Boosting mass spectrometry-based analytics for biopharma

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Chapter 8

Affinity capillary electrophoresis – mass spectrometry as tool to unravel proteoform-specific antibody-receptor interactions

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8.1 Abstract

Monoclonal antibody (mAb) pharmaceuticals consist of a plethora of different proteoforms with different functional characteristics, including pharmacokinetics and pharmacodynamics, requiring their individual assessment. Current binding techniques do not distinguish between coexisting proteoforms requiring tedious production of enriched proteoforms. Here, we have developed an approach based on mobility shift-affinity capillary electrophoresis - mass spectrometry (ACE-MS), permitting to determine binding of co-existing mAb proteoforms to Fc receptors (FcRs). For high-sensitivity MS analysis we used a sheathless interface providing adequate mAb sensitivity allowing functional characterization of mAbs with high sensitivity and dynamic range. As a model system, we focused on the interaction with the neonatal FcR (FcRn), which determines the half-life of mAbs. Depending on the oxidation status, proteoforms exhibited different electrophoretic mobility shifts in presence of FcRn which could be used to determine their affinity. We confirmed the decrease of FcRn affinity with antibody oxidation and observed a minor glycosylation effect, with higher affinities for galactosylated glycoforms. Next to relative binding, the approach permits determination of individual K_d values in-solution resulting in values of 422 nM and 139 nM for oxidized and non-oxidized variants. Hyphenation with native MS provides unique capabilities for simultaneous heterogeneity assessment form mAbs, FcRn and complexes formed. The latter provides information on binding stoichiometry revealing 1:1 and 1:2 for antibody:FcRn complexes. The use of differently engineered Fc-only constructs allowed to distinguish between symmetric and asymmetric binding. The approach opens unique possibilities for proteoform-resolved antibody binding studies to FcRn and can be extended to other FcRs and protein interactions.

8.2 Introduction

Monoclonal antibodies (mAbs) have demonstrated to be beneficial for treating various diseases ranging from oncology over cardiovascular to infectious diseases [1]. Most therapeutic strategies using mAbs require binding to Fc receptors (FcRs) to trigger an immune response and for efficient recycling [2, 3]. The neonatal Fc receptor (FcRn), in particular, is involved in antibody recycling and transport through polarized membranes [3]. Recycling of mAbs from circulation determines the half-life of antibodies in serum (i.e. pharmacokinetics), whereas transcytosis is crucial for neonates as they are not able to produce enough IgGs during their first months [4]. This cellular transport and recycling mechanism is based on a pH-dependent binding of the antibody to the FcRn.

Antibody therapeutics contain not only one defined molecule but several different proteoforms of the same antibody. These proteoforms range from different glycoforms attached to the conserved N-glycosylation site at Asn297 [5,6] to additional posttranslational modifications (PTMs) on the protein backbone such as oxidation or deamidation. Antibody binding to FcRn is proteoform-dependent, and proteoforms that are not able to bind to the receptor (e.g. due to a modification close to the binding site) are directed to the lysosome for degradation [7]. Binding to FcRn occurs via the CH2-CH3 domain of human IgG [8, 9]. In this region two Met are present in human IgG1 (Met252 and Met428) which are crucial for the binding. Oxidation of Met252 (and to a minor extent Met428) drastically affects the binding to FcRn reducing its serum half-life [10-12]. The role of Fc glycosylation in FcRn binding has been more controversial. While some studies showed distinct FcRn binding between different glycoforms (e.g. deglycosylated, galactosylated or sialylated) [13-15], others reported no binding difference [16-18]. Recently, glycosylation of the Fab region has also been shown to influence the binding with the FcRn due to a decreased complex association [19]. However, most of these reports focus on specific features and do not consider potential confounding by other proteoforms (e.g. influence of oxidation during glycosylation studies). This highlights the importance of proteoform-selective binding assessment to draw reliable conclusions.

For studying the interaction between mAbs and FcRn enzyme-linked immunosorbent assays (ELISA) [20] and surface plasmon resonance (SPR) [21, 22] are the most widely employed techniques. In SPR, quantitative information on the antibody and Fc receptor affinity can be obtained (K_d, K_{on}, K_{off}) [23]. With ELISA an affinity value (EC_{50}) can be obtained, which describes the concentration necessary to obtain half of the maximum binding effect [24]. However, the major drawback of these techniques is that no distinction between different proteoforms can be made and an overall K_d or EC_{50} value for all the proteoforms of an antibody sample is obtained. Therefore, binding information on single proteoforms requires tedious proteoform isolation or engineering procedures [24,25] and yet faces the problem of potential confounding by modifications that go unnoticed. In an effort to

overcome this challenge, the use of affinity liquid chromatography hyphenated with mass spectrometry (affinity LC-MS) using FcRn columns has been proposed [26]. In this approach the antibody is injected at the binding pH and eluted by gradually increasing the pH to induce dissociation. Proteoforms with lower affinity will elute earlier than higher affinity ones, permitting to study differences in binding in a straightforward way. However, no K_d or EC_{50} values can be determined and, therefore, only relative differences in retention time under stress conditions can be studied [27]. Furthermore, immobilization of the FcRn receptor to a stationary phase does not permit further structural characterization of the interaction regarding e.g. stoichiometry and limit their application to FcRn, as columns with other immobilized FcRs are not commercially available. In short, currently there is not an optimal solution for proteoform-resolved study of antibody binding to FcRn and other FcRs.

Capillary electrophoresis (CE) separates analytes in an open-tube capillary without stationary phase. As separations occur in-solution, non-covalent (protein) interactions can be maintained during the analysis opening great possibilities to study protein binding under physiologically relevant conditions. One approach that has shown immense potential to study the affinity of individual proteoforms is the mobility-shift approach. In this approach, the capillary is filled with one (free) interaction partner (e.g. receptor), whereas the second one is injected in the system (e.g. mixture of proteoforms). During separation, proteoforms interact with the receptor influencing their effective electrophoretic mobility which can be exploited to determine K_ds [28, 29]. Traditionally, affinity CE is performed with UV detection, bringing significant limitations as many proteoforms often comigrate in one peak. Hyphenation with MS overcome this issue as structural heterogeneity is assessed and resolved. Mobility shift ACE-MS has so far only been employed to study the interaction of very small peptides with antibiotics [30-32] or stromal cell-derived factor-1 with different sulfated oligosaccharides [33]. Recently, we have for the first time shown the capabilities of mobility-shift ACE-MS for monitoring protein-protein interactions using two small model proteins, i.e., trypsinogen (24 kDa) and aprotinin (6.5 kDa) [29]. Hyphenation with MS was done using a sheath-liquid interface resulting in a limited sensitivity and hampering the possibility to apply the approach to antibodies and FcRs (approx. 150 and 50 kDa, respectively).

