Chapter

Analytical Characterization of Monoclonal Antibodies with Novel Fc Receptor-Based Chromatography Technique

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Abstract

Most clinically approved large biotherapeutics are monoclonal antibodies (mAbs), primarily belonging to immunoglobulin G subclass-1 (IgG1) and, to a lesser extent, IgG2 and IgG4. Glycosylation is the main source of post-translational heterogeneity of mAbs, impacting their drug therapeutic mechanism of action (MOA). Glycosylation is also one of the critical factors in drug product solubility, kinetics, stability and efficacy. Thus, monitoring glycan critical quality attributes (CQAs) is an essential part of any biopharmaceutical development. The binding affinity of an IgG to its cellular Fc receptor (FcR) depends on both its IgG subclass and Fc domain glycosylation pattern. Since composition of the N-glycans also correlates to the Antibody-Dependent Cellular Cytotoxicity (ADCC), the glycosylation pattern needs to be monitored for consistency in potency and efficacy. This applies for the original mAb biologics as well as biosimilars. In this chapter, we present a truly novel way to assess the variances in mAb glycoforms using FcγRIIIa-based affinity chromatography. First, a brief overview of the Fc receptor function is presented. Then, the principle of FcR-based affinity chromatography is explained including how this column's potential to analyze a variety of mAbs according to their N-glycan content is highly selective and robust. Finally, we provide examples of the FcR column's potential to improve analytical characterization of mAbs with practical applications such as effective cell line screening, monitoring of glycoengineering, process development and process control in manufacturing.

Keywords: FcR, glycoform, N-glycan, monoclonal antibody, mAb, biosimilar, affinity chromatography, antibody-dependent cellular cytotoxicity, ADCC

1. Introduction

Affinity chromatography is a popular method for the purification of biomolecules. The purification involves interaction of the biomolecule with a ligand covalently immobilized to a solid stationary phase. Elution occurs as a function of binding strength of the biomolecule to the stationary phase, tighter the binding later the elution time in a linear gradient. Due to high selectivity and fast separation, affinity chromatography is regarded as the most widely used purification method for capture step in biopharmaceutical industry. Different types of affinity

chromatography ligands are available as resins and pre-packed columns for purification at various scales. Similarly, different types of U/HPLC affinity chromatography columns are available for analytical characterization and quality control. This chapter focuses on a recently introduced novel FcR-based affinity column, TSKgel-FcR-IIIA-NPR, that enables chromatographic characterization of mAbs based on their N-glycan content attached to a highly conserved Asn-297 in Fc region.

2. Brief overview of Fc receptor

A brief overview of the Fc Receptor (FcR) structure and function is provided to best understand the chromatography principle of the column discussed in this chapter. FcR proteins belong to immunoglobulin (Ig) superfamily [1]. Interest in FcRs for biotherapeutic research has gained momentum since 1980s. The purification of FcR from the glycoprotein fraction of the placental membranes by chromatography was reported in 1982 [2]. A functional 40 kDa FcR, with low affinity for native IgG, was purified from the human peripheral nerve extract using F(ab)$_2$ fragments of mAb against placental FcR as affinity agent in 1989 [3]. Important role of FcR in IgG distribution to the brain [4], inhibition of cell activation [5] and in enhancement and suppression of the effector function [6] have also been reported. Overall, it became evident that FcRs are important for numerous biological functions.

2.1 FcγRs

The FcRs binding to immunoglobulin G (IgG) are known as Fc-gamma receptors (FcγR). FcγRs play essential role in immunity, inflammatory and infectious diseases [7]. Immune enhancement and suppression are influenced by binding to these FcγRs [6]. Additional interaction between hyaluronic acid (HA) and sialic acids on immune cells helps to optimize the FcR-mediated effector function [8]. Fcγ receptors do not bind to IgA or IgM [9].

2.2 Fcγ receptor binding to IgG

Typical IgG is Y-shaped protein of ~150 kDa in size, containing two heavy chains and two light chains (**Figure 1**). The heavy chain (HC) contains three constant domains (CH1–CH3) and a variable domain (VH) with three complementarity-determining regions (CDRs). The light chain (LC) has only one constant domain (CL) and a variable domain (VL) with CDRs. The Fd consists of VH and CH1. LC and Fd together form the antigen binding fragment (Fab). The CH2 domain of each heavy chain of IgG has a highly conservative asparagine (N) residue at position 297 (Asn297 or N297) that is almost invariably glycosylated. Fcγ receptor binding site is located near the hinge region of IgG, close to N297 in the CH2 domain. The most flexible portion of the hinge region is between CH1 and CH2 domains of a heavy chain. The four chains are covalently connected via disulfide bridges [10]. Fraction crystallizable (Fc) is composed of CH2 and CH3 domains of the two heavy chains. The highly conserved glycan moiety at position N297 infers structural changes to the Fc-region required for binding to FcγR. Subtle differences in the glycan composition at this site, thus, can affect the conformational rigidity of the Fc-structure, and may also alter the interaction with FcγR by direct contact [11].

2.3 FcγR subclasses

FcγRs are divided into three subclasses, abbreviated as FcγRI, FcγRII and FcγRIII. Extracellular regions of all the FcγRs are extremely homologous, whereas the
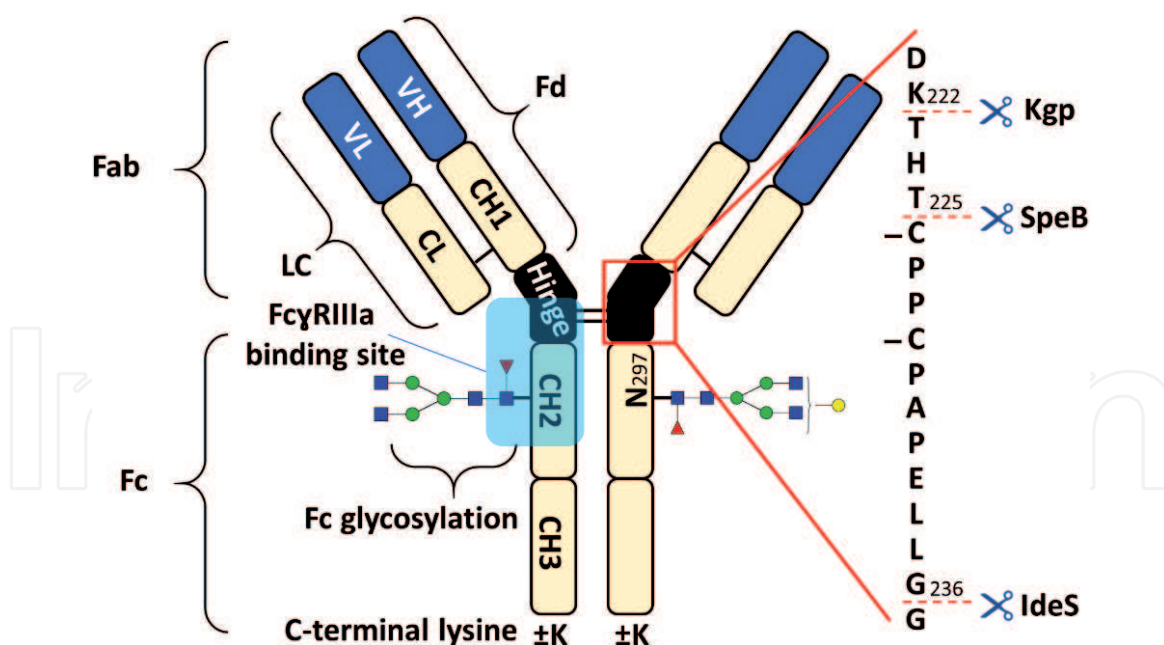
Figure 1.
Schematic overview of human IgG1 with a detailed sequence view into the hinge region [10] (reprinted with permission).

cytoplasmic domains differ considerably from each other [12]. FcγRI exhibits the highest affinity for IgG, K_a 10^8–10^9 M^{-1} whereas FcγRII and FcγRIII show a weaker affinity [13] for monomeric IgG, $K_a \leq 10^7$ M^{-1}. Receptor clustering is essential for FcγR signaling. FcγRIII (also known as CD16) is a cluster of differentiation molecule found on the surface of natural killer (NK) cells, neutrophils, monocytes and macrophages.

2.4 FcγRIII isoforms

FcγRIII exists in two different isoforms, (a) FcγRIIIa or CD16a and (b) FcγRIIIb or CD16b. Both forms take part in intracellular signal transduction. Two nearly identical genes in human encode these two isoforms. FcγRIIIa is a 50–65 kDa type-1 transmembrane protein whereas FcγRIIIb is a 48 kDa glycosylphosphatidylinositol (GPI)-anchored protein. This chapter focuses on the modified recombinant FcγRIIIa protein ligand, immobilized on a polymethacrylate stationary phase and packed into an analytical chromatography column that can be used for characterization of antibodies based on their N-glycan content on Asn297.

2.5 FcγRIIIa and glycosylation mode of IgG

Post-translational modifications, particularly glycosylation, of both IgG antibodies and Fcγ receptors modulate the affinity of their interaction. N-glycan (**Figure 2**) is a well-defined complex biantennary structure composed of a core hepta-saccharide, made up of N-acetylglucosamine (GlcNAc) and mannose, followed by variable additions of galactose, sialic acid (N-acetylneuraminic acid), fucose, and bisecting GlcNAc residues [11].

The attached glycans play various crucial roles on the function of immunoglobulins. Fc sialylation prolongs serum half-life of therapeutic antibodies [14]. **Figure 3** shows details of the structure of the glycosylated Fc fragment complexed to a FcγRIIIa receptor [15]. In non-fucosylated mAb, the carbohydrate-carbohydrate interactions increase binding affinity between N-Glycan of IgG-Fc and N-Glycan of FcR ($K_D = 7.2 \times 10^{-9}$ M) while in fucosylated mAb the carbohydrate-carbohydrate interaction is weekened or non-existent depending on the extent of steric hindrance of the fucose moiety ($K_D = 3.0 \times 10^{-7}$ M) [15].
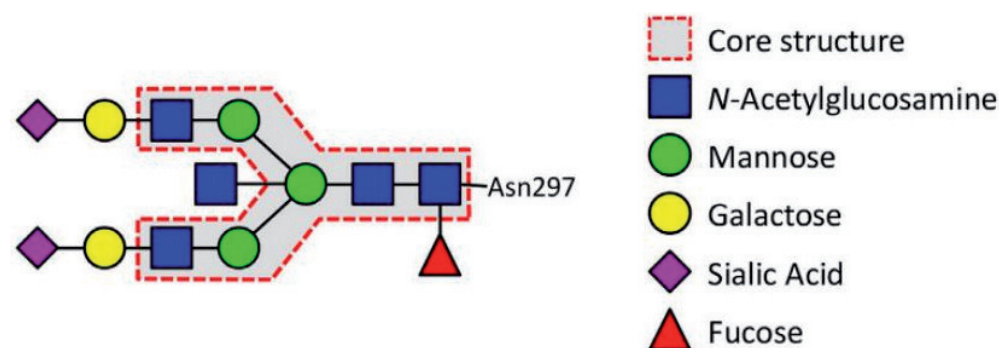
Figure 2.
A schematic representation of a common N-glycan structure where blue squares denote to GlcNAc, green circles denote to mannose, yellow circles denote to galactose, purple diamonds denote to sialic acid (N-acetylneuraminic acid) and red triangle denote to fucose (reprinted with permission).
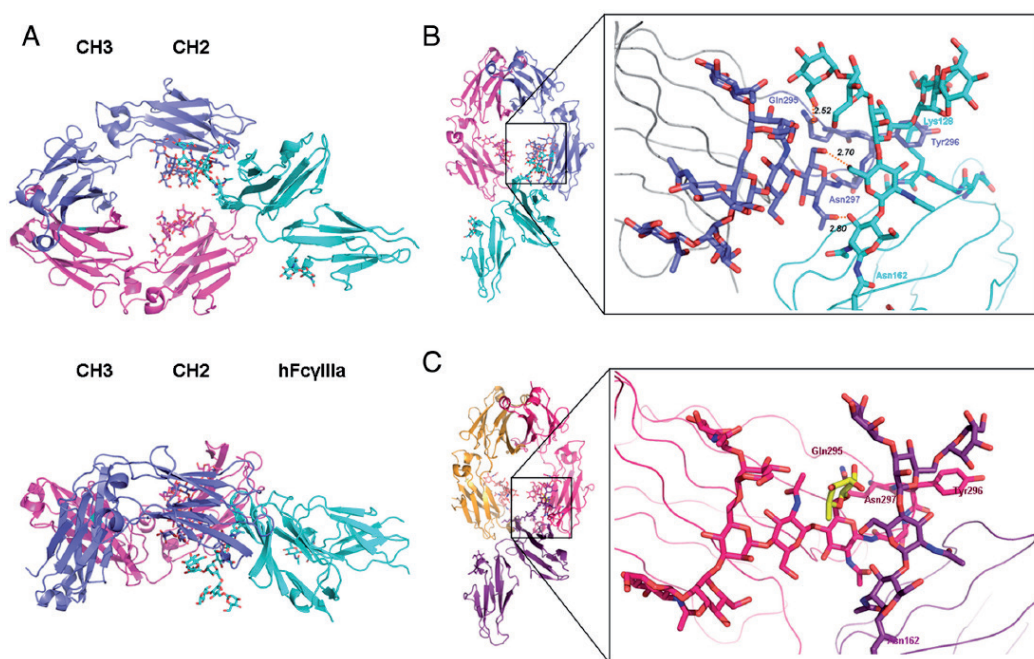

Figure 3.
Crystal structure of glycosylated Fc-FcγRIIIa complex. (A) Top and side views of the structure of the glycosylated Fc-FcγRIIIa complex. The Fc chains are shown in blue and magenta and the receptor in cyan. The oligosaccharides are depicted as ball-and-stick representation. (B) View on the interaction interface between afucosylated Fc fragment and glycosylated Fc receptor. Chain A of the Fc fragment is shown in blue, the Fc receptor in cyan. Hydrogen bonds are presented as dashed lines with distance between donor and acceptor atoms. (C) View on the interaction interface between fucosylated Fc fragment and glycosylated Fc receptor. Chain A of the Fc fragment is shown in magenta, the Fc receptor in dark violet. Core fucose of fucosylated Fc is highlighted in yellow [15]. (Reprinted with permission).

