

Single System for Physicochemical and Biological Minor Variant Characterization of Biotherapeutics: A Proof of Concept

By

Alexander Kozintsev

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Chairperson Dr. Christian Schöneich

Dr. Cinzia Stella

Dr. John Stobaugh

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The Thesis Committee for Alexander Kozintsev
certifies that this is the approved version of the following thesis:

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ABSTRACT

Late stage analytical development activities for recombinant monoclonal antibody therapeutics necessitate the physicochemical and biological characterization of product variants and impurities in order to determine their relative risks to patient safety and efficacy. In particular, a minor variant characterization study is performed in order to obtain and demonstrate a thorough understanding of the structure and function of product variants, and the study is achieved through the application of a suite of analytical methods targeting common product and process variants such as charge and size variants. However, the execution of such studies relies primarily on costly and lengthy product variant enrichment processes followed by the execution of many physicochemical and biological assays on an assortment of instruments. In this work, size and charge LC techniques are combined for the first time with functionally relevant Fc receptor affinity chromatographies and mass spectrometry on a single, modular, multi-dimensional system capable of executing online structural and functional characterization of most recombinant monoclonal antibody product variants. Coupling size, charge, and Fc receptor-affinity chromatographies to mass spectrometry in an online, multi-dimensional fashion resulted in significant time savings and enabled direct identification of heterogeneities observed in the chromatographic profiles without the need for additional enrichment or isolation. The sensitivity and efficiency of this system for investigating relevant product variants was demonstrated via online and offline characterization of variants from several pipeline biotherapeutic molecules, including a standard N-glycosylated IgG1 monoclonal antibody and an aglycosylated IgG1-like bispecific antibody. The single-system, physicochemical and biological analytical suite and its applications as described herein demonstrate an important proof of concept and novel direction for rapid and efficient characterization of product variants for biotherapeutics.

Keywords: recombinant monoclonal antibody (mAb), bispecific antibody (BsAb), minor variant characterization, post-translational modification, critical quality attributes (CQAs), two-dimensional liquid chromatography (2D-LC), Fc receptor affinity chromatography, cation exchange chromatography (CEX), size exclusion chromatography (SEC), mass spectrometry (MS), neonatal Fc receptor (FcRn), Fc γ receptor IIIa (Fc γ RIIIa), antibody dependent cellular cytotoxicity (ADCC)

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INTRODUCTION

Monoclonal antibody (mAb) biotherapeutics are susceptible to a host of post-translational modifications⁽¹⁾, potentially leading to minor variants with differences in charge⁽²⁾ and size⁽³⁾ distribution, which have an impact on the overall therapeutic efficacy and safety of the biotherapeutic product. Though critical quality attributes (CQAs) are identified early on in the biological drug development process and remain conspicuous throughout the drug's developmental lifecycle, the comprehensive characterization of minor variants typically takes place later in the development. This sub-phase of late stage development, commonly referred to as minor variant characterization (MVC), involves the physicochemical characterization of charge and size minor variants alongside panels of artificially degraded or stressed materials⁽⁴⁾. In addition to the investigation of structural attributes via physicochemical assays, MVC also necessitates biological characterization of enriched minor variants and degraded materials containing elevated or decreased levels of CQAs in order to correlate observations from structural characterization to functional impacts (eg, potency, pharmacokinetic clearance, and antibody-dependent cell cytotoxicity). Due to the material quantities necessary for biological characterization using cell-based bioassays (tens of milligrams), this material is typically generated using offline fraction collection on preparatory chromatographic systems followed by separate offline physicochemical characterization of the isolated.

Although cell-based bioassays are the preferred method of interrogating impacts of minor variants on biological activities, they are often only developed and validated in later stages. In earlier stages of product development, immobilized receptor or target binding affinity assays are instead used. While target binding assays are typically product-specific, platform methods for interrogating Fc (Fragment Crystallizable region) receptor binding affinity are readily available and allow researchers to target specific binding interactions in Fc-related biological pathways.

Common examples of such targets include the mediation of pharmacokinetic clearance (PK) by endosomal recycling via the binding of FcRn (neonatal Fc receptor) to the Fc, or the mediation of antibody-dependent cellular cytotoxicity (ADCC) by the binding of FcγRIIIa (membrane receptor critical to triggering lysis by Natural Killer cells) to the Fc.