Sheathless interfaces for CE-MS operate with nano-ESI and have demonstrated to provide better ionization efficiency and suffer less from ionization suppression opening possibilities to application to larger proteins such antibodies [34]. In this work, we developed a new approach based on mobility-shift ACE-MS using sheathless interfacing for the proteoform-specific assessment of the interaction between mAb and the FcRn receptor. Different engineered antibodies and oxidized samples were employed to develop and demonstrate the capabilities of the approach. We show that we can simultaneously monitor the heterogeneity of the antibody and the FcRn and specifically assess their binding in-solution. This allowed us to determine specific K_d values for the different proteoforms in a mixture

without the need of isolation or proteoform engineering. Furthermore we determined the antibody:FcRn complex stoichiometry 1:2 with symmetrical FcRn binding.

8.3 Experimental section

8.3.1 Chemicals

All reagents employed were of analytical grade. Ammonium hydroxide, dithiothreitol (DTT), 7.5 M ammonium acetate (AmAc) solution, hydrogen chloride, guanidinium hydrochloride (Gua-HCl), tris-hydroxymethyl aminomethane (Tris), calcium chloride and H_2O_2 were obtained from Sigma Aldrich (Steinheim, Germany). Formic acid (FA), acetonitrile (ACN) and iodoacetic acid (IAA) were purchased from Thermo Scientific (Pittsburgh, PA). NAP-5 columns were obtained from GE Healthcare (Chicago, IL). Deionized water was obtained from a Milli-Q purification system (EMD Millipore, Berlington, MA). Bio-Spin 6 columns were purchased from Bio-Rad (Hercules, CA). 10 kDa Vivaspin MWCO filters were provided by Sartorius (Göttingen, Germany). Trypsin proteomic-grade was obtained from Roche Diagnostics (Mannheim, Germany).

8.3.2 Samples

Standard and engineered mAbs, Fc-only constructs and FcRn were provided by Roche Diagnostics (Penzberg, Germany). The FcRn consisted of the human beta-2-microglobulin with a (G4S)4 linker [35], followed by the extracellular domain of the human IgG receptor FcRn large subunit p51, a His 10-tag and an Avi-tag. Oxidation of mAb samples (1 µg/µL) was achieved by adding different amounts of a 1% H2O2 solution resulting in a final H2O2 concentration ranging from 0 to 0.2% and incubation for 24 h at 25°C. mAbs and Fc-only samples were buffer exchanged to 50 mM AmAc pH 6.0 using NAP-5 columns. The columns were equilibrated with a complete buffer fill four times. Next, 300 µL (1 mg) of mAb samples were loaded onto the column and allowed to drip through. After adding an additional 350 µL of 50 mM AmAc pH 6.0, the sample was eluted with 500 µL 50 mM AmAc pH 6.0. The concentration of the samples was determined using a NanoPhotometer (Implen, Munich, Germany) and diluted to obtain a concentration of 1 µg/µL for mAb samples or mixtures of oxidized and non-oxidized mAbs and 0.75 µg/µL for Fc-only samples prior injection. Lysozyme from chicken egg (Sigma Aldrich) was desalted and buffer exchanged to 50 mM AmAc pH 6.0 using 10 kDa VivaSpin columns. Lysozyme was diluted to a final concentration of 2 µg/µL. Similarly, the FcRn receptor (4.9 µg/µL) was desalted and buffer exchanged to 50 mM AmAc pH 6.0 using 10 kDa VivaSpin columns and diluted to the desired concentration (0.5 to 12 µM).

8.3.3 Affinity CE-MS

Affinity CE experiments were carried out on a Sciex (Framingham, MA) CESI 8000 instrument

using OptiMS neutrally-coated capillaries (Sciex) with a porous tip (length, 91 cm; 30 µm i.d.; 150 µm o.d.). The capillaries were prepared following the instructions of the manufacturer. The capillary was flushed for 5 min (100 psi, forward) with 0.1 M HCl, 10 min (100 psi, forward) with 50 mM AmAc (pH 3.0) and 30 min (100 psi, forward) with MilliQ, followed by an equilibration for 16-18 h. This procedure was performed only priot first injection. Before each run, the capillary was flushed for 2 min with 0.1 M HCl (100 psi, forward pressure), 2 min with MilliQ (100 psi, forward pressure), 2 min with 50 mM AmAc pH 6.0 (100 psi, reverse pressure) and 2 min with 50 mM AmAc pH 6.0 (100 psi, forward pressure). Following the capillary was filled for 2 min (100 psi, forward pressure) with 50 mM AmAc pH 6.0 containing different concentrations of FcRn receptor or without receptor. Afterwards, a marker protein (lysozyme) was injected (1.5 psi, 15 s), followed by the antibody sample (2.5 psi, 15 s) and a plug of BGE with or without receptor (1 psi, 25 s). The separation was carried out for 45 min with 20 kV at 25° C. After the separation was complete, the voltage was ramped down to 1 kV in 5 min.

The outlet of the separation capillary was placed in a nano-electrospray ionization source at the entrance of an Orbitrap Exactive Plus Extended Mass Range mass spectrometer (Thermo, Waltham, MA). The mass spectrometer was operated in positive ionization mode using the following parameters: capillary voltage 1.8-2.2 kV; ion injection time 200 ms; HCD energy 100 eV; skimmer voltage 15 V and S-Lens voltage 15 V. Mass spectra were recorded in a mass range of 1,000 to 15,000 m/z with a resolution of 17,500 at m/z 200. MS control and data acquisition were performed using the Xcalibur (Thermo Fisher Scientific) software. For data analysis, the Intact Mass software from Protein Metrics (Cupertino, CA) was used.

For calculation of the K_ds, the electropherograms were aligned using the marker protein (lysozyme) and the electrophoretic mobility shift compared to the measurement without FcRn was calculated for each proteoform. Following data were plotted using the software GraphPadPrism using the one side specific binding model.