The glycosylation of Fc part is prerequisite for its affinity to FcR. Therapeutic monoclonal antibodies recognize specific cell surface-expressed antigens in malfunctioning cells (e.g. cancer cells) and elicit immune effector functions such as Antibody-Dependent Cell-mediated Cytotoxicity (ADCC) (**Figure 4**). Glycan composition at conserved N297 in IgG largely affects the binding affinity, thus regulating ADCC activity.

FcγRIIIa protein contacts Fc portion of IgG (to both CH2 regions) and to attached glycans. The glycans from FcγRIIIa side are also apparently in contact with glycans from IgG-Fc. The binding is asymmetrical in nature as revealed from the co-crystal structure of Fc-FcγRIII complex [16], although the stoichiometry of binding is 1:1. Lack of fucosylation in core Fc-glycan dramatically increases the ADCC activity due to enhanced binding affinity of FcγRIIIa to IgG [17].
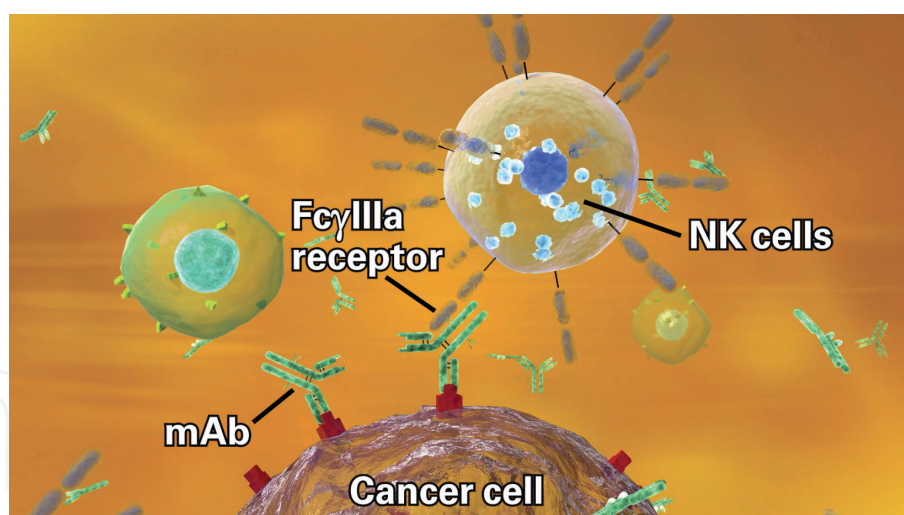
Figure 4.
Schematic representation of the antibody-Fc receptor interaction resulting in ADCC activity.

3. TSKgel FcR-IIIA-NPR affinity column

TSKgel FcR-IIIA-NPR affinity column contains non-porous polymethacrylate base beads as stationary phase. The ligand is a modified recombinant non-glycosylated FcγRIIIa of 20 kDa, produced in *E. coli* expression system. Recombinant FcγRIIIa ligand has eight amino acid substitutions as compared to its wild-type form. These changes were necessary for stabilization of the ligand structure [18]. The 2.7 Å crystal structure of recombinant FcγRIIIa protein verifies the molecular basis of the IgG-FcR complex formation. No significant difference was found between the crystal structures of glycosylated wild-type FcγRIIIa expressed in human embryonic kidney (HEK) cells vs. non-glycosylated recombinant FcγRIIIa from *E. coli* (**Figure 5**). This confirms suitability of *E. coli*-produced non-glycosylated FcγRIIIa to be used as an affinity ligand in a chromatography resin. Notably, no direct contact of the terminal Asn297 N-glycan galactose of IgG and modified non-glycosylated FcγRIIIa was observed [19]. Both proteins were crystallized as complex with Fc [19].

The dimension of the TSKgel FcR-IIIA-NPR column is 4.6 mm ID × 7.5 cm (l) with a total bed volume of 1.25 mL. Polymethacrylate-based matrix is composed of non-porous material with ~5 μm particle size. Maximum pressure limit of the column is 90 bar (9 MPa). The column is suitable for both HPLC and UHPLC instrument settings. The recommended run temperature is 15–25°C. The operational pH range is from pH 4 to 8. In general, most monoclonal antibodies bind effectively on the column at pH 6.5. Typically, 50 mM ammonium citrate (or ammonium acetate) buffer is used. A linear pH gradient from pH 6.5 to 4.5 over 16 column volumes (CV) at the flow rate of 1.0 mL/min is recommended. **Figure 6** shows a typical three-peak chromatographic profile for a monoclonal antibody in these settings. Sodium chloride can be added to buffer to enhance the separation if needed. Longer retention time indicates stronger mAb affinity to the ligand. However, as to the general composition of glycans, it should be noted that all the three peaks still contain a mixture of glycoforms with variable amounts of galactose and other sugar molecules (**Figure 7**).

From a related experiment (**Figure 7**), the three peaks were collected for glycan analysis and ADCC activity assay. Determination of the glycan structures revealed that the retention time increase correlates with increased number of the terminal galactose. Terminal galactose tends to stabilize conformation of the Fc region, providing tighter binding onto FcγRIIIa affinity ligand [20]. However, FcR column is not designed for quantitation of only galactose but to obtain a more general understanding of the variation in distribution of the glycan content among the
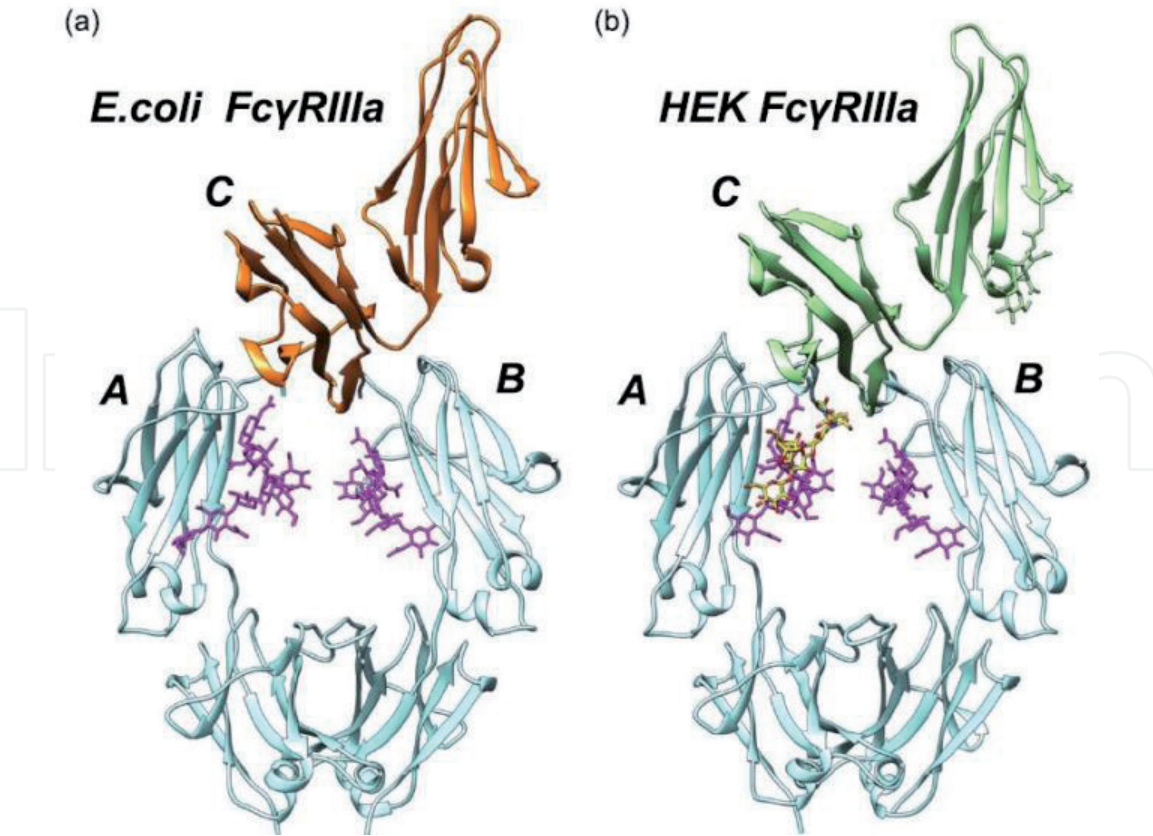
Figure 5.
Crystal structures of a recombinant non-glycosylated FcR ligand (Panel a) and a glycosylated native form (Panel b) (reprinted with permission).
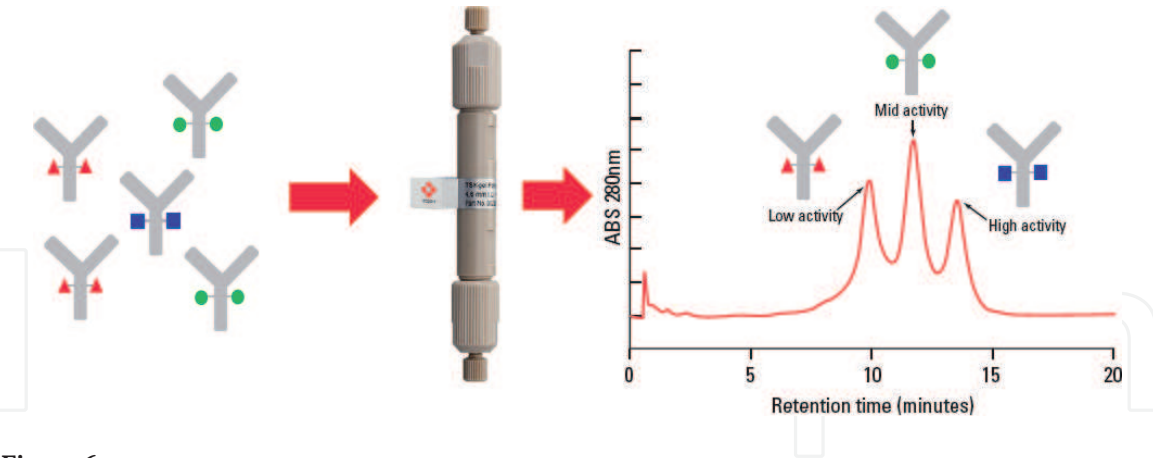

Figure 6.
A typical 3-peak elution profile for a monoclonal antibody using a TSKgel FcR-IIIA-NPR column with correlation to ADCC activity.

three peaks. Although added galactose increases retention time, other factors (as explained below) also affect the mAb binding affinity.

