coli* and yeast which does not bear the O-glycan elicits neutralizing antibody response. Moreover, O-glycan structures of endogenous and recombinant human EPO displayed significant differences. Early studies showed that CHO and BHK-derived recombinant EPO carried predominantly mono- and di-sialyl T structures [32, 43] whereas the human urinary EPO were predominantly modified by sialyl- or asialo-Tn O-glycans [44].

The future promise of mucin-type O-glycosylation in biopharmaceutical research comes from the observation of mucin O-glycosylation change in cancer. Mucins are a family of large, heavily O-glycosylated proteins normally expressed on apical surface of glandular cells [40]. The first and best characterized mucin molecule, human mucin 1 (MUC1), was found to be extensively decorated with branched O-glycans. However, in many cases of adenocarcinoma, the expression of MUC1 is elevated [40]. In addition, the attached O-glycans are truncated, with Tn, STn and T being the predominant structures. It has been shown that in many cases of breast and ovarian cancer, such aberrant O-glycans provoked immune response with the generation of circulating autoantibodies against MUC1 O-glycopeptides [45]. Moreover, higher levels of such autoantibodies were shown to be correlated to better prognosis of breast cancer [46]. Based on this rationale, researchers have been investigating the possibility of producing recombinant MUC1 protein/peptide carrying cancer-like O-glycans as a prophylactic or therapeutic vaccine candidate [47, 48]. Success of such ideas relies on two critical aspects: i) an appropriate host cell line that is capable of producing tumor-associated O-glycans; and ii) a robust analytical technique that can reliably profile the O-glycan structures associated with the protein. Several reports have shown the differential site occupancy of MUC1 by O-glycans in different cell lines, ranging from about 2 to more than 4 O-glycans per repeat [48–50]. Thus, we postulate that site-specific analysis of MUC1 O-glycosylation will be necessary for optimal and consistent therapeutic performance of MUC1.

Due to the lack of enzymatic release method, structural analysis of O-glycans is more challenging as compared to N-glycans. Various chemical release methods have been developed but all have their own limitations [51-53], including the conversion of reducing end sugar to alditol by reductive β-elimination [51], the involvement of the hazardous hydrazine by hydrazinolysis [53], as well as the degradation of the reducing end monosaccharide of the O-glycans (an effect termed as "peeling") [54]. Although alternative chemical release methods have been proposed, more investigations are needed to check the release efficiency and integrety of O-glycans [55]. Analysis of released O-glycans can be performed by chromatographic or mass spectroscopy (MS) approaches similar to that of N-glycans, given the availability of O-glycan standards and knowledge of the biosynthetic pathway in a particular cell type, respectively [56]. Therefore, the subsequent review on analytical methods can serve as a guideline for both types of glycosylation analysis.

3 Analytical methods

3.1 Isoelectric focusing

Isoelectric focusing (IEF) separates proteins based on their isoelectric points, or *p*I values. As a result, glycoproteins can be resolved into bands representing differentially charged glycoforms on an IEF gel. This method has been extensively used as a qualitative or semi-quantitative method for anti-doping, quality control or comparison purposes. For example, it has been utilized by the World Anti-Doping Agency to distinguish endogenous and administered recombinant human EPO in athletes based on the difference in sialylation of the two forms of EPO [57]. This method was also employed to compare the innovator product of EPO with its biosimilars [35]. In addition to EPO, IEF analysis of IgG coupled with lectin blotting suggested an association of glycosylation change with rheumatoid arthritis [58]. The afore-mentioned examples highlight the sensitivity and reliability of the IEF technique in the analysis of glycoproteins.

MS and chromatography-based methods for glycan analysis can be costly and time-consuming. The IEF technique, on the hand, eliminates the need of protein purification when coupled with affino- or immunoblotting. It also allows parallel analysis of multiple samples, thus significantly increasing the throughput for sample analysis [59]. Because IEF readout on unpurified samples often depends on affino- or immunoblotting detection, sensitivity of this assay is primarily determined by the latter. In the case of EPO glycoform profiling, 20–50 mIU (fmol range) of protein has been routinely analyzed by IEF followed by immunoblotting [60, 61].