8.3.4 Assessment of mAb oxidation levels by LC-MS/MS

To determine the oxidation levels of the mAbs and Fc-only samples, 50 µg (50 µL) of sample were denatured with an equal amount of denaturation buffer (8 M Gua-HCl, 0.4 M Tris/HCl, pH 8.5). Afterwards, the samples were reduced by adding DTT to a final concentration of 20 mM and incubated for 60 min at 50°C. Following, the samples were alkylated with IAA in a final concentration of 50 mM and kept for 30 min in the dark. For buffer exchange, prior to trypsin digestion, the samples were loaded on Bio-Spin 6 columns. Beforehand the columns were 2 min centrifuged at 1000xg to remove the storage liquid and three times equilibrated with 500 µL digestion buffer (50 mM Tris/HCl, 2 mM CaCl2, pH 7.5) and centrifuged for 2 min at 1000xg in between. The reduced and alkylated samples were loaded onto the column and centrifuged for 4 min at 1000xg collecting thereby the eluate. 25 µg trypsin were dissolved in 100 µL 10 mM HCl solution. 3 µL of trypsin solution were added to each sample and

incubated for 18 hours at 37ºC. To stop the digestion, 17 µL of 10% TFA solution were added and the samples were diluted 1:1 (v/v) with MilliQ prior to LC-MS measurement. 10 µL sample were injected into an Acquity UPLC (Waters, Milford, MA, USA) equipped with a CSH C18, 1.7 µm, 130 Å, 2.1x150 mm column (Waters). The separation was performed using 0.1% FA in H2O as mobile phase A and 0.1% FA in ACN as mobile phase B at a column temperature of 65ºC. A linear gradient from 1% B to 35% B in 45 min was used for the separation of peptides, followed by a cleaning of 3 min at 8% B and a re-equilibration for 4 min at 1% B. The LC was coupled to an Orbitrap Velos mass spectrometer operated in positive ionization mode. The isolation width for CID fragmentation was 1 m/z, the fragmentation energy was set to 35 eV, the activation q was 0.25 and the activation time 10 ms. The resolution was set to 30000, and an m/z range was 200 to 2000 in full scan mode was monitored. The amount of oxidation for the sites Met252 and Met428 were calculated by determining the area under the curve of the non-oxidized and oxidized peptide extracted ion chromatograms and determining the relative ratio of oxidized peptide in each sample.

8.4 Results and Discussion

8.4.1 Development of a mobility-shift ACE-MS approach for proteoform-resolved antibody - FcRn binding

The binding of antibodies to FcRn takes place in the endosome at a pH between 5.5 and 6.0. Mobility shift affinity approaches rely on the different mobility of the protein and receptor and in consequence, the protein-receptor complex. To study which conditions show the largest different in mobility between FcRn and mAb1, a mixture of both proteins was injected (1 µg/µL each) and analyzed using a background electrolyte consisting of 50 mM AmAc at pH 5.5 or 6.0. At these pHs, both FcRn receptor (calculated pI 6.24) and mAb1 (calculated pI 8.47) are positively charged. Therefore, separations were performed using a neutrally-coated capillary to avoid adsorption to the capillary wall. 50 mM AmAc at pH 5.5 resulted only in a minor difference in mobility between FcRn and mAb1 (**Figure S1**). Using AmAc at 6.0, FcRn exhibited significantly lower mobilities than mAb1 (**Figure S1**), indicating that a shift in the mobility could be observed for the FcRn-antibody complex.

For affinity CE experiments, FcRn was added to the BGE, and after filling the capillary with the receptor, the mixture of antibody proteoforms was injected. Antibody proteoforms with high affinity towards FcRn would interact with the receptor during the separation and exhibit lower effective mobility, while proteoforms with no interaction should not change their effective mobility compared to their analysis in absence of receptor. To correct for changes in the ionic strength and viscosity of the BGE with addition of different amounts of FcRn, a marker protein showing no interaction with FcRn (lysozyme, molecular weight 14.3 kDa, calculated pI 10.36) was employed.

Initial experiments were performed with an engineered antibody which bears no glycans in the Fc domain (NGmAb). Oxidation of Met252 it is known to influence the binding between the antibody and the FcRn receptor. Therefore, to fully explore the capabilities of the approach, analyses were performed with NGmAb material which was intentionally oxidized as confirmed by bottom-up proteomics. For NGmAb oxidation levels for Met 252 were 41%, comprising a mixture of antibodies containing 0, 1 or 2 oxidations at this site. First, the mixture of oxidized and non-oxidized species were analyzed in absence of FcRn receptor using 50 mM AmAc at pH 6.0.

Upon oxidation, NGmAb showed unchanged electrophoretic mobility under standard CE conditions (**Figure 1A**) which was in line with expectations as oxidation comes with only a relatively small increase in intact mass while not affecting mAb charge.

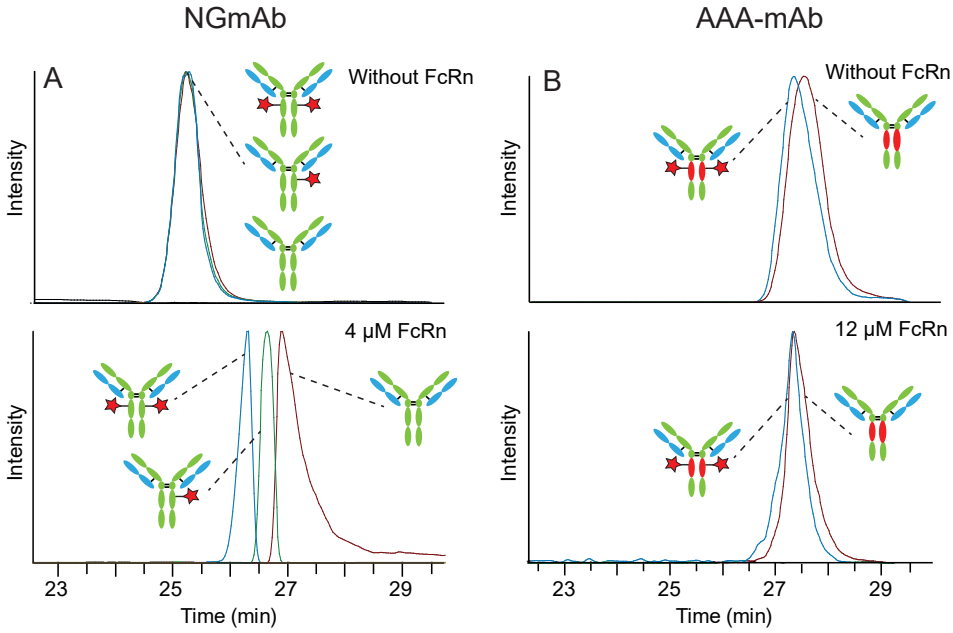

Figure 1. Sheathless CE-MS separation obtained for a H_2O_2 stressed A) NGmAb or B) AAA-mAb sample using a BGE without FcRn (upper panel) and containing FcRn (lower panel). The blue trace corresponds to the extracted ion electropherograms (EIEs) of the double-oxidized (two red stars), the green trace corresponds to the EIEs of mono-oxidized (one red star) and the brown trace shows the EIEs of the non-oxidized mAbs present in the mixture. Signal intensities are normalized.