The results shown in the **Figure 7** support the binding model presented for galactose in the **Figure 8**. The crystal structure, basis of a cartoon model in the **Figure 8**, surprisingly did not show direct contact of the galactose units with the receptor that could more easily explain galactose effect on the affinity for FcγRIIIa. Instead, based on the evidence reported in literature so far, it has been proposed that the galactose moiety can influence the dynamic and conformational assembly of IgG-Fc. Hydrogen-deuterium exchange mass spectrometry
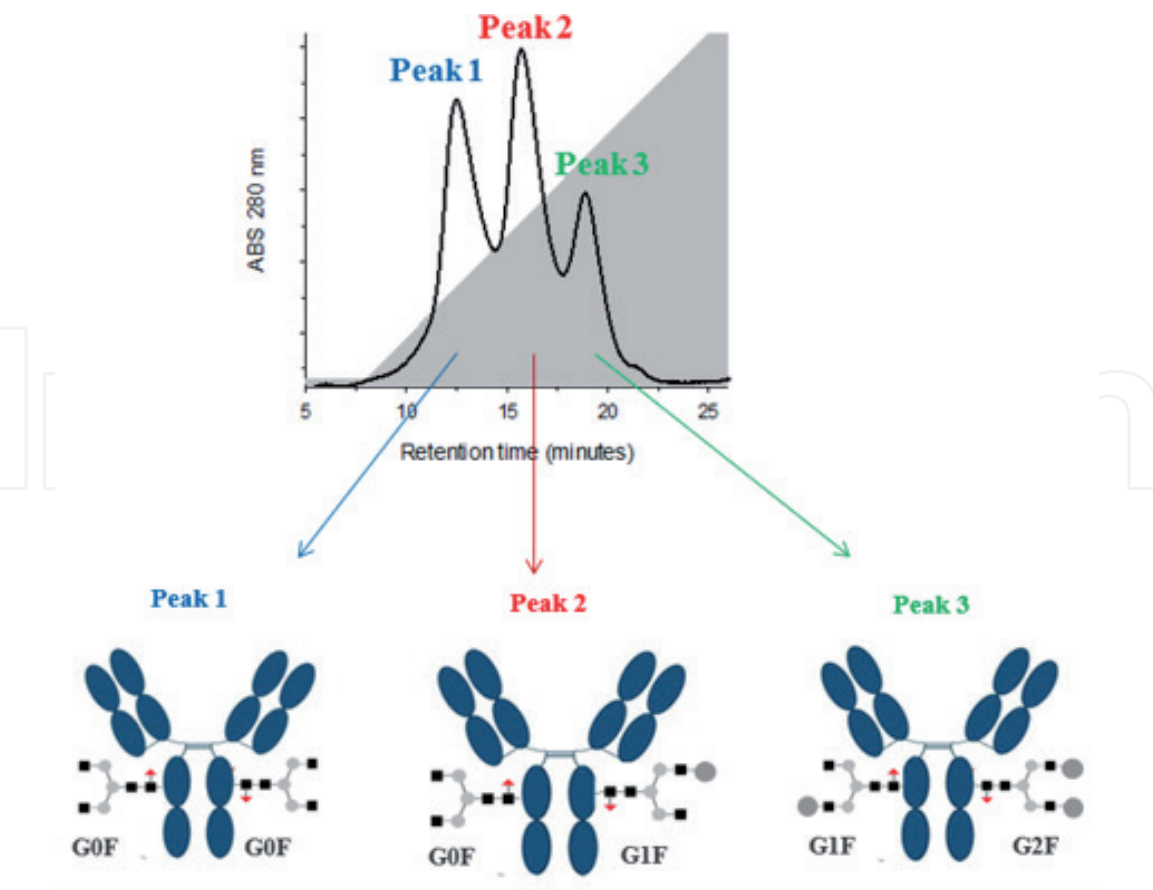
Figure 7.
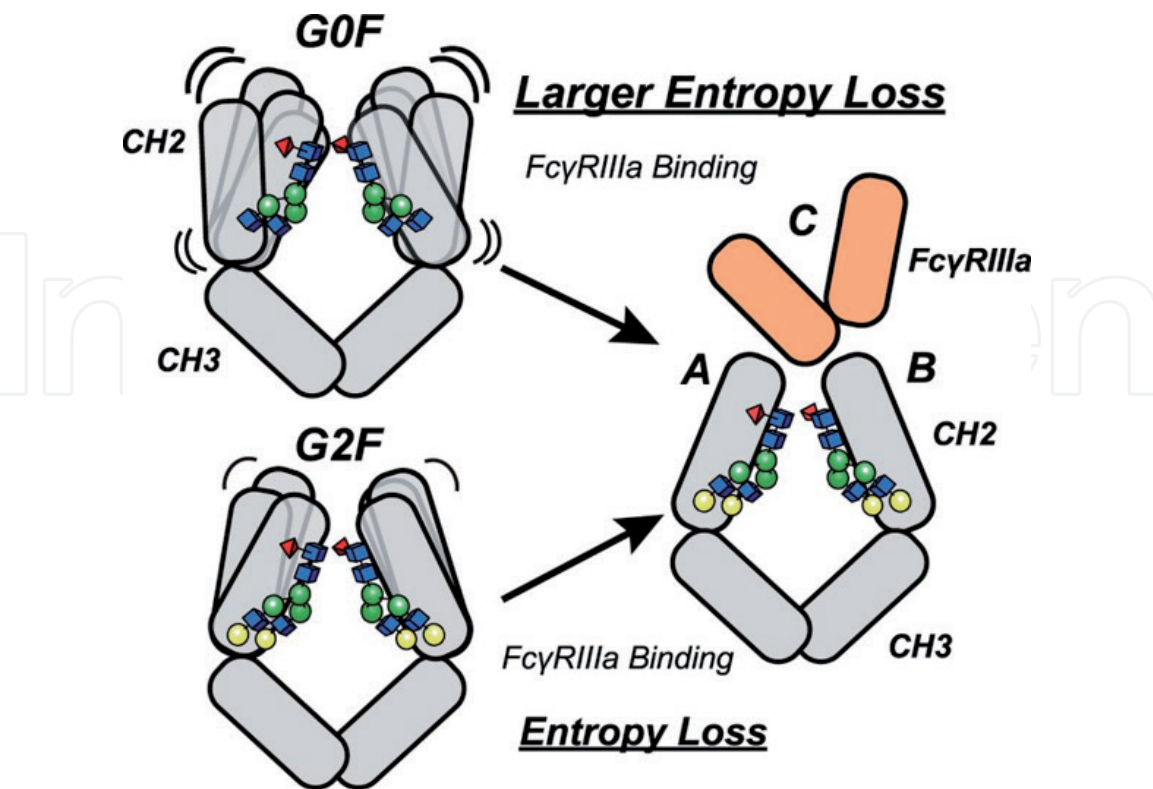*Correlation between the number of galactose units and retention time [20] (reprinted with permission).*

Figure 8.
Conformational entropy modulated by galactose content controls the binding affinity of IgG to FcγRIIIa [19]. (reprinted with permission).

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(HDX-MS) study using purified IgG glycovariants support this hypothesis. By the deuterium exchange mass spectrometry, it was noticed that the deuterium uptake increases in the peptide ranging from 245 P to 256 T in the following order: Peak 1 > Peak 2 > Peak 3. This result implicates that this particular peptide exhibits a more rigid conformation as the fraction of galactose units increase. Differential Scanning Calorimetry (DSC) experiment also proved that the peak 3 contains antibodies with the highest galactose content, and it exhibited the greatest denaturation enthalpy. This result thus suggests that the terminal galactose engages in non-covalent interaction with surrounding residues leading to increased conformational stability. The value of entropy change decreased as the content of galactose increased, suggesting a reduction of the conformational entropy of the antibody. More specifically, terminal galactose moiety seems to especially stabilize the mAb hinge region. In N-glycans containing galactose, the CH2 domain remains in more rigid conformation as compared to the agalactosylated (G0F) glycoform (i.e. no galactose). Overall, the number of terminal galactoses have the greatest impact on the binding affinity of mAb onto the column [19]. However, the other types of glycans such as fucose, mannose and sialic acid also affect the binding.

4. Correlation between mAb retention time and ADCC activity

In order to confirm correlation with ADCC activity and retention time, Rituximab as an example [20], was analyzed and three successive peaks from TSKgel FcR-IIIA-NPR column were collected. ADCC bioassay was performed for these peaks using the Promega ADCC reporter assay kit (**Figure 9**). In the assay, higher luminescence (RLU units), as compared to mAb concentration, denotes to stronger ADCC activity. As a measure of ADCC potency, EC50 values (mAb concentration with 50% of the maximal ADCC activity) were determined. RLU units for Rituximab sample prior to load and for three fractions were plotted as a function of their concentrations (μg/mL) (**Figure 9**, Panel B). The order of ADCC activity in the peaks is as follows: peak 3 > peak 2 > mAb > peak 1. The result thus clearly indicates that the peak 3 has the highest ADCC activity as compared to the other two peaks. This study proves that the increased retention
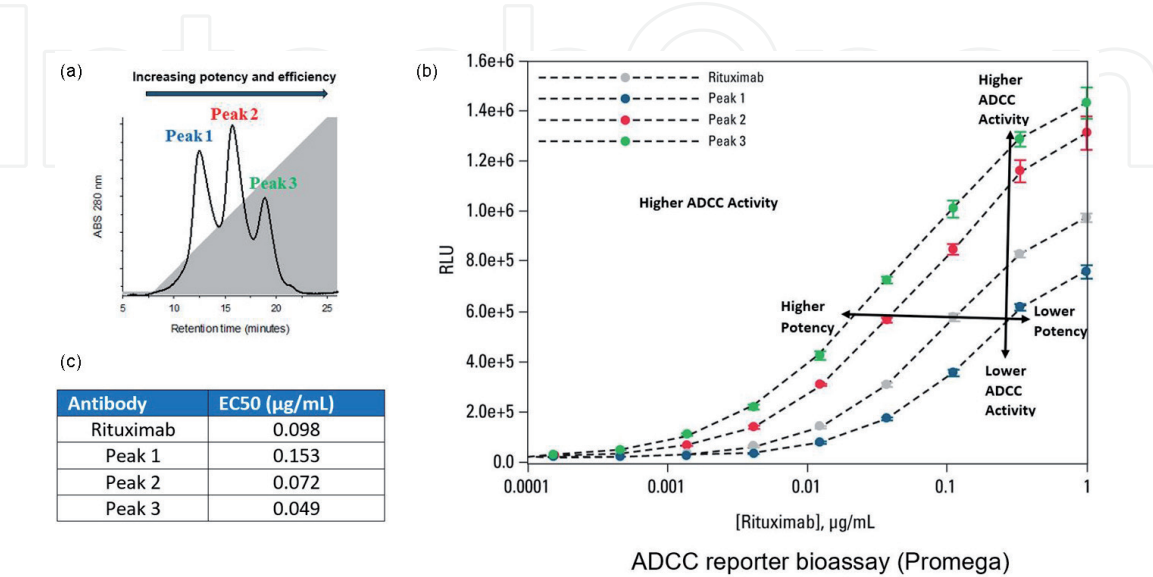

Antibody	EC50 (μg/mL)
Rituximab	0.098
Peak 1	0.153
Peak 2	0.072
Peak 3	0.049

ADCC reporter bioassay (Promega)

Figure 9.
ADCC reporter bioassays for Rituximab and its glycoforms separated on TSKgel FcR-IIIA-NPR. Panel A: A typical 3-peak elution pattern for the mAb. Panel B: ADCC reporter bioassay for the mAb control and collected peaks from panel A. Panel C: EC50 values for the three peaks. (reprinted with permission).

time for the peaks obtained from the U/HPLC assay indeed correlates with the higher ADCC activity.

The core fucose can also exert a strong modulatory effect on the affinity for FcγRIIIa. **Figure 10** illustrates the location of a fucose molecule in the glycan moiety. In the mAb, the core fucose inhibits the carbohydrate contacts and decreases the binding affinity. Fucosylation of N-glycan thus reduces the affinity of mAb-FcR interaction by steric hindrance within the Fc cavity, obstructing the carbohydrate–carbohydrate interaction [15].

Afucosylated mAb binds effectively to FcγRIIIa ligand [19]. Subsequently, fucosylated and non-fucosylated mAbs were compared on the column where it was shown that deletion of core fucose significantly increased both retention time and the ADCC activity (**Figure 11**).