Major limitations of IEF include the inability to resolve different neutral glycoforms. Among the charged glycoforms, IEF alone cannot distinguish the type of sialic acids (Neu5Ac vs. Neu5Gc). The charge can be confounded by the potential interference of phosphorylation and sulfation which also give negative charges to glycans. Quantification of different bands is also challenging. However, these limitations can be resolved by sialidase treatment prior to IEF to rule out phosphorylation/sulfation interference and by using complementary approaches such as determination and quantitative sialic acid assays (see below). Nevertheless, IEF still remains the method of choice for screening and initial comparison of acidic glycoforms of recombinant proteins given its capacity for fast, sensi-

tive medium-throughput sample analysis without any need of purification.

3.2 Sialic acid assay

Because sialic acid can critically determine the in vivo bioactivity especially the circulatory half-lives of glycoprotein drugs, sialic acid content represents a CQA for many biopharmaceuticals, and thus requires quantitative analysis. The traditional thiobarbituric acid (TBA) assay for sialic acid quantification requires purification of glycoprotein samples and are typically lengthy (~ 1 day) [62]. Recently, Markely et al. [63] developed a high-throughput method (HTM) that allows for rapid quantification of sialic acids carried by glycoproteins in crude culture supernatants. Briefly, this method involves chemical reduction of interfering molecules in the supernatant, followed by enzymatic release of sialic acids which are then derivatized by malononitrile for fluorescent detection. The reduction step prior to sialic acid release removes the interference of culture medium components and therefore gives the HTM assay higher specificity than the TBA assay [63]. The whole procedure can be done in microplate format within 15 min and was shown to be at least 10 times more sensitive (2 μM quantification limit) than other sialic acid quantification kits [63]. Due to these advantageous features, the HTM assay represents a method of choice for quantitative monitoring of glycoprotein sialylation throughout product development and optimization processes. On the other hand, the HTM assay – as a "pan-sialic acid" assay – is unable to discriminate different types of sialic acid (Neu5Ac vs. Neu5Gc) on a glycoconjugate. If more detailed information about the sialic acids (Neu5Ac and Neu5Gc) is required, chromatographic methods can be employed. In the case of biopharmaceuticals, it is crucial to determine the amount of Neu5Gc, since antibodies against Neu5Gc have been detected in humans [64].

One chromatographic method available utilizes a fluorescent tag (DMB, 1,2-diamino-4,5-methylenedioxybenzene) to both retain sialic acid species in reversed phase chromatography medium and allow detection with high sensitivity. Even though initially described more than a decade ago [65], it is still commonly used for determining the quantity of Neu5Ac and Neu5Gc in recombinant glycoproteins [66]. The method requires the acidic or enzymatic release of sialic acids and subsequent derivatization by DMB, prior to the analysis by reversed phase high performance liquid chromatography (RP-HPLC) or LC-MS. This approach is characterized by good resolution and excellent sub-picomolar sensitivity, but also requires a time consuming derivatization step [65, 67].

An alternative chromatographic method, high pH anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD), was developed in 1998 [68]. While the method still requires the release of sialic acids, it omits the derivatization step. HPAEC-PAD makes use of the weakly acidic properties of sugars at high pH values [68, 69]. It is commonly used for the separation of monosaccharides and does not require derivatization, since the detection is based on electrocatalytic oxidation on gold electrodes in basic conditions. Specific anion exchange columns are used with isocratic or gradient elution in NaOH buffers with or without acetate as a pushing agent with run times in the range of 30 minutes. The limit of quantification is slightly higher than that of the DMB method (10–20 pmol) but the resolution and the preparation time are superior [67]. Standard curves for each substance are necessary for the quantification due to the different response of analytes on the detector. This should not be considered as a serious drawback for biopharmaceutical analysis, since Neu5Ac and Neu5Gc standards are available. This method has excellent resolution, good sensitivity and, due to the lack of labeling, is faster than the DMB method. However, it requires equipment that can operate at very high pH.