Reported K_ds for antibodies and FcRn are between 3.2–284.7 nM [36]. Therefore, for affinity experiments FcRn was added to the BGE at concentrations in the range of nM to low µM. Analysis of the oxidized NGmAb sample using 4 µM FcRn in the BGE resulted in a clear shift of the effective electrophoretic mobility for all the species indicating that all of them interact with FcRn (**Figure 1A**). More interestingly, the different species start to separate indicating

different binding affinities. The double oxidized form of the NGmAb migrated in the first place indicating that it has the lowest binding affinity while the unmodified NGmAb which migrates the latest has the highest binding affinity. The mono-oxidized NGmAb showed an intermediate shift between the double and non-oxidized antibody indicating a decrease on binding affinity. However, is important to note that the observed decrease corresponds to the inaccessibility of one of the Fc chains for binding to FcRn in solution, but does not imply in-vivo half-life decrease. In addition, even if a decrease on binding affinity was observed for the oxidized forms, still binding to FcRn was observed for all the species.

To confirm that these observations correspond to the specific binding of the antibody to the FcRn and are not the result of non-specific interactions or any other artifact, we analyzed an antibody which has been silenced for binding with FcRn (AAA-mAb or triple-A mutant). The antibody was intentionally oxidized prior analysis resulting in oxidation levels of 99.6% for Met252 (i.e. double oxidized antibodies). Analysis of a 1:1 mixture between oxidized and non-oxidized AAA-mAb in absence of FcRn resulted in two overlapping signals at 27.5 min resulting from the double-oxidized and the unmodified triple-A antibody (**Figure 1B**). Of note, different antibodies (e.g. triple-A and NGmAb) exhibit different electrophoretic mobilities and therefore, the obtained migration times can not be taken as absolute values of affinity but relative with their analysis in absence of receptor (i.e. mobility shift). Addition of up to 12 µM FcRn receptor to the BGE did not induce any shift on the mobility or additional separation indicating that the AAA-mAb is not binding to FcRn (**Figure 1B**). These results confirmed that the shift on the mobility observed for the NGmAb antibody, indeed is induced by the specific binding of the antibody with the FcRn receptor.

To demonstrate the applicability of the method for simultaneous binding assessment of multiple proteoforms, a standard glycosylated mAb was employed. As in previous cases, a 1:1 mixture of oxidized (97.7% for Met252) and non-oxidized mAb1 was analyzed, resulting in co-migration in absence of FcRn (**Figure 2A**). Addition of 0.25 and 1 µM of receptor in the BGE induced a shift on the mobility for both species with a partial separation between the double-oxidized and the non-oxidized mAb for the latest (**Figure 2A**). Further increase on the concentration (up to 6 µM) did not result in a higher mobility shift and/or further separation suggesting that a plateau is reached above 1 µM **(Figure 2A**). One characteristic of mobility shift affinity CE approaches is their capability to provide quantitative information about binding. Next to monitor differences in relative binding for different proteoforms we also wanted evaluate the capabilities of the method to calculate their absolute binding – i.e. determination of affinity constants in a proteoforms-selective manner. After determining and plotting the shift on the effective electrophoretic mobility for the measured concentrations of FcRn a binding curve was obtained. By fitting the curve using nonlinear regression a K_d value of 139 (± 67) nM for the non-oxidized and 422 (± 157) nM for the oxidized mAb was obtained **(Figure S2**). Reported K_d values are in the range of 3.2–284.7 nM [36] depending in the specific structural features of the antibody (Fc and Fab portion) which are in line with the

obtained values. However, the errors associated to our K_d determinations were significantly elevated indicating that more points and further correction of electrophoretic mobilities would be needed for accurate K_d determinations but this initial exploration shows that the current approach could be used to determine K_ds of different antibody proteoforms.

In addition to oxidation different glycoforms can potentially can affect the binding with FcRn. mAb1 comprise different glycoforms dominated by complex-type glycans containing different core fucosylation and galactosylation levels. The influence of these glycans on FcRn binding was investigating by looking at their shift on their electrophoretic mobility (**Figure 2B**). While different glycoforms do not influence electrophoretic mobility (**Figure 2B, upper panel**), shifted profiles were observed in presence of FcRn **(Figure 2B, lower panel**). The shift difference between glycoforms was significantly lower than the observed for oxidoforms but indicate some degree of influence on FcRn binding. In line with recent publications we did not observe any effect on fucosylation in binding while higher galactosylation slightly increased binding affinity [13-15].

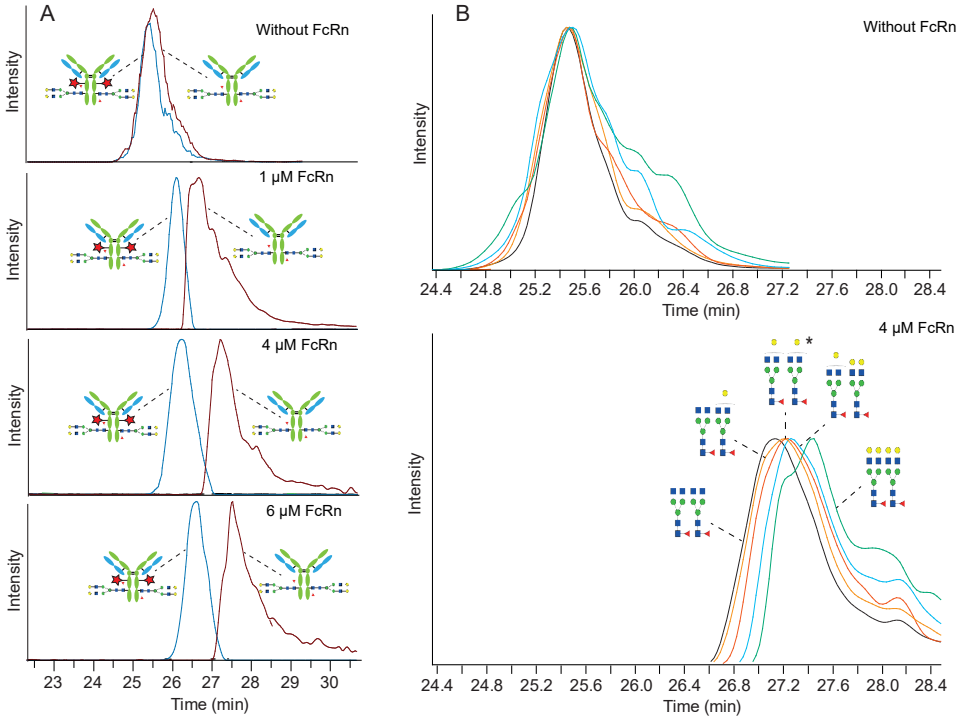

Figure 2. Sheathless CE-MS separation obtained for a 1:1 mixture of a H2O2 stressed and reference mAb1 using a BGE without or with different amounts of FcRn. A) Illustration of the effect of oxidation. The blue trace corresponds to the EIEs of the double-oxidized (two red stars) and the brown trace shows the EIEs of the non-oxidized mAbs. In both cases the antibody with G0F and G1F glycoforms were extracted. B) Illustration of the effect of glycosylation. In different colors EIEs of different glycoforms of the non-oxidized antibody. Signal intensities are normalized. *Either G1F/G1F or G2F/G0F.