In the past decade, multi-dimensional liquid chromatography (mD-LC) setups have been increasingly developed and described in the literature for the rapid physicochemical characterization of mAb minor variants and degradation products⁽⁵⁾, including a recent fully-automated multi-dimensional MVC physicochemical characterization setup⁽⁶⁾. Though present day multi-dimensional setups appear to achieve incredible characterization throughput for a variety of applications⁽⁵⁾, these setups typically operate at an analytical scale and have thus far continued to rely on offline preparatory isolations and biological assays in order to support biological characterization as part of MVC. The offline isolation processes, which require preparative isolations of sufficient sample mass for subsequent bio-assays, can end up gating the completion of the minor variant characterization exercise despite the efficiencies gained from developments in multi-dimensional physicochemical characterizations.

One strategy to overcome the sample mass limitations of traditional biological characterizations could involve coupling preparatory isolation and physicochemical characterization techniques as part of a multi-dimensional chromatographic system. In their review of very recent developments and applications of techniques in multi-dimensional liquid chromatography, *Pirok et al.* acknowledge the possibility of “heart-cutting” techniques to be used for preparative separations⁽⁵⁾; however, no such examples in the biopharmaceutical field are mentioned. Considering the state of art in multi-dimensional chromatographic separations⁽⁵⁾, the requirements of the industry in regards to product minor variants⁽⁴⁾, and the wide availability of

semi-preparative versions of common chromatographic columns (eg. ProPac from ThermoFisher, SRT from Sepax), it could be feasible to integrate a semi-preparative isolation dimension into a multi-dimensional minor variant characterization setup. Such a system would also have practical applications in any study which could benefit from the online collection of characterization-representative material, such as for the generation of material for minor variant biological characterization, or for performing characterization techniques that are non-amenable to multi-dimensional systems (eg. imaged capillary isoelectric focusing). The current limiting factor for the industry implementation of such a system is the lack of commercially-available preparatory-scale fraction collection modules which also have the capability of analytical-scale re-injection. Even if or when such a system became available and established, however, it would not eliminate the material need for cell-based bioassays but would instead serve to satisfy them more efficiently.

Another, presently feasible strategy for expediting MVC lies in lowering the material need for biological characterization by directly integrating common biological characterization modes into multi-dimensional physicochemical analysis systems via immobilized-receptor affinity chromatography. In vitro analyses of Fc Receptor-biotherapeutic interactions to investigate biological effects such as pharmacokinetic clearance (PK) and antibody-dependent cell cytotoxicity (ADCC) are already commonly being performed using surface plasmon resonance (SPR) technology^{(7),(8)}. However, SPR-based receptor affinity measurements, by mode of action, are neither descriptive of the heterogeneity of analyte forms nor amenable to coupling with multi-dimensional LC systems. More-recently, immobilized Fc receptor columns have been used to assay the heterogeneity of Fc interactions using both immobilized FcRn⁽⁹⁾ and FcγRIIIa⁽¹⁰⁾. FcRn and FcγRIIIa affinity chromatography methods are amenable to analytical

scale physicochemical characterization and, when integrated into multi-dimensional systems, these techniques can be applied to expedite structure-function investigations, such as for CQA investigations in biopharmaceutical technical development⁽¹¹⁾. Immobilized antigen/target affinity columns, although currently uncommon, are being developed in industrial applications and may be applied similarly in the future to investigate the mechanism-of-action-related biological effects of analyte forms.

The work presented here describes the setup of a two-dimensional liquid chromatography (2D-LC) system paired to a time-of-flight (ToF) mass spectrometer, modularly capable of physicochemical and biological characterization through the combination of typical LC modes with immobilized receptor affinity chromatography. Integration and optimization of common physicochemical characterization techniques, such as ion exchange and size exclusion chromatography techniques, into the two-dimensional system is described and applied to MVC of recombinant monoclonal antibody variants from different formats. The MVC physicochemical toolkit is further expanded through the offline development and evaluation of a native SEC-extended mass range mass spectrometry method, intended to serve as a backend to the two-dimensional LC system. Finally, FcRn and FcγRIIIa affinity chromatography techniques are developed and applied, in both single and multi-dimensional modes, to the functional characterization of various isolated forms and impurities from glycosylated IgG1 monoclonal antibodies (mAb1, mAb2) as well as an IgG1-like aglycosylated bispecific antibody (bsAb1). The collection of individual physicochemical LC modes with functionally-representative affinity chromatography techniques into a modular analytical toolkit on a single system is a novel and important proof of concept for biotherapeutic product variant physicochemical and functional

characterization. This system also holds promise as a backend characterization workflow to preparatory variant enrichment technologies in development.

EXPERIMENTAL SECTION

Materials. mAb1, mAb2, BsAb, homodimer and half-ab were manufactured at Genentech (South San Francisco, CA); enriched minor variants