3.3 HPLC profiling of released glycans

Information about the monosaccharide content and composition holds only part of the information about protein glycosylation. For more information about the glycan structure, including branching and site occupancy, HPLC and MS methods are the most important to date, and are often used in tandem. A powerful approach consists of the release of the glycan, either enzymatically (preferred for N-glycans) or chemically, and the subsequent separation and detection of the free glycan by chromatography after fluorescent labeling. The hydrophobicity of the aromatic labeling tag allows RP-HPLC to be employed for separation. Alternatively, the hydrophilic nature of the glycan can be harnessed by using normal phase (NP)-HPLC or hydrophilic-interaction liquid chromatography (HILIC). All three chromatographic methods utilize volatile buffer substances and can therefore be directly linked to mass spectrometric detection.

Peptide N-glycanase F is the standard approach of releasing N-glycans from the protein [70]. After release of the glycan and clean up, the oligosaccharides are labeled with an aromatic fluorescent dye [71], but 2-aminobenzamide (2-AB) is considered the gold standard [72]. The reaction is non-selective of particular structures, allowing for the quantification of the detected glycans without bias. If sialic acids are present in the sample the temperature during labeling should not exceed 70°C to avoid hydrolysis. Since the labeling process is a first order reaction, care has to be taken to achieve full derivatization under long enough reaction times [73]. If mass spectrometry is to be used as a subsequent detection step, 2-AB labeling has the added advantage of increasing the sensitivity of MALDI-MS analysis compared to native glycans [74].

Sensitivities of 10 fmol for 2-AB labeled glycans have been reported, but for routine analysis sensitivities in the range of 100 fmol are more common [75].

HPLC with fluorescence detection might be the best mode of quantification, since there is no possibility of ion-suppression as compared to HPLC-MS. Due to insufficient resolution of some glycans using HPLC-MS, there can be peak overlays, which will complicate quantification and lead to missing detection of minor species. An alternative method is the use of HILIC columns. HILIC retention correlates highly with mass and as such is not fully orthogonal to MS. New chromatographic materials with small average bead diameters (1.7–3 μm) can increase peak resolution. Analysis times under 30 minutes can be achieved if appropriate UPLC systems are used. Together, this makes HILIC an attractive alternative to RP-HPLC [76–78]. Additionally, porous graphitic carbon (PGC) may be promising stationary phase for glycosylation analaysis, allowing separation of underivatized glycans. However, this method is still lacking reproducibility and robustness, making it unattractive for industrial use at the present time [79].

Apart from being popular for the analysis of monosaccharides, HPAEC-PAD can also be used for separation of released glycans, and offers the unique ability to separate glycan isomers according to their linkage [80, 81]. The method is performed analogous to the HPAEC-PAD method mentioned earlier. When utilized to analyze released glycans it offers sensitivity of around 300 fmol, comparable to aromatically labeled HPLC [80, 82]. The major drawbacks of this technique are long analysis time and the need for glycan standards for the identification and quantification of each peak.

3.4 Mass spectrometry characterization of released glycans

Mass spectrometry has proven to be an essential tool for glycobiologists since the advent of soft ionization methods, namely matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI). Since a great deal of information lies in the mass of a glycan, MS alone can reveal a lot of qualitative information about a glycan profile. There are different types of MS systems that mainly differ in their ionization methods. The two state-of-the-art methods at the moment are MALDI and ESI. MS can be performed on native glycan, but permethylation is regularly performed, especially for samples of mammalian origin, in order to neutralize acidic glycans and erase the different ionization efficiency between neutral and acidic glycans [83-85]. This also increases the chances of ring cleavage during MS/MS, thus providing additional structural information [86]. MS/MS can provide very useful information about the structure of the glycan, but the evaluation of fragmentation spectra is time consuming. For well characterized structures, a more automated approach can be employed by spectral matching of multi-stage mass spectrometry (MS^n) data [87].