8.4.2 Structural assessment by on-line native MS

Affinity separations were performed with online native MS detection permitting, next to functional assessment, structural characterization. The use of 50 mM AmAc as BGE provided good signal intensities for the antibody proteoforms. For the NGmAb, which is non-glycosylated, next to main form, two additional signals corresponding to the antibody with one (+162 Da) and two times glycation (+324 Da) were observed. After addition of the receptor to the BGE, additional signals appeared in the mass spectra corresponding to the free FcRn and in complex with the antibody, which were more perceptible with increasing amounts of FcRn in the BGE. The constant presence of FcRn in the MS also caused a slight decrease of antibody signal intensity. The plateau was reached at around 4 µM of FcRn depending on the antibody. Still, at higher concentrations of FcRn (up to 12 µM was tested) the ionization suppression was moderate and good sensitivity was obtained for the detection of the antibody proteoforms and their complexes. The constant FcRn signal, on the other hand, permitted the simultaneous characterization of the receptor heterogeneity during the affinity experiments. FcRn was detected as a complex pattern of signals at around 49 kDa, corresponding to different glycoforms at the N-glycosylation site at position 220 on our FcRn construct (**Figure 3** and **Figure S3**). The glycoforms were assigned based on their average mass and on previously reported glycopeptide data [37] (**Figure S3**). Next to the antibody and the receptor, NGmAb in complex with FcRn was detected in a stoichiometry of 1:1 or 1:2 (**Figure 3**). At concentrations of FcRn of 2 µM only NGmAb in complex with 1 FcRn molecule (**Figure S4A**) was observed while at 4 µM NGmAb in complex with 2 FcRn molecules was also noticeable (**Figure S4B**). Further increase of FcRn concentration up to 12 µM higher resulted in an increase of the signals in particular for the corresponding to the NGmAb-FcRn 1:2 complex (**Figure S4C, D**). **Figure 3** shows the zoomed spectra of the complex of the NGmAb with one FcRn molecules. Several signals were observed corresponding to the antibody in complex with different FcRn glycoforms (**Figure 3**) which can be clearly represented after simulation of the glycoprofile of the NGmAb-FcRn for the most abundant FcRn glycoforms (**Figure 3**, red traces). The NGmAb in complex with 2 FcRn was also observed at around 243 kDa, as a complex pattern of signals where the main glycoforms were detected (**Figure 3**).

Using the glycosylated standard mAb1 resulted in a more complex pattern of signals for the complex formed between the mAb1 and FcRn. Still, we were able to detect the antibody in complex with one FcRn (**Figure S5**). The deconvoluted mass spectrum of this complex is dominated by the glycosylation of the antibody (G0/G0 to G2F/G2F) (**Figure S5**) which still contains different glycoforms of the receptor resulting in broader signals. The complex (**Figure S5** red lines) was simulated using the two mayor glycoforms of the FcRn receptor resulting in a good match between the theoretical and the observed spectra. The complex of mAb1 with two FcRn molecules could not be detected, most probably due to the high complexity arising from the glycosylation of the antibody and the two FcRn molecules resulting in a higher spread of the signals and, therefore, reduced signal intensity.

Monitoring the heterogeneity of all the species including the antibody, receptor and the complex results multiple benefits. Next to establishment of the stoichiometry, it permit to study if the variability of the FcRn receptor (e.g. different glycoforms) have an influence in the binding with the antibody -i.e. it permits to study the interaction in a receptor proteoform specific manner. For our particular example on FcRn we could not observe any clear preference of certain FcRn glycoform in binding with NGmAb based on the obtained data, suggesting no influence of the FcRn glycosylation on the binding with the antibody. The observed glycation on the NGmAb (**Figure 3**) did also not show any shift in mobility as expected.

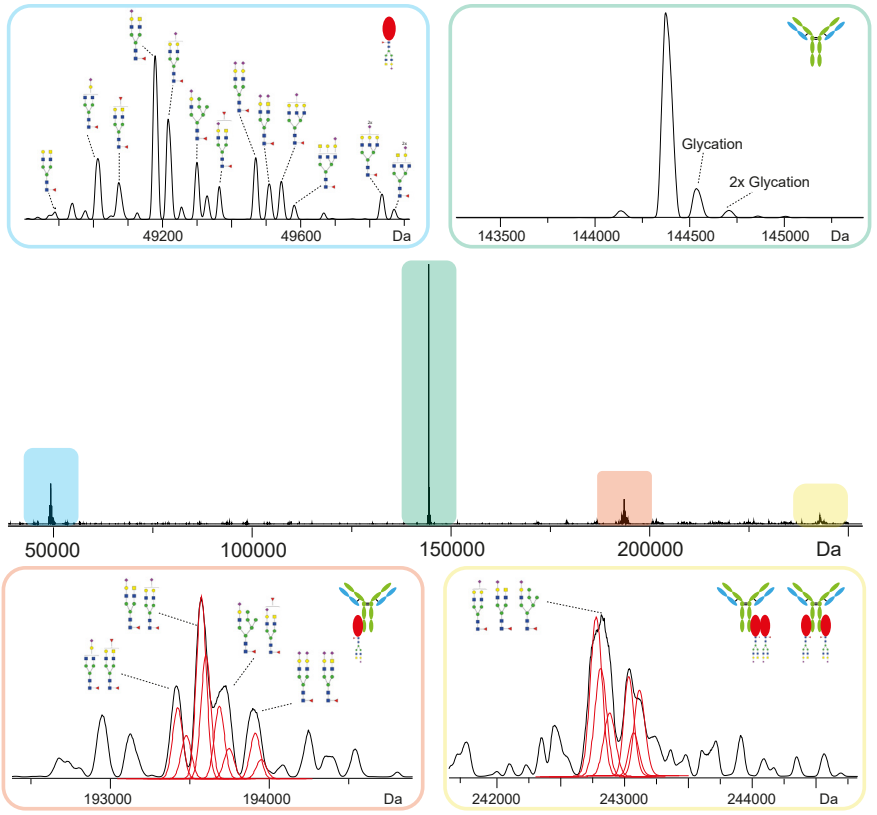

Figure 3. Deconvoluted mass spectrum of the non-stressed NGmAb using a BGE containing 12 µM FcRn. Blue, zoom of FcRn containing several different glycoforms. Green, zoom of NGmAb containing 0, 1 or 2 glycations. Vermillion, zoom of NGmAb in complex with 1 FcRn. Yellow, zoom of NGmAb with 2 FcRn molecules. Complexes heterogeneity comes from different glycoforms of the FcRn (most abundant ones are simulated in red.