Sialic acid (N-acetylneuraminic acid) has a role in ADCC activity. Sialylation in the context of core fucosylation significantly decreased ADCC activity [21] as the sialic acid lowers the binding affinity to Fc receptor [19]. In absense of core fucosylation, sialylation doesn't have any significant impact on ADCC activity [21]. FcγRIIIa affinity chromatography yields longer retention by core fucosylation, terminal galactosylation and sialylation [18]. Overall, the effect
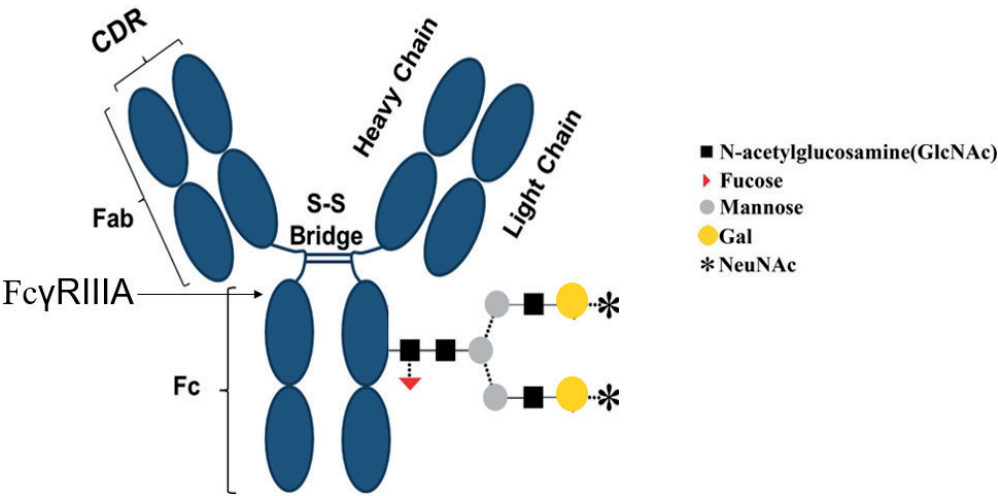

Figure 10.
Schematic representation of the core fucose location in the N-glycan moiety (reprinted with permission).
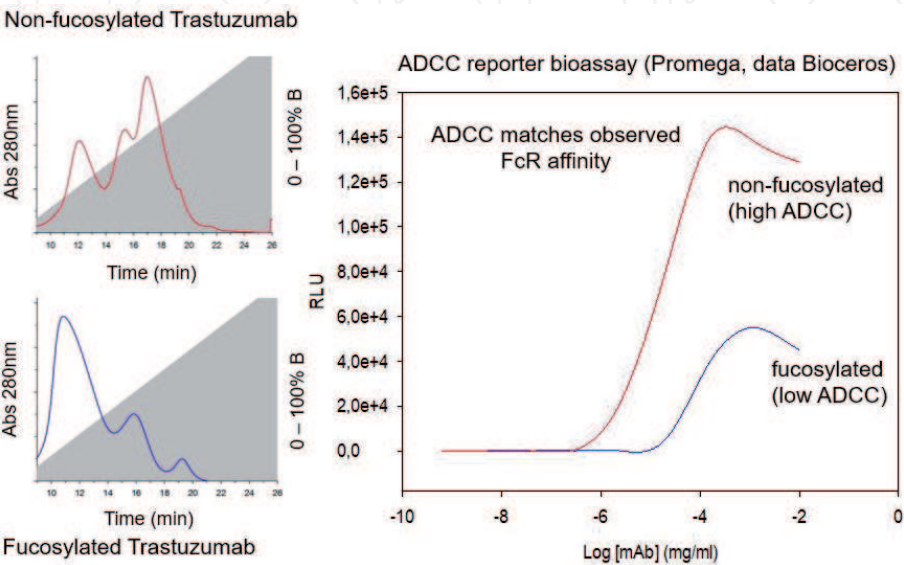

Figure 11.
Effect of the core fucose on ADCC activity [20] (reprinted with permission).

of different glycan molecules on the binding strength to Fc receptor can be arranged in the following order: Galactosylated (terminal) > Afucosylated > Sialylated (terminal) N-glycans in mAb. Similalry, retention time and ADCC activity is expected to be in the following order in the other forms of glycosylation patterns; A2G2 > A2G2S2 > A2G0, High mannose (HM) > FA2G2 and FA2G2S2 unless otherwise affected by any other factor. High mannose and A2G0 may be of similar activity. Complement-dependent cytotoxicity (CDC) is not significantly related to sugar structure [18].

5. Glycosylation profiling of a variety of monoclonal antibodies

IgG1 is the most abundant antibody in human body. Other antibodies are IgG2, IgG3, and IgG4 (**Figure 12**). Generally, IgG1, IgG3, and to some extent, IgG4 are formed against protein antigens. IgG2 is the major subclass formed against repetitive T cell-independent polysaccharide structures found on encapsulated bacteria [22]. Most of the biotherapeutics predominantly belong to IgG1 subclass. The antibodies from subclasses other than IgG1, as well as numerous engineered forms, are also gaining plenty of interest for use as biotherapeutics (**Figure 12**).

Several commercially available IgGs were recently analyzed in-house using TSKgel FcR-IIIA-NPR column to compare their elution profiles. Almost all IgGs yielded the typical 3-peak separation profile although there was substantial difference in each peak height between IgGs. Generally, IgG1, IgG3 and IgG4 subclasses bind to TSKgel FcR-IIIA-NPR whereas IgG2 does not have affinity. Due to
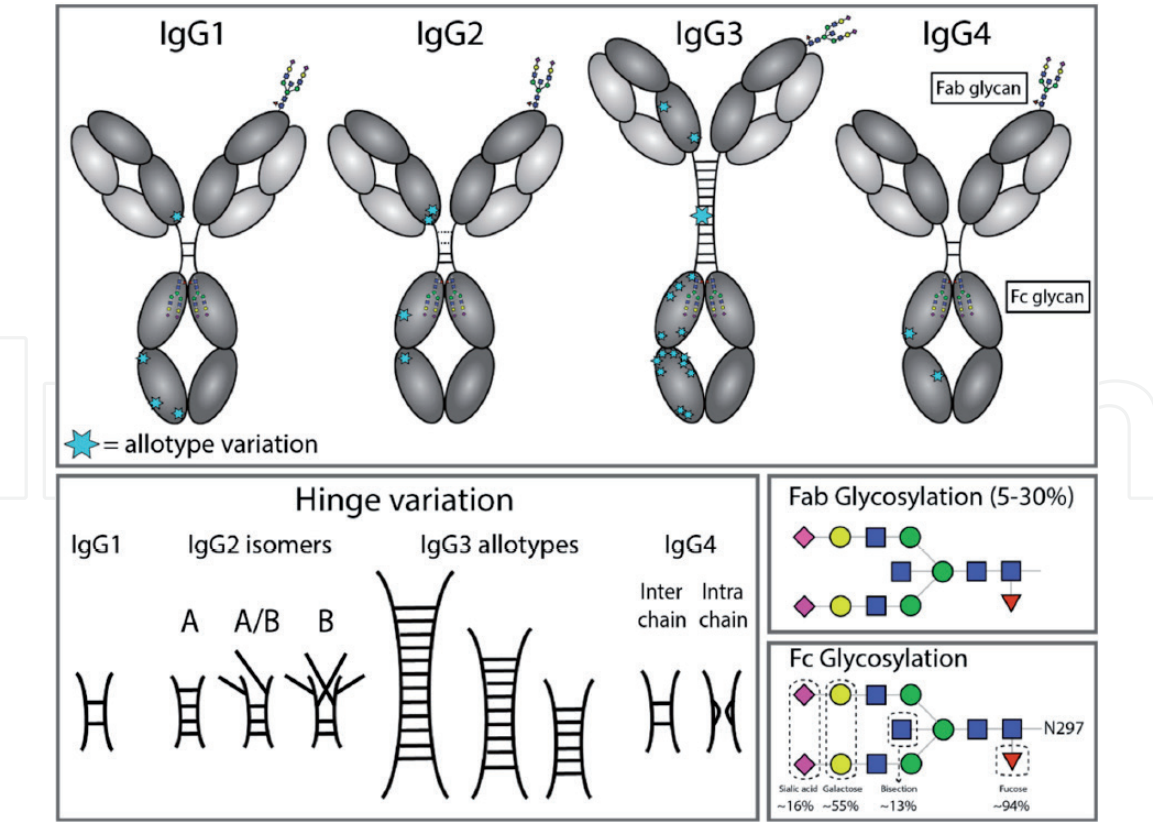

Figure 12.
A structural representation of the IgG subclasses and the variation within these subclasses, including allotypes, hinge variation, and glycosylation. The variation originating from allotypic polymorphisms in the immunoglobulin heavy gamma (IGHG) Fc domain is indicated with blue stars. Except for the star representing the variation in hinge length between IgG3 allotypes, each smaller blue star indicates amino acid variation at one particular residue in the constant domain. Glycans attached to N297 in Fc region are highly variable and the frequency of each glycan moiety on IgG antibodies in human serum is indicated [22] (reprinted with permission).

individual peak size differences, each mAb is indicative of its unique separation profile (**Figure 13**). This study thus shows that TSKgel FcR-IIIA-NPR column can be used for the analysis of a variety of IgG subclasses for glycosylation profiling. The results typically well correlate to ADCC activity.

Since the first FDA approvals of biosimilars in the USA in 2015, the interest continues to increase toward biosimilars. They are, in general, less costly to develop than the original or innovators. However, the biosimilar manufacturers are required to confirm the extent of similarity with the corresponding innovator. In the recent literature report [23], it was indeed confirmed that glycan microheterogeneity may play a critical role in effector function between the originals and biosimilars. In the study, it was shown that a biosimilar had a higher level of afucosylated glycans, resulting in a stronger FcγRIIIa binding affinity and increased ADCC activity. The study in the **Figure 14** also shows that the TSKgel FcR-IIIA-NPR column yielded dissimilar chromatographic pattern for biosimilars as compared to its innovator. Thus this column can be used for monitoring biosimilar consistent with the innovator biomolecule.
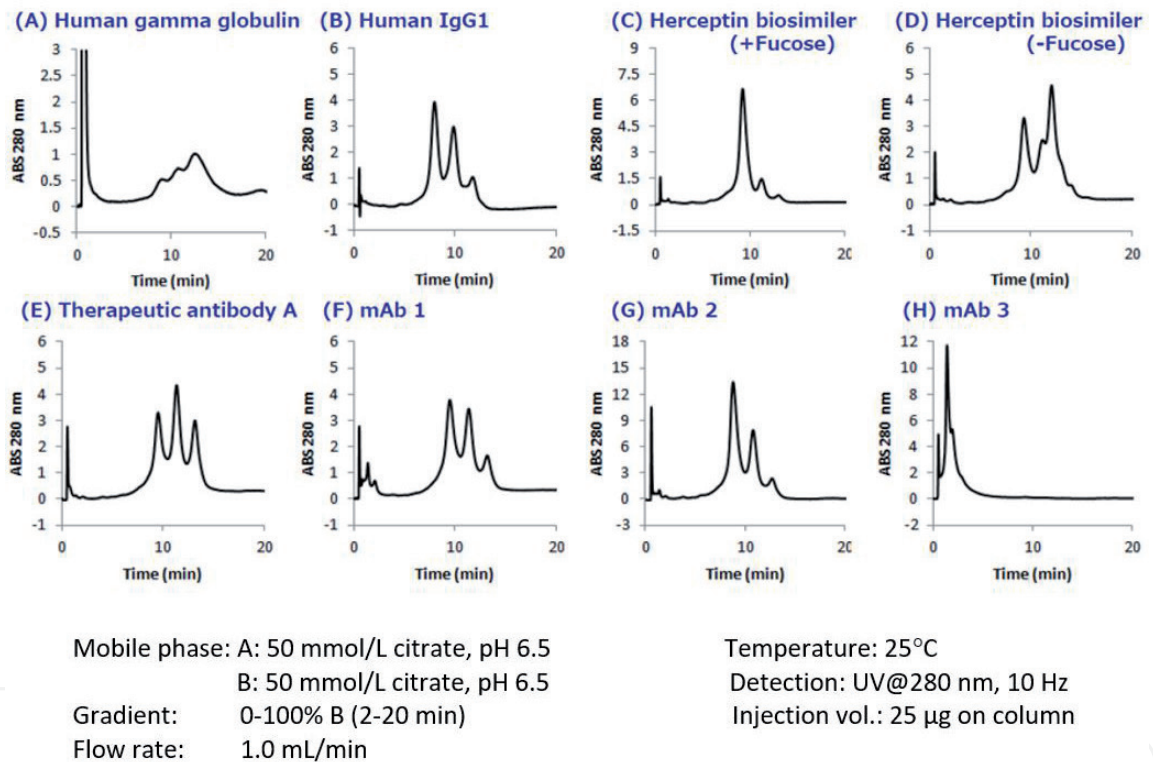

Mobile phase: A: 50 mmol/L citrate, pH 6.5
                   B: 50 mmol/L citrate, pH 6.5
Gradient: 0-100% B (2-20 min)
Flow rate: 1.0 mL/min

Temperature: 25°C
Detection: UV@280 nm, 10 Hz
Injection vol.: 25 µg on column

Figure 13.
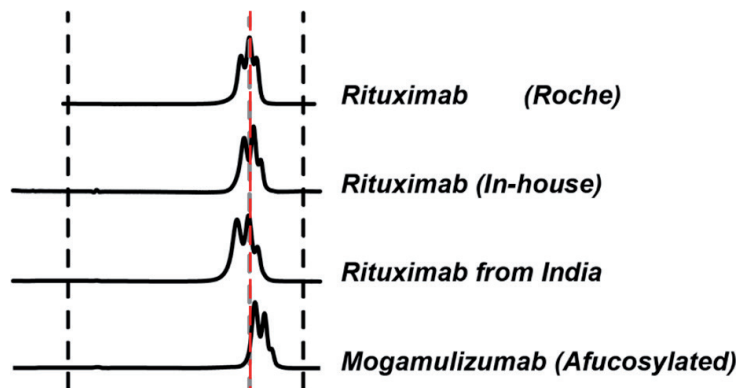*Analysis of a variety of monoclonal antibodies on TSKgel FcR-IIIA-NPR.*

Figure 14.
Comparison of Roche's innovator Rituximab to its two biosimilar biotherapeutic forms. The figure also includes mogamulizumab (Poteligeo™) as an example of a completely afucosylated mAb.