Moreover, permethylation treatment makes the glycans more hydrophobic and removes charge, decreasing ion-suppression issues [88], thus allowing for relative quantification by MALDI-TOF (matrix-assisted laser desorption ionization coupled with a time-of-flight analyzer) MS [84, 85]. MALDI-TOF is a valuable method in the glycobiologists tool kit for glycan profiling. Another advantage of MALDI-TOF is that the ionization always results in singly charged ions, making the mass spectrum easier to evaluate.

ESI based ionization has inherently better resolution, because of the absence of matrix adduct peaks; however, it also creates multiply charged peaks, making spectral evaluation more troublesome [88]. It is mainly used in conjunction with a LC to separate glycans and hence simplify the spectrum, but can also be used as a standalone method [89].

A general problem of MS-based identification methods remains: the fact that isobaric glycan variants cannot be distinguished by mass alone. These can be topoisomers, branch isomers or linkage isomers, and must be resolved prior to MS analysis by an orthogonal method prior to identification [90]. This is usually achieved by coupling MS detection to a liquid chromatography separation method. A complementary application is the use of tandem MS (or MS/MS or MS^n), in which the ions are fragmented in multiple steps of MS to discriminate between isobaric structures. MS/MS allows the specific linkage position in the glycan to be obtained [70, 91].

3.5 LC-MS approach for site-specific glycosylation analysis

Site-specific analysis of glycosylation, mainly driven by LC-MS based data, has significantly contributed to the field of glycoproteomics. The integrated LC-MS approach has allowed for the identification, characterization and quantification of glycans, as well as the peptides that they are attached to. Such site-specific analysis is crucial in the context of biopharmaceuticals. In fact, it forms a key part of the ICH Q6B guidelines which require information on the glycosylation site in addition to glycan structures [11]. Examples of LC-MS based site-specific glycosylation analysis include EPO [92], the mAb C5-1 produced in transgenic plants [93] and a hemagglutinin-based influenza vaccine candidate [94]. Recently, Dell and colleagues [95] have successfully extended the LC-MS technology to the site-specific analysis of O-glycosylation of the recombinant α-dystroglycan. Use of LC-MS as a quantitative tool was also demonstrated in the context of prokaryotic glycosylation [96].

LC-MS characterization of glycopeptides often involves initial enrichment or fractionation by lectin affinity column [97], RP [93] or NP chromatography [98] to sepa-

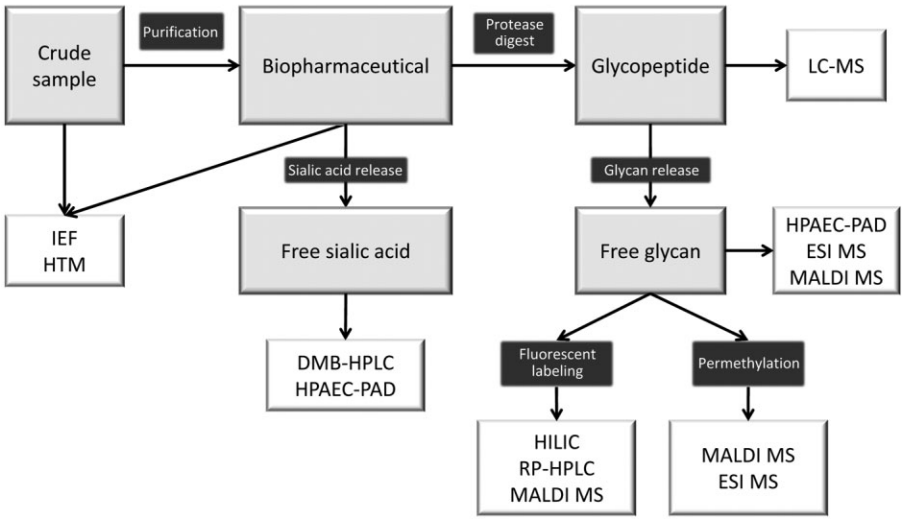

Figure 3. Workflow diagram for glycosylation analysis of biopharmaceuticals. Choices of analytical methods (in open boxes) are assigned to different samples (in grey boxes) derived from different treatment steps (in dark boxes).