8.4.3 Evaluation of antibody:FcRn binding stoichiometry and symmetry using Fc-only constructs

Unlike other Fc receptors, the binding of antibodies to FcRn occurs in a 1:2 stoichiometry. This has been demonstrated by crystal structures of rat FcRn and rat IgG2a [38]. However, whereas some reports suggest a dimerization of the rat FcRn and binding only to one side of the Fc portion [38, 39] other studies using column binding assays [40] or using mutated Fcs [41] suggested binding of one FcRn per Fc chain. For human FcRn similar opposite observations have been made. While no FcRn dimers could be observed in crystallization experiments [42] recent publications show strong evidences on self FcRn interaction in a pH dependent manner [43]. Recently, cryo EM analysis of mAbs:FcRn showed 1:1 and 1:2 stoichiometry with one FcRn molecule binding to each side of the mAb-Fc [44].

Our MS results show 1:2 binding for NGmAb but still this does not answer the question on binding symmetry. To test whether we can also study if we have an asymmetric or symmetric binding of the FcRn with our mobility-shift CE-MS approach, we analyzed two different engineered Fc constructs [45]. These constructs consist on the Fc portion (two Fc/2 chains) connected by disulfide bridges. One of the constructs comprised two unmodified Fc/2 chains (wt/wt) and should allow for FcRn binding on both sides. The second construct carried a triple-A mutation in one of the Fc/2 chains (wt/AAA) permitting only binding of the FcRn to one of the two Fc/2 chains. Injection of a mixture of both constructs without FcRn led to a separation of both Fc constructs due to their different pI (and therefore, different electrophoretic mobility) with the wt/wt migrating earlier than the wt/AAA (**Figure 4A**). After filling of the capillary with 6 µM FcRn receptor a clear shift on the mobility for both constructs was observed (**Figure 4B**). For wt/AAA the mobility shift was minor while for the wt/wt constructs a larger shift was observed.

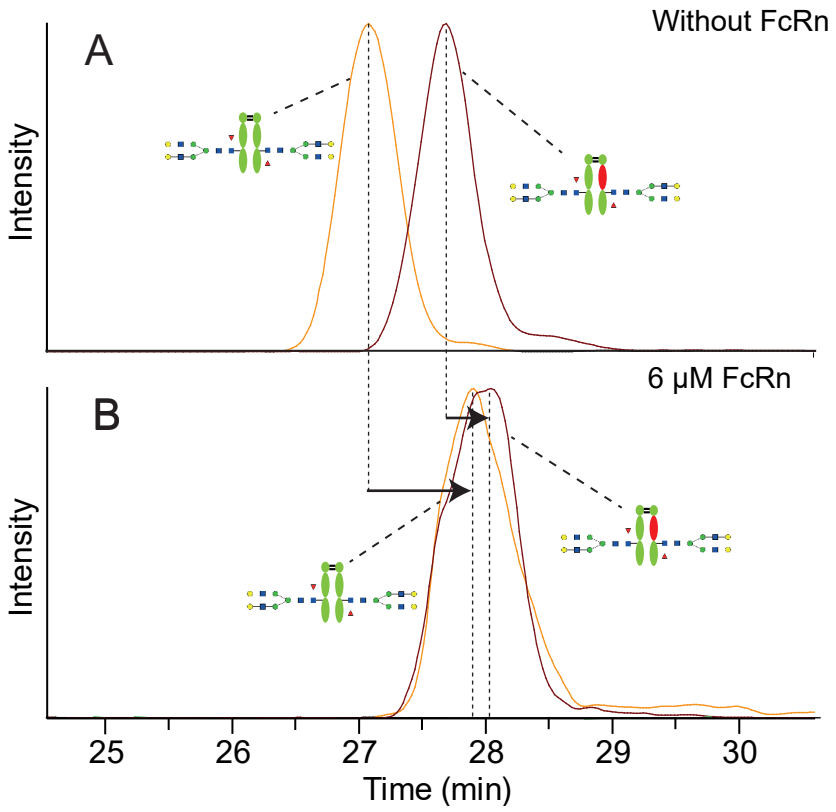

Figure 4. Sheathless CE-MS separation obtained for a 1:1 mixture Fc constructs (wt/wt (green) or wt/AAA (green and red)) using a BGE A) without FcRn or B) containing 6 µM FcRn. The orange trace represent the EIE of the wt/wt Fc-construct and brown trace shows the EIE of the wt/AAA Fc-construct. Signal intensities are normalized.

These results indicates, that the construct which allowed a two side (symmetrical) binding showed a larger shift than the one where only one FcRn molecule can bind. These results suggest that under these conditions the observed 1:2 complex correspond most likely to the one FcRn molecule bound to each side of the mAb-Fc as reported recently [44]. Looking at the mass spectra no dimers of the FcRn were observed supporting this observation. However, dissociation during ionization can not be excluded. Regarding the complexes we could only observe the complex between the Fc constructs (wt/wt and wt/AAA) and one FcRn (**Figure S6**). As for mAb1, the complex with two FcRn receptor for the wt/wt construct could not be detected due to the large heterogeneity coming from the glycosylation of the Fc construct and the two glycosylated FcRn molecules. Furthermore, from this experiment it can be concluded that the missing Fab portion do not abrogate binding of the Fc to FcRn and still a mobility shift can be observed. Although the absolute affinity of this molecules would be different from their complete counterparts containing the Fab domain, the proposed

approach can be applied to monitor the effect of Fc modifications in FcRn binding opening possibilities to application to polyclonal samples.

8.5 Conclusion

We developed a unique approach based on mobility shift ACE-MS to study the affinity of individual antibody proteoforms in a sample to FcRn. This approach represents the first affinity ACE-MS for functional studies on mAbs and overcome most of limitations faced with current binding techniques. We have focus in FcRn antibody interaction, which is of special interest for antibody recycling and thereby important for antibody therapeutic pharmacokinetics. We demonstrated that proteoforms with different binding affinities (i.e. oxidoforms) exhibit different shift in the electrophoretic mobility in presence of FcRn. This permits to determine their individual binding affinity without necessity of enrichment or purification of different forms. We also showed that K_d values could be individually determined for the oxidized and non-oxidized mAb1 in mixture, providing lower K_d for the non-oxidized mAb1. The mass spectrometric detection allows not only to characterize the antibody and its proteoforms, but also the heterogeneity of the receptor and the complexes formed. Another advantage of MS detection is the possibility to also characterize and study binding affinity of overlapping species. Analyzing a standard glycosylated mAb showed slight difference in binding of Fc-glycoforms to FcRn with galactosylated variants showing higher binding as recently suggested in few reports. Similar observations were made through MS monitoring of the complex between mAb and FcRn receptor where no preferred binding of FcRn or antibody glycoforms could be monitored. In the particular case of the FcRn receptor heterogeneity our data did not show specific FcRn glycoforms in higher abundance in the complex compared to the background FcRn signal but more work would be needed to confirm this due to the complexity of the spectra. Reversing the approach (i.e. adding the mAb in the BGE and inject the receptor) could provide information on proteoform receptor binding in a more accurate way, and will be further studied. Furthermore we investigated the binding stoichiometry between mAbs and the FcRn. Our MS spectra revealed 1:1 and 1:2 mAb:FcRn complexes which under the applied conditions seems to be symmetrically bound. We used two different Fc-only constructs which allowed one or two side binding of the FcRn. We found binding of both Fc-only construct to FcRn with the one allowing both sides binding providing a larger mobility shift compared to the construct allowing only one side binding.