6. Factors affecting the chromatographic separation

6.1 Salt concentration

Salts affect the separation of mAb on TSKgel FcR-IIIA-NPR. To best control the pH in the linear gradient, mobile phase should consist of a buffer with suitable buffering capacity whereas neutral salts are used to increase the ionic strength. Both components affect the binding affinity. Buffer provides pH control and salt ions provide charge shielding or stoichiometric ion bonding on the stationary phase and mAbs. Salts impart specific or non-specific effects by modulating protein–protein and protein-surface interactions. Binding affinity to the column depends on the binding constant. Increasing salt concentration have shown to lead to the elution at earlier retention time (**Figure 15**, Panel A), although the intensity of the effect probably is also related to the individual mAb studied. The binding strength is also dependent on the buffer used such as sodium citrate or sodium acetate. Citrate yielded stronger binding and hence higher retention time. Acetate buffer instead yielded better resolution of the peaks as compared to citrate (**Figure 15**, Panel B).

In the affinity chromatography, the optimum flow rate of elution may be dependent on the molecule-specific interaction with the ligand. Irrespective of the flow rates (**Figure 16**), all the three glycoform peaks eluted within 67% of mobile phase B when the analysis was carried out using a linear gradient of 50 mM sodium citrate buffer from pH 6.5 to 4.5 over 50 minutes at 20°C. Although flow rate did not have effect on elution pH, lower flow rate may be used to increase the sensitivity due to longer residence time in the flow cell.
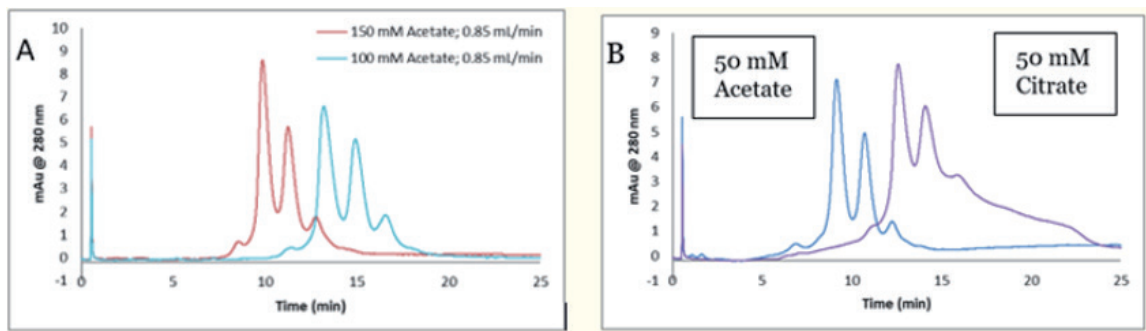

Figure 15.
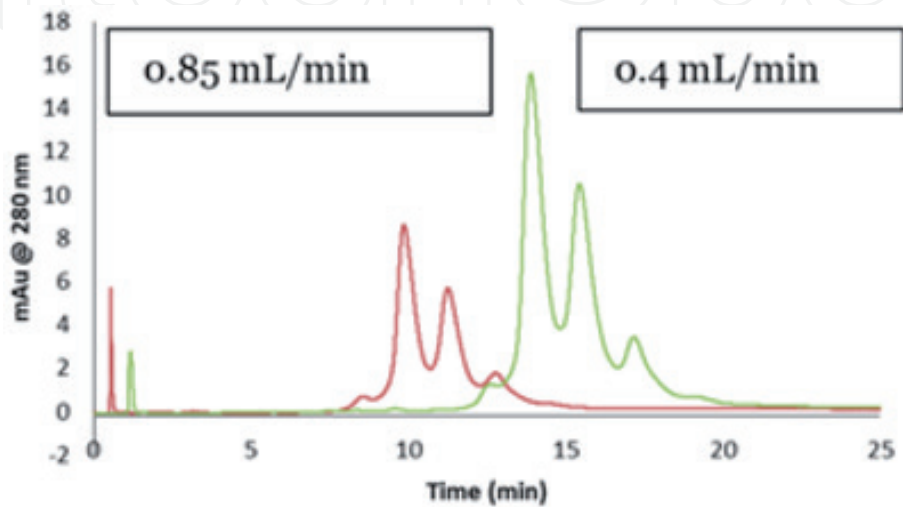*Effect of salt (Panel A) and buffer (Panel B) on the separation of mAb glycoforms.*

Figure 16.
Increased sensitivity at lower flow rate.

6.2 Gradient slope

The gradient elution method is common for the separation of species of different binding strength. **Figure 17** shows the effect of a gradient slope on the separation mAb on TSKgel FcR-IIIA-NPR. As expected, longer gradient time increased the resolution between the peaks whereas the overall peak area and relative ratio of the peak areas remained unchanged. **Figure 17** indicates how shallower slope increased the resolution between the three peaks. This is particularly noticeable between peaks 2 and 3. On the other hand, it should be noted that, for any further analytical
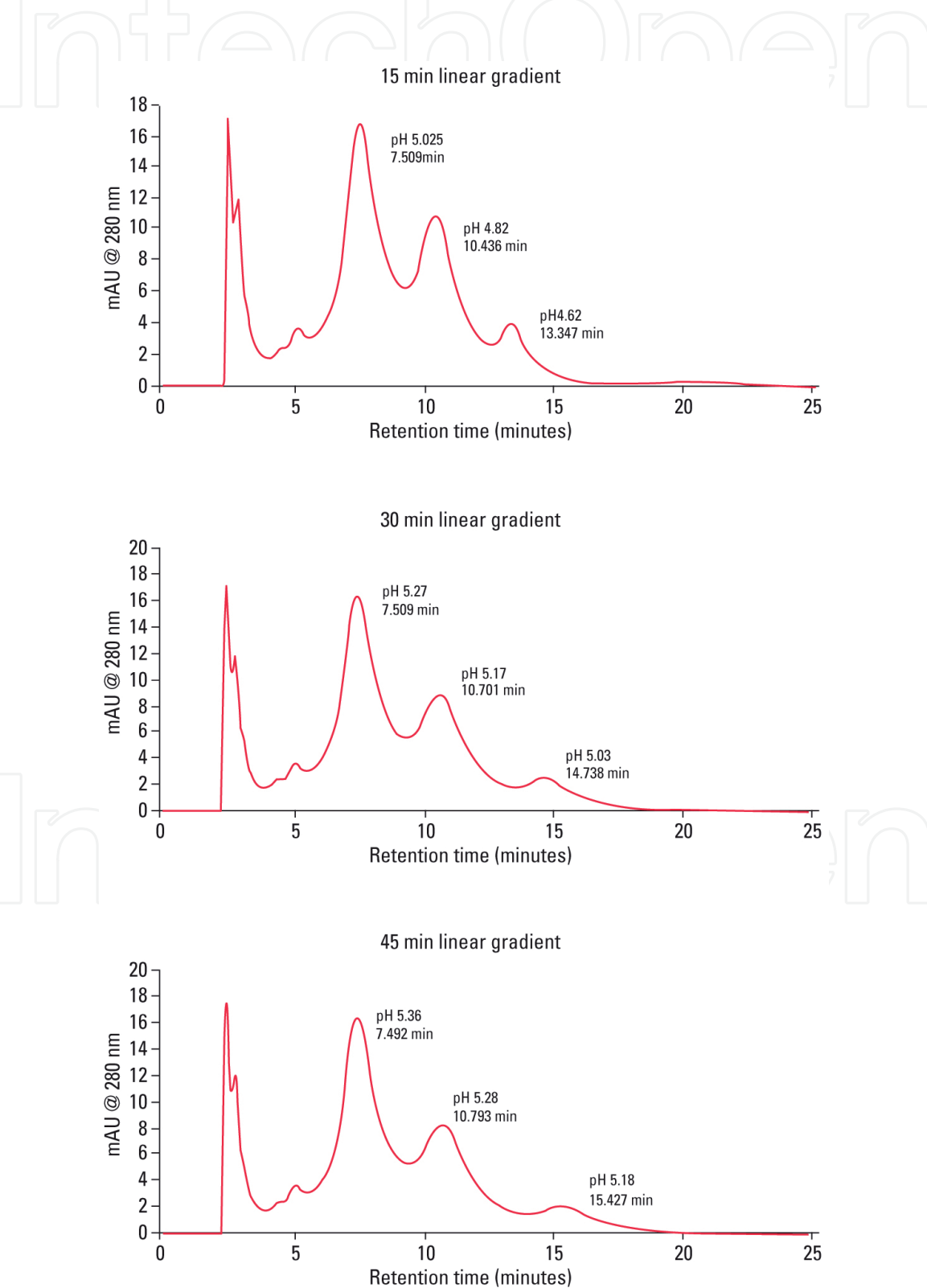

Figure 17.
Effect of gradient slope on the separation efficiency of peaks.

work (e.g. for mass spec), longer gradient increases the peak volumes and thus peak fractions will be more diluted.

6.3 Temperature

The ligand in FcR-IIIA-NPR column is a 20-kDa folded polypeptide and thus special care is to be considered to maintain protein conformation intact with proper run temperature. **Figure 18** shows the separation of mAb at four different temperatures (5°C, 15°C, 20°C, 25°C) at flow rate of 0.2 mL/min. As the temperature increased, the retention time of the three peaks decreased, indicating somewhat lower binding affinity as a function of higher temperature. However, importantly, overall peak profile at each temperature remains unchanged. Thus, for practical reasons, temperature range from 15 to 25°C is recommended for most analytical work. Following the completion of the analysis the column needs to be stored at 2–8°C.

6.4 Sample load

Figure 19 shows the effect of load amount of mAb on the separation profile. The limit of detection was determined as 1.5 µg as per USP definition S/N of 2–3. A load of 3.16 µg could still be easily quantified (LOQ). The analysis was repeatable, robust and the total peak area increased proportionately as the load amount was
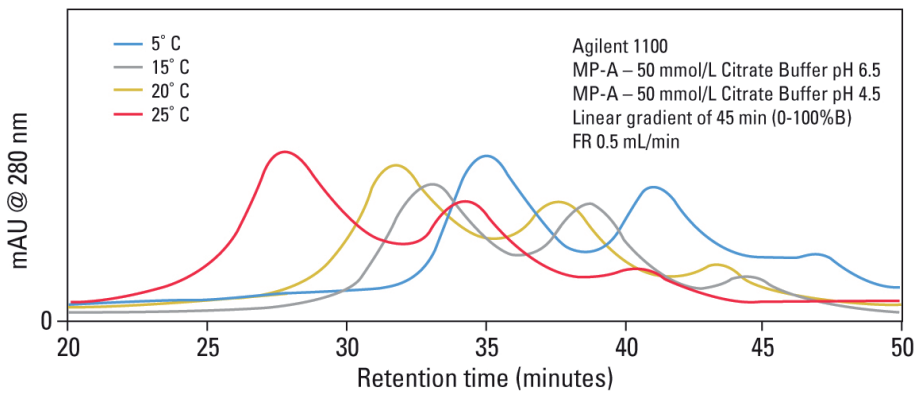

Figure 18.
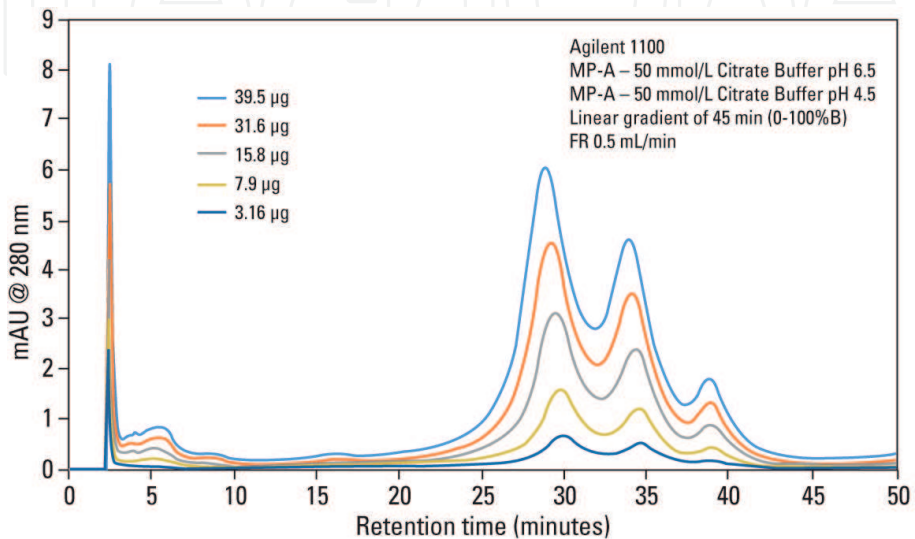*Effect of temperature on the separation of mAb.*

Figure 19.
Effect of sample load on the separation of mAb.

increased in a linear manner in consecutive injections. Relative ratio of the individual peak areas in the three peaks remained constant. The column can generally be used up to 100 μg protein load. However, 5–50 μg load of mAb is recommended for the best resolution and for maintaining the lifetime of the column.