rate glycopeptides from non-glycosylated peptides. MS characterization of the enriched glycopeptide fractions can be performed offline using a MALDI-MS, or online using an ESI-MS. Due to the in-source and post-source decay of underivatized sialylated species in MALDI, online LC-ESI-MS offers more intact structural information over LC-offline MALDI-MS, as shown by the analysis of O-mannosylated and O-GalNAcylated recombinant α-dystroglycan glycopeptides [95]. In addition, online coupling of LC with ESI-MS automates the MS analysis of the fractionated glycopeptides and therefore increases the sample throughput. Structure assignment can be achieved through tandem MS (often MS/MS or MS^n). The first MS yields a series of ions among which different glycoforms of the same peptide are characteristically separated by the m/z values of monosaccharides. Depending on the MS instrumentation, subsequent fragmentation by collision-induced dissociation (CID) or electron-transfer dissociation (ETD) often yields unique groups of fragment ions which allow the assembly of intact precursor structures both of the glycans and the peptides [99].

With the impressive throughput and the wealth of information delivered by the LC-MS, the bottleneck in glycoproteomic analysis now lies in the interpretation of mass spectra. Unfortunately there is currently no commercially available software which allows for automatic annotation of glycopeptide peaks. This is in part due to the heterogeneity of glycans carried by the same peptide, as well as different fragmentation patterns generated by different instrumentations and experimental conditions. The availability of such software in future will greatly accelerate the characterization of glycopeptides for better understanding of biopharmaceutical glycosylation.

4 Conclusion

Effective implementation of quality by design (QbD) requires predefined QTPPs and identification of CQAs. This review highlighted the importance of glycosylation in the determination of several efficacy parameters, including bioactivity, in vivo circulation, and drug targeting. Monitoring the CQA-related glycan structures for consistent QTPPs relies on sensitive and accurate analytical methods. Here we summarise current analytical methods for sialylation comparison, glycan profiling and site-specific analysis of glycosylation, and reviewed the advantages and disadvantages of each method. At present, no single method can cover all the analytical aspects required during process development, optimization and manufacturing. Instead, combinations of orthogonal methods are often needed for different CQAs and biomanufacturing stages. One example to demonstrate such a combination in the context of recombinant EPO production is IEF and HTM assays for the rapid monitoring of its sialylation extent in crude culture media, followed by post-purification glycan profiling by MALDI-MS and relative quantification of sialylated glycoforms by HPAEC-PAD. Figure 3 shows an integrated workflow for a comprehensive analysis of recombinant protein glycosylation.

The past decade has seen tremendous progress in glycosylation analysis, mainly driven by HPLC and MS instrumentations. While such advancements have led to many exciting discoveries, progress in glycoanalytics of biopharmaceuticals can be further accelerated by breakthroughs in the automation of sample preparation and data analysis, as well as increase in sample throughput and sensitivity of detection.

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Dr **Muriel Bardor**'s PhD thesis focused on the humanization of the N-glycosylation of recombinant mAb produced in transgenic plants. After her graduation in 2001 she joined Prof Ajit Varki's group at the Glycobiology Research and Training Center, University of California San Diego, USA where she demonstrated the uptake mechanism of the non-human sialic acid N-glycolylneuraminic acid into human and mammalian cells. At the end of 2003, she obtained an Associate Professor position at the University of Rouen, France. There she initiated and led a project regarding the characterization of the N-glycosylation processing in microalgae. She is currently group leader of the Analytics department at Bioprocessing Technology Institute, A*STAR, Singapore. Her group is developing analytical tools for the glycoanalysis of recombinant proteins produced in mammalian cells and for the characterization of the glycosylation of the host producer cells.

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