The flexibility and simplicity of switching receptors (just changing the BGE) next to the low amount of receptor necessary compared to classical affinity LC, makes this approach very attractive for effector function antibody monitoring in a proteoform selective manner. Due to the binding similarity of FcRn to other FcRs we believe that this approach could be extended to study their binding. More studies are warranted on the interaction of mAbs with other Fc receptors.

8.6 Supporting Information

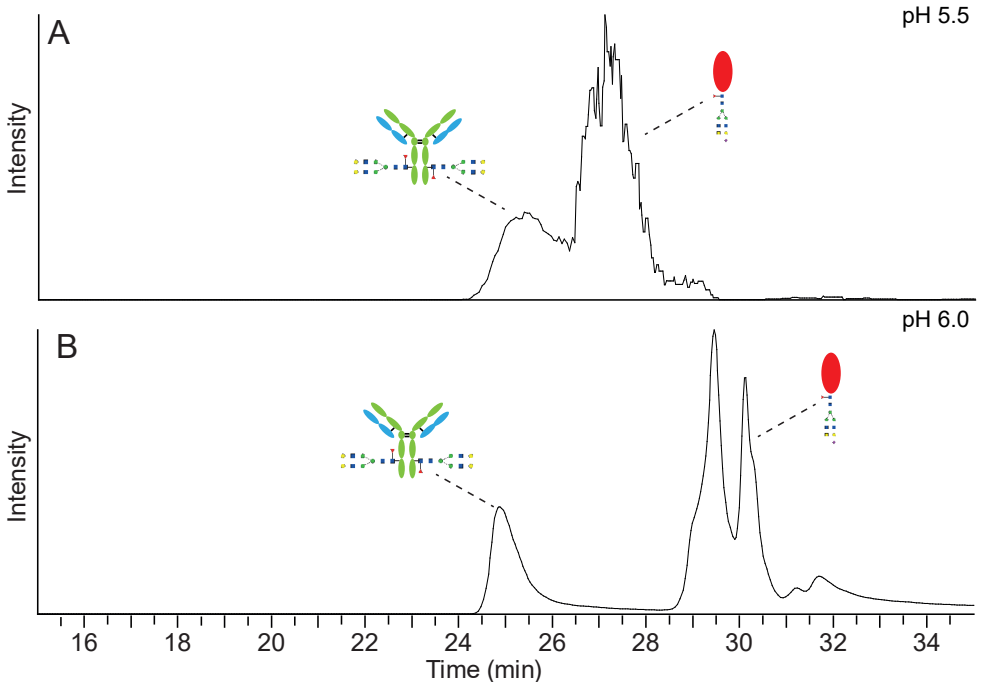

Figure S1. Sheathless CE-MS separation obtained for mAb1 and FcRn using A) 50 mM AmAc pH 5.5 and B) 50 mM AmAc pH 6.0.

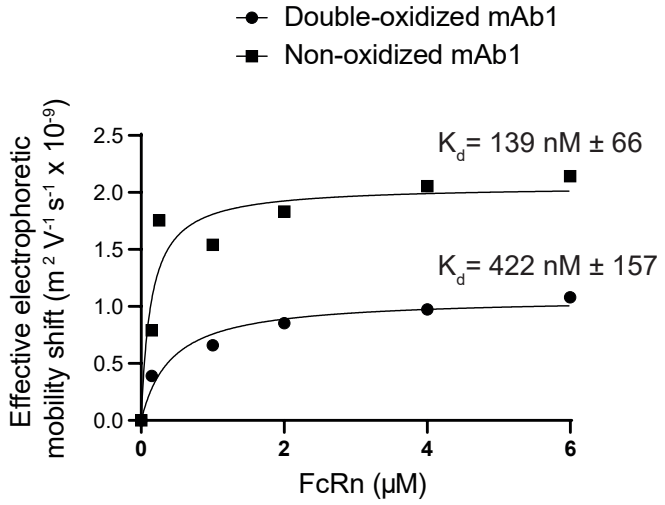

Figure S2. Binding curves obtained by plotting the mobility shift for non-oxidized and oxidized mAb at different concentrations of FcRn.