Presence of aggregates in IgG samples impact the binding to the Fcγ receptors. A recently published article reports that deamidated IgG samples caused aggregation or formation of higher molecular weight (HMW) species and thereby impacted the binding affinity. Asparagine deamidation led to reduced binding of IgG to the low affinity FcγRIIIa receptor [24]. IgGs may also be more prone to aggregation when glycans are absent, which in turn has an effect on Fc effector functions. Lack of glycan and its effect on binding is explained below in Section 7.1. IgG dimers and aggregates may also bind stronger to different types of Fc receptors and thus have significant impact on affinity determination. Accumulated strength of multiple non-covalent affinities between the ligand and the receptor is known as avidity effect. This effect can alter the binding to the receptor and should be considered during the analysis mAb with dimer and higher order aggregates. The interaction, if any, needs to be evaluated in case-by-case basis. Up to 5% of aggregates in IgG samples changed the binding and kinetics to each of the Fc receptors [24]. Methionine is easily oxidized to methionine sulfoxide which can also lead to light chain aggregation. Oxidation has impact on the binding to the Fcγ receptors and depends on the extent of oxidation. As reported [24], methionine oxidation below 7% did not impact on binding to the receptors. Taken together, all above factors should be considered when using this column, especially during analysis method development for mAbs containing any amount of aggregates or oxidized forms.

7. Robustness of TSKgel FcR-IIIA-NPR affinity column

7.1 Selectivity

The usefulness of any affinity chromatography column depends on several robustness factors. Here, selectivity is dependent on the nature of N-glycan. This is clearly demonstrated by analyzing enzymatically deglycosylated mAb. PNGase-F deglycosidase reacts between asparagine residue and the innermost N-acetyl glucosamine (GlcNAc) of the complex oligosaccharide or high mannose content. **Figure 20** shows that enzymatically deglycosylated NIST mAb does not bind to the column and thus elutes in void volume.

7.2 Lot-to-lot variability

Scope of quality control of the therapeutic antibodies is expanding rapidly due to the emergence of biosimilars, "biobetter" forms and numerous other kinds of biologics in the biotherapeutic market. Lot-to-lot difference in the activity of innovator mAb may vary up to 20% in the manufacturing process [25]. Although substantial improvement has been attained in CHO cell engineering during recent years, and different strategies are there e.g., to produce afucosylated antibody drugs, still not enough technology is available to fully control *in vivo* glycosylation during production [26]. The lot-to-lot difference in N-glycan content may give rise to a wide variety of risk and thus N-glycan heterogeneity is a key factor to be monitored in quality control.

To demonstrate importance of the mAb lot-to-lot quality control, two manufacturing lots of mAb were analyzed using the TSKgel FcR-IIIA-NPR column (**Figure 21**). Both lots yielded a similar 3-peak elution profile. However, when percentual peak areas of the individual peaks were compared to check the consistency between the two mAb lots. Lot B showed a higher glycan percentage in peak 1 (42% versus
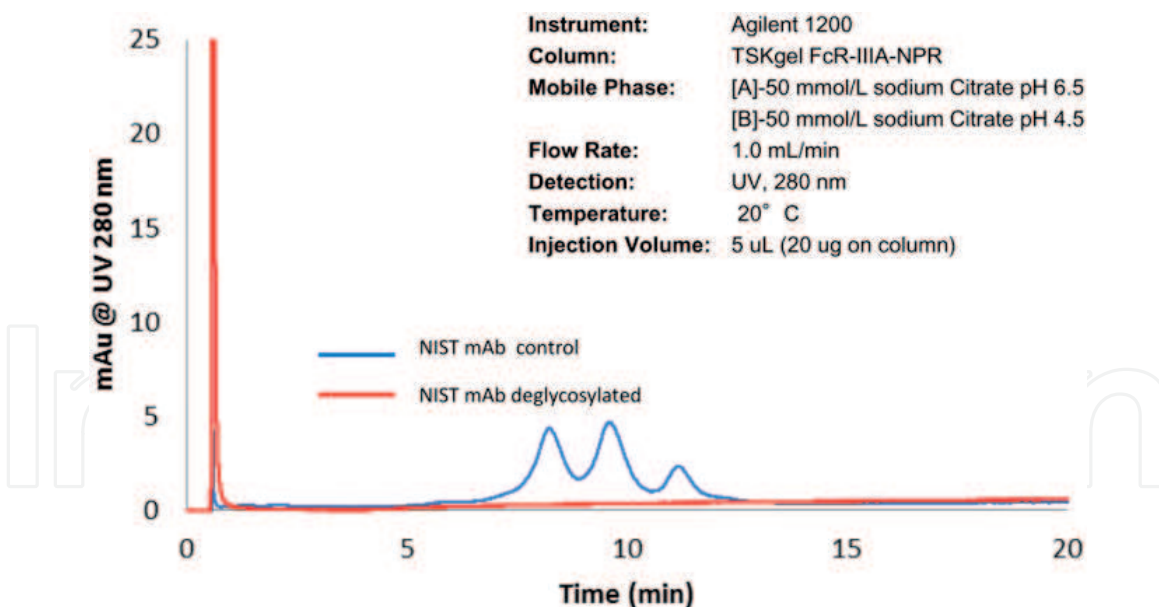
Figure 20.
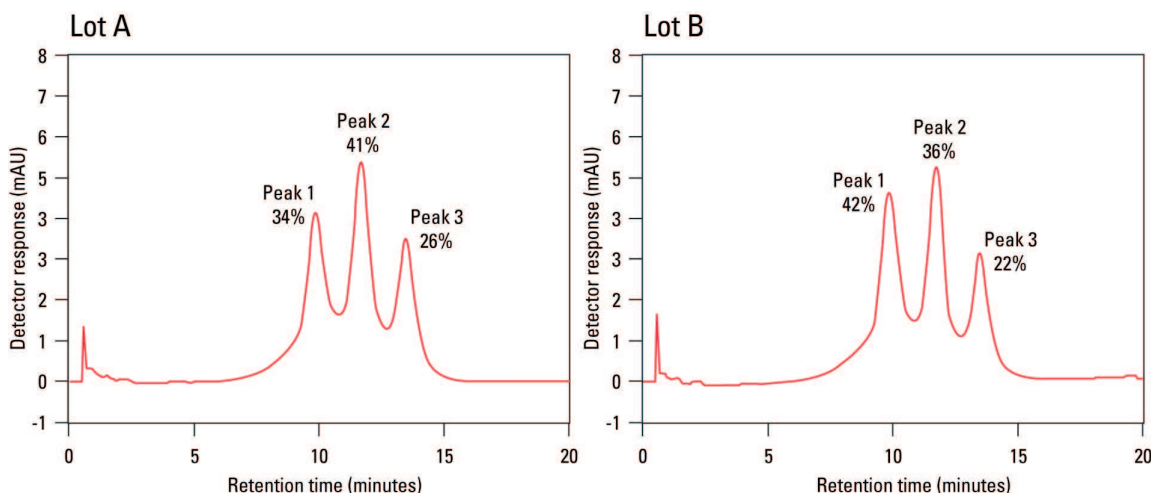*Deglycosylated mAb does not bind to the column.*

Figure 21.
N-glycan analysis of two manufacturing lots of a therapeutic antibody on TSKgel FcR-IIIA-NPR.

34%) and lower percentual amounts in peaks 2 and 3. In a subsequent ADCC assay, this also correlated with lower ADCC activity in the lot B. This experiment thus supports the notion that FcR affinity chromatography is suitable for lot-to-lot quality control of therapeutic mAbs.

To confirm consistency in FcR column manufacturing is also equally important for quality control. Three different lots of TSKgel FcR-IIIA-NPR column (Lots A, B, and C) were tested using reference mAb sample under identical chromatographic conditions (**Figure 22**). No significant variation in 3-peak profile was noticed between the three different column lots.

7.3 Effect of host cell proteins on the separation of mAb

Most mAb pharmaceuticals are produced in CHO cell culture system. Host cell proteins (HCPs), or host cell impurities, are collectively recognized as several forms of host cell products such as DNA, proteins, endotoxin and, if contaminated, viruses. These together are considered as process-related contaminants. They often have antigenic or pyrogenic effects in human and thus must be removed during downstream processing.

With regard to QC characterization, it is also necessary to assess if host cell proteins can interfere mAb binding on a TSKgel FcR-IIIA-NPR column. In the following study, CHO cell culture supernatant ("feedstock") was directly used for FcR column analysis and the results were compared to a previously purified mAb in the same assay (**Figure 23**, Panel a). However, no significant difference was noticed between the two profiles. This indicates that the HCPs had no significant impact on the mAb affinity to FcR column and about 5 μg of mAb in a feedstock was enough to obtain a suitable signal for monitoring process development in a bioreactor. The robustness of the assay was further tested using the mAb in CHO cell supernatant with 200 consecutive injections (**Figure 23**, Panel b). The total peak area remained constant with a % RSD (n = 10) of 0.79. It was also noticed that addition of NaCl to minimize unwanted non-specific interactions further improved durability at 20°C. The **Figure 23** (Panel c) shows how FcR column can be used for cell line selection and upstream monitoring. In this case, samples from CHO cell culture supernatant were collected, filtered, captured on protein A, and then injected to a TSKgel FcR-IIIA-NPR column. NaCl was added to improve separation. Glycoform changes in mAb were monitored over 14 days. The proportion of the intensities and peak areas of the three peaks significantly changed over the days that can be correlated to indicate changes in ADCC activity during
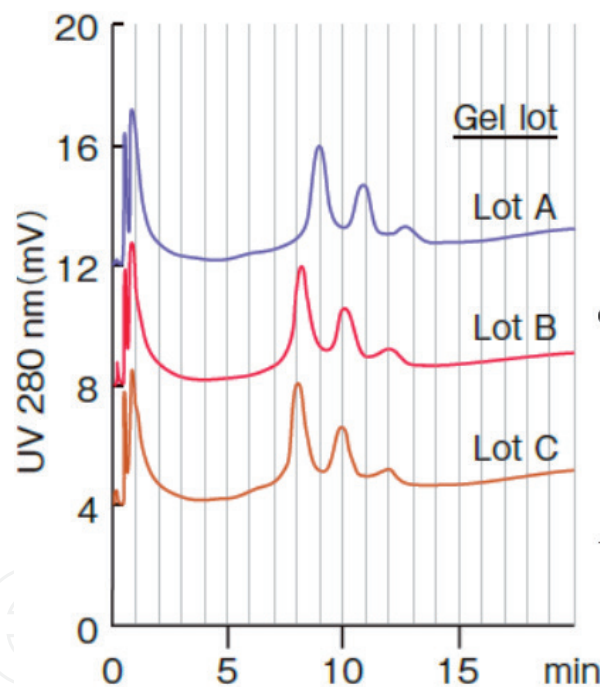

Figure 22.
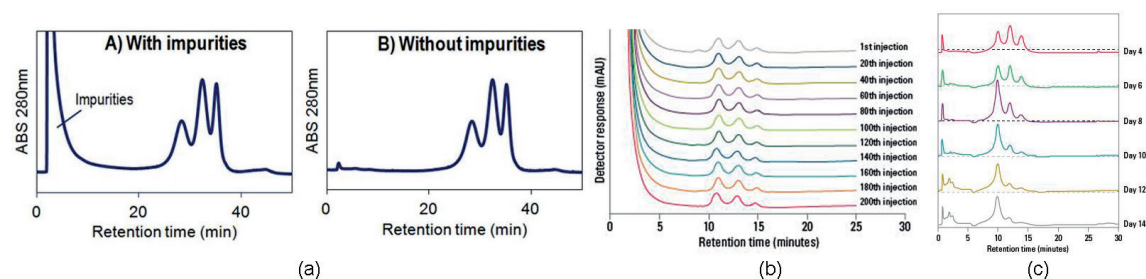*Lot-to-lot consistency in TSKgel FcR-IIIA-NPR column manufacturing.*

Figure 23.
(a) Analysis of CHO cell feedstock containing mAb versus purified mAb. (b) Assessment of FcR column stability over 200 injections. (c) Monitoring of glycan composition changes during fermentation.
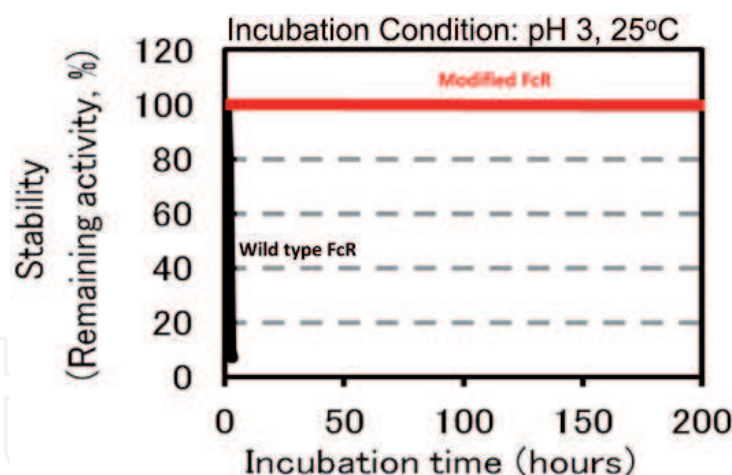
Figure 24.
Acid stability of a recombinant FcγRIII ligand as compared to a wild type ligand.

fermentation. Thus, by monitoring samples from the bioreactor using TSKgel FcR-IIIA-NPR, process engineers can approximate the optimal day for desired yield and ADCC activity.

7.4 Column pH stability and cleaning

Recommended working pH for the FcR column as mentioned in operational conditions and specifications (OCS) is from pH 8 to 4. As mentioned earlier, the protein ligand contains eight amino acid substitutions for improved stability. To further test acid stability, the column was held at pH3 for 200 hours. The modified ligand did not lose its binding affinity and the selectivity while the wild-type lost the binding affinity and selectivity within one hour (**Figure 24**). Based on this and other studies, a pH range of 3–8 is can be used for short term and pH 4.5–7 for long term usage. Due to a protein nature of the ligand, acetonitrile and other organic solvents are not suitable for the column. For cleaning, 3–5 injections of 0.5–2 mL of a buffer containing 500 mM NaCl or 20% ethanol can safely be used in reverse direction at half the normal flow rate. Once the cleaning procedure is complete, it is necessary to equilibrate the column in mobile phase for at least 45 minutes. Cleaning with alkalic solutions above pH 8 are not recommended since this will denature the protein ligand. Sodium azide (0.05%) can be used in the mobile phase as antibacterial agent.

8. Mass spectrometric characterization of glycoform peaks separated by TSKgel FcR-IIIA-NPR column

Mass spectrometric characterization is becoming an integral part of the liquid chromatography analysis. As an example how TSKgel FcR-IIIA-NPR column can be utilized in mass spec work, we describe here an in-line LC–MS intact mAb analysis of trastuzumab (Herceptin Biosimilar). The analysis was carried out using 100 mM volatile ammonium acetate buffer and a linear pH gradient from pH 6.5 to pH 4.5 at the flow rate of 0.4 mL/min. The wavelenght of detection was 280 nm. The column temperature during the analysis was maintained at 20°C. Three glycoform peaks could be detected by UV detector. Mass spectrometric detection was carried out using SCIEX X500B Q-TOF in ESI positive mode, within mass/charge (m/z) range of 5000–7000. Ion source gases 1 and 2 were maintained at 50 psi, curtain gas at 30 psi, CAD gas at 7 psi and temperature at 450°C. Spray voltage was maintained at 5200 V, declustering
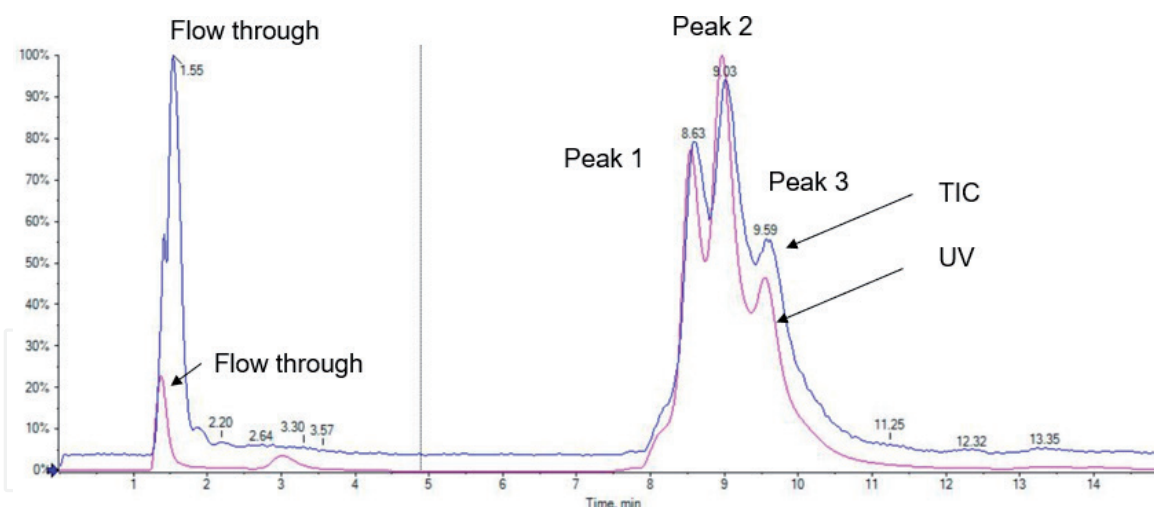
Figure 25.
Overlay of UV spectrum and Total Ion Chromatogram (TIC) of Herceptin Biosimilar.

potential at 275 V, and collison energy at 20 V. Time bins to sum was set at 120. For the automated characterization of the data acquired on the X500B QTOF, SCIEX Biotool kit software was used. Total Ion Chromatogram (TIC) was obtained by summing up intensities of all mass spectral peaks belonging to the same scan. An overlay of UV profile and TIC profile is shown in **Figure 25**. For further analysis of glyosylation profiles of these three peaks, SCIEX BioPharmaView™ software can be used.

As mentioned earlier, organic solvents such as acetonitrile are not suitable for the column and vapor pressure of water is very low. Thus, volatile salts such as 100 mM ammonium acetate or ammonium formate are used. To avoid ion source contamination during prolonged use, molarity should be kept at low (preferably <50 mM). Depending on the need for further optimization for different mAbs, volatile salt ammonium bicarbonate can also be used as such or in combination with other volatile salts.

9. Analysis of Fc fragment on TSKgel FcR-IIIA-NPR

The C-terminal part of the heavy chains contains the Fc fragment which is responsible for cellular effector functions, essential for proper function of most therapeutic mAbs. In some cases, it is desirable to express fragment antibodies that are smaller than intact mAbs but still are capable of eliciting their therapeutic function by activation of the immune system. Literature reports that both glycoengineering and protein engineering have rendered Fc domains with enhanced Fc receptor binding. In general, Fc fragments and their numerous variants are rapidly gaining interest as a platform in the development of efficient biotherapeutics.

The binding efficiency of the Fc fragment was tested in-house to assess suitability of TSKgel FcR-IIIA-NPR column on the characterization of smaller fragment antibodies. In short, trastuzumab was fragmented with papain that cleaves IgG at His228 forming two Fab parts and one Fc part (**Figure 26**). The reaction mixture was incubated at 37°C for 15 minutes to activate papain followed by mAb addition and further incubation overnight at room temperature. Papain activity was stopped with 5 mM iodoacetamide. A control a sample (no papain during incubation) and a sample from papain digestion were used for this study. Size exclusion chromatography followed by mass spectrometric analysis confirmed >95% cleavage of mAb to Fc and Fab fragments (data available by request).

Both the control mAb and fragments were analyzed on TSKgel FcR-IIIA-NPR column. As expected, Fab did not bind to the FcR column but eluted in
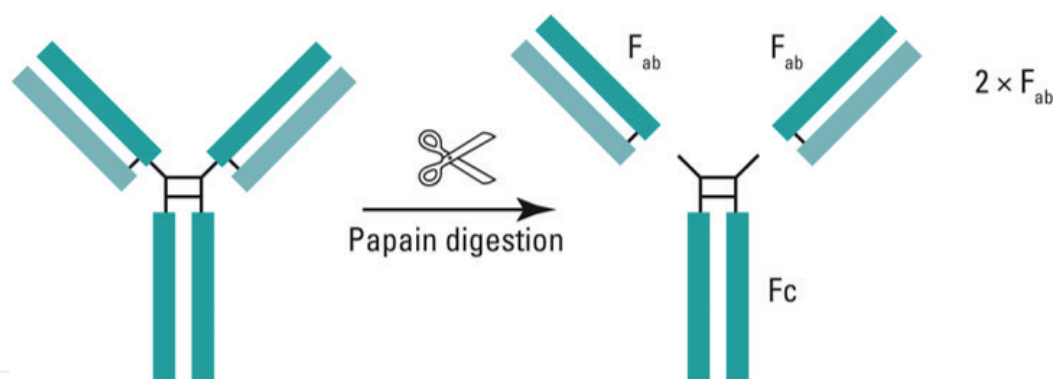
Figure 26.
Schematic representation of monoclonal antibody fragmentation with papain.

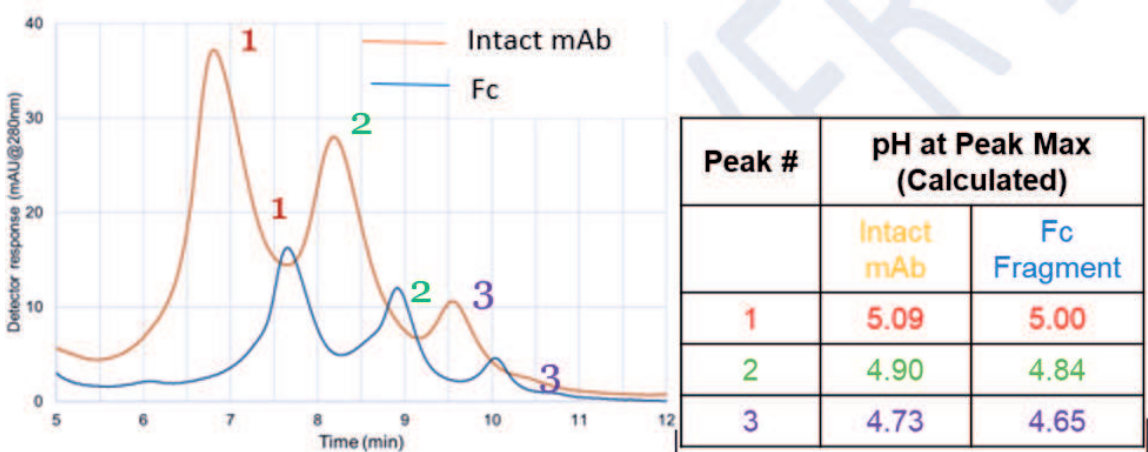| Peak #	pH at Peak Max (Calculated)	
	Intact mAb	Fc Fragment
1	5.09	5.00
2	4.90	4.84
3	4.73	4.65

Figure 27.
Analysis of intact mAb and Fc fragment on TSKgel FcR-IIIA-NPR column.

flow-through. Fc fragment efficiently bound to the column and yielded three glycoform peaks similar to intact mAb (**Figure 27**). Same sample volumes from the control sample and digestion reaction mixture were loaded onto the column. Lower peak heights for the Fc fragment were due to loss of Fab (2 x 48 kDa) from the protein mass during analysis. Interestingly, slightly longer retention times were detected for Fc fragment peaks, thus suggesting more rigid conformational stability for the Fc fragment leading to stronger binding as compared to the intact mAb. In summary, this experiment confirms that fragment antibodies, as long as they contain intact unobstructed Fc region, can be tested using the FcR column.