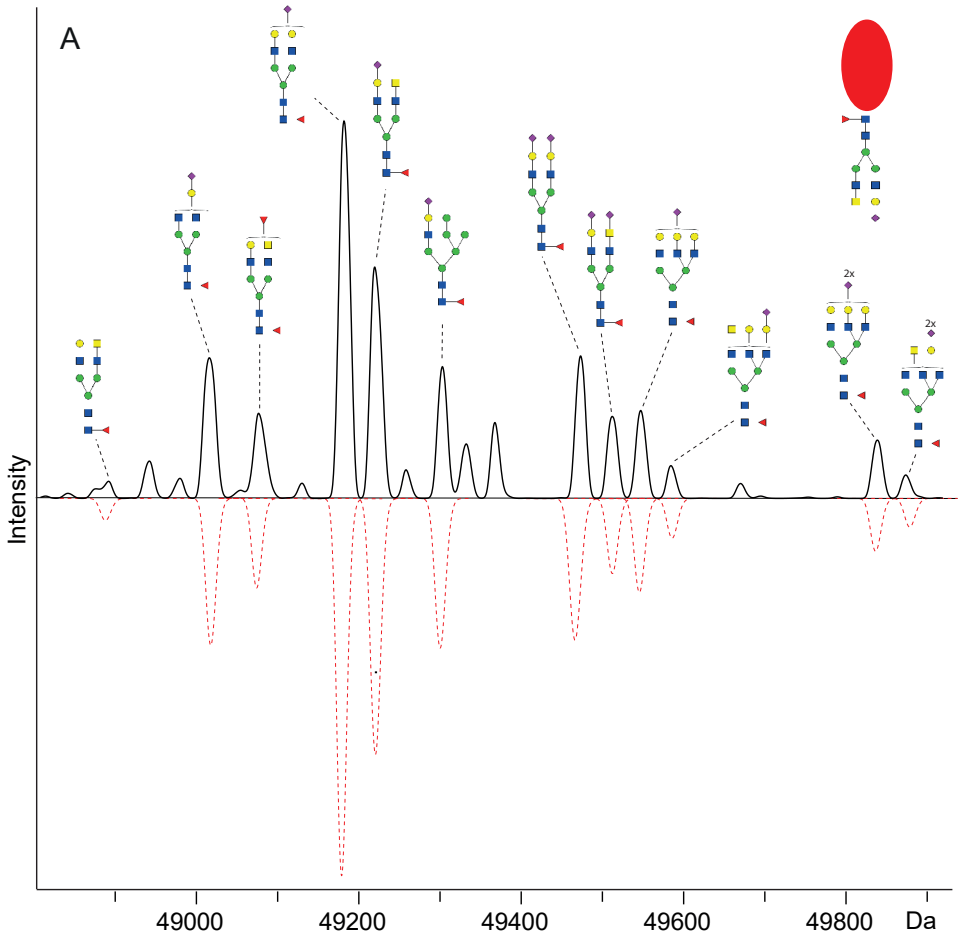

Figure S3. Glycosylation of FcRn receptor (black line) and simulated glycoforms in dashed red line.

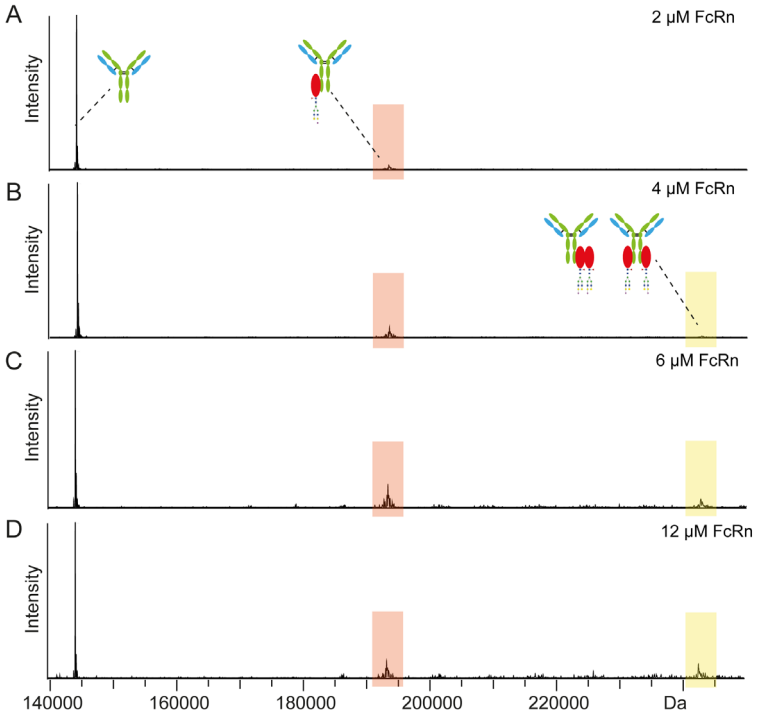

Figure S4. Deconvoluted mass spectra after ACE-MS separation of NGmAb using different amount of FcRn receptor in the BGE. A) 2 µM FcRn receptor, B) 4 µM FcRn receptor, C) 6 µM FcRn receptor and D) 12 µM FcRn receptor. Complex between NGmAb and 1 FcRn receptor is highlighted in vermillon and NGmAb in complex with 2 FcRn receptors highlighted in yellow.

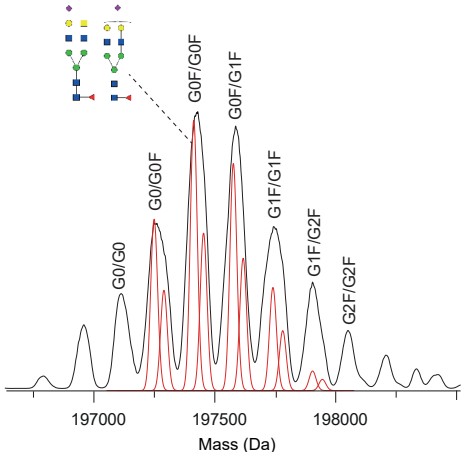

Figure S5. Deconvoluted mass spectra of the non-oxidized peak highlighting the area where the complex between mAb:FcRn appear and simulation of the mass spectra for the complex with the two major glycoforms of the FcRn receptor (red lines).

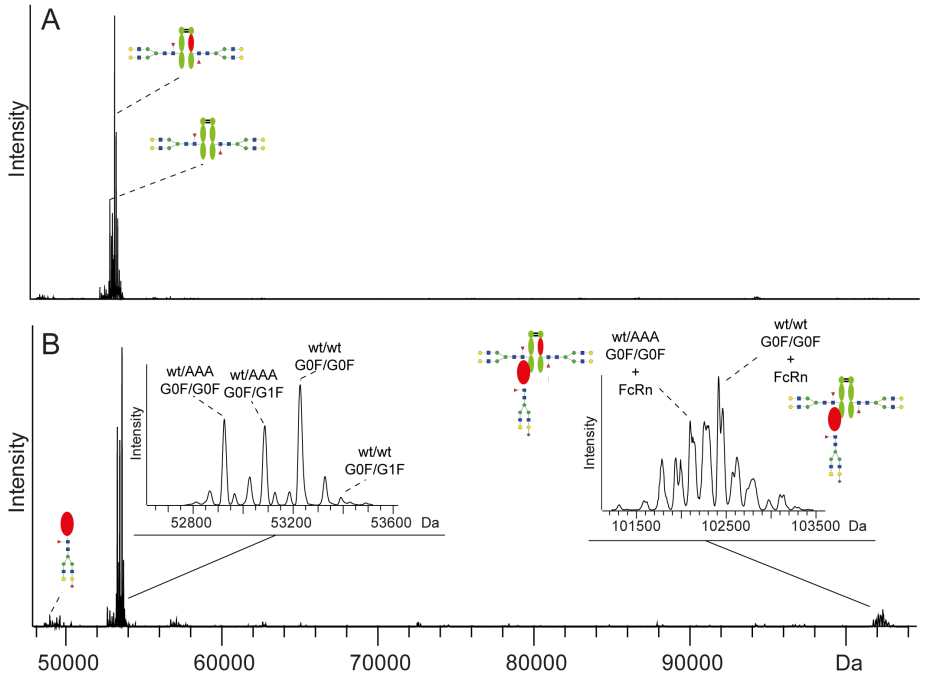

Figure S6. Deconvoluted mass spectra after ACE-MS separation of a 1:1 mixture Fc constructs (wt/wt (green) or wt/AAA (green and red)) using a BGE containing different amounts of FcRn. A) No FcRn and B) 6 µM FcRn. Shown are the free FcRn, the Fc constructs and the complex of the Fc constructs with one FcRn.

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