10. Novelty of TSKgel FcR-IIIA-NPR column

The mechanism of binding for IgG and other Fc engaging molecules is shown in the **Figure 28**. Complement component (C1q), Fc gamma receptors (FcγR), the Neonatal Fc receptor (FcRn), Tripartite motif 21 (Trim21), and Fc receptor-like (FcRL) molecules bind to various locations of mAb for the exertion of biological activity. For each ligand, the stoichiometric ratio of binding is also reported (Panel a). Recently, a biotinylated recombinant human FcRn immobilized to a Streptavidin Sepharose matrix and packed in a low pressure FPLC column has been introduced by Roche. A prepacked analytical protein-A affinity column (TSKgel ProteinA 5-PW) marketed by Tosoh also interacts with Fc region. Site of interaction of FcγR is separate from the site of interaction for FcRn or Protein A as seen in the **Figure 28** Panel b.
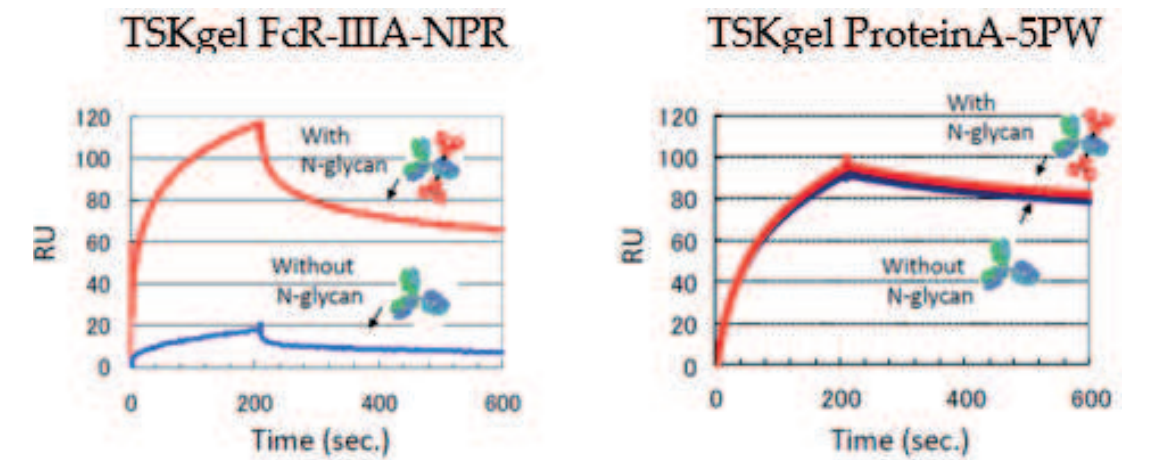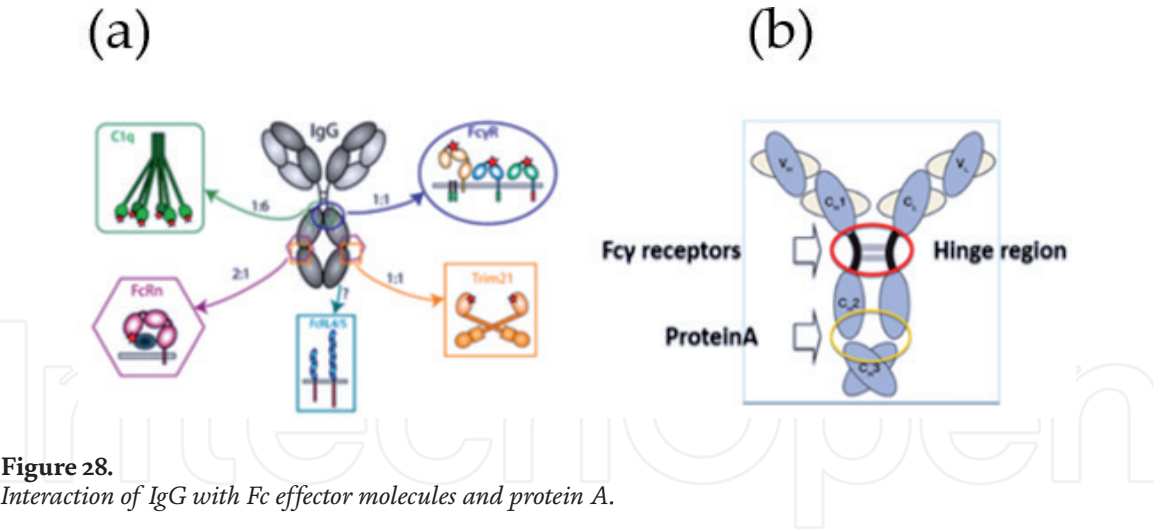
Figure 28.
Interaction of IgG with Fc effector molecules and protein A.

Figure 29.
Selectivity of the modified recombinant FcγRIIIa ligand vs. Protein A ligand.

TSKgel FcR-IIIA-NPR and TSKgel Protein A-5PW columns were tested for binding affinity of mAb with and without N-glycan using surface plasmon resonance technique (**Figure 29**). Protein A affinity chromatography column showed similar binding to mAb regardless of N-glycan whereas TSKgel FcR-IIIA-NPR column did not bind to mAb without N-glycan, similarly to the result in the **Figure 20**. Thus, the FcR column is unique due to its capability to analyze mAbs solely on the basis of their glycosylation.

11. Preparative scale purification of antibodies using FcR-based chromatography technique

Here, we provide a quick preview of the preparative scale TSKgel FcR-IIIA-5PW column that will be commercially available soon. The preparative column is manufactured using the same recombinant FcγRIIIA protein ligand, however, the ligand in this column is bound to porous (~100 nm nominal pore size) polymethacrylate polymer base beads. The column is suited for mAb purification in a significantly larger scale (loading 0.5–5 mg) as compared to the analytical FcR column (loading ≤100 μg). Chromatographic profile in the **Figure 30**, panel A was obtained using U/HPLC instrument and the analytical FcR column whereas the panel B shows peak separation with the preparative FcR column connected to a FPLC instrument. The peak separation profiles are closely similar with both columns. However, the preparative scale column allows collection of much more material for further experimentation such as glycan release, labeling and HILIC analysis, among other assays.
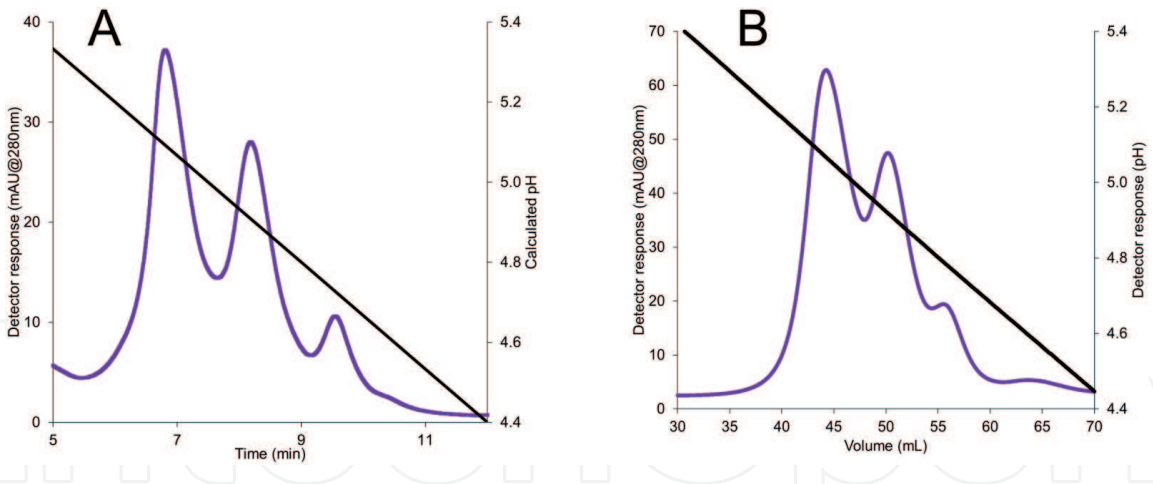
Figure 30.
Separation of mAb glycoforms using (A) analytical FcR column and (B) preparative FcR column.

12. Conclusions

TSKgel FcR-IIIA-NPR affinity chromatography column is a unique tool separating monoclonal antibodies into three peaks based on their glycosylation profile. Selectivity of the genetically engineered FcγRIIIa ligand is very specific to the mAb based on its glycan composition on highly conserved Asn-297 residue. IgG1, IgG3 and IgG4 subclasses bind to the FcR column whereas IgG2 subclass does not have affinity. IgA and IgM also don't bind to this column. Non-glycosylated mAb also does not bind to the column. Importantly, this column can be used for fast evaluation of antibody's ADCC effector function since the peak profile correlates well with the ADCC activity. Longer the retention time, higher is the ADCC activity.

The generally accepted workflow for mAb characterization, based on its glycan content typically follows the three different pathways (**Figure 31**, panel A). These are reporter bioassay for monitoring ADCC activity, Surface Plasmon Resonance (SPR) for measuring FcR affinity and U/HPLC-MS analysis for characterization of the glycan structure. Characterization of mAb on TSKgel FcR-IIIA-NPR column can combine these three pathways to one workflow (**Figure 31**, Panel B) in most circumstances.

The column is expected to be useful in several application areas (**Figure 32**) including (a) early screening of ADCC activity, (b) upstream (cell culture) optimization, (c) quality control of the mAb lot-to-lot consistency and (d) comparison between innovator and biosimilar products. Overall, this novel FcR affinity column is anticipated to be useful in research and development, characterization, manufacturing and quality control.
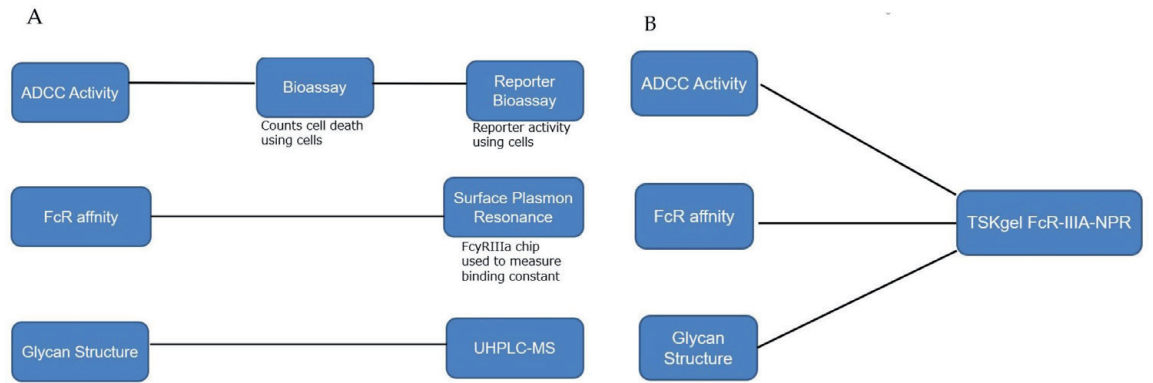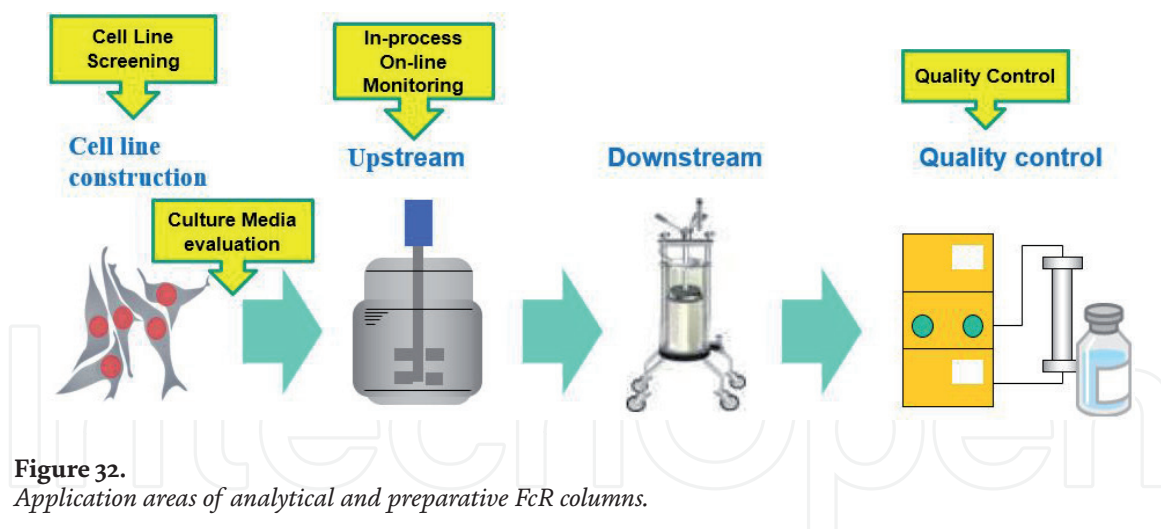

Figure 31.
Workflow for the characterization of mAb.

Figure 32.
Application areas of analytical and preparative FcR columns.

Acknowledgements

Conflict of interest

All the authors are current employees of Tosoh Bioscience, part of the Tosoh Corporation, that markets the TSKgel FcR-IIIA-NPR affinity column. Beyond this, the authors are not aware of any affiliations, memberships, funding, or financial holding that might be perceived as affecting the objectivity of this work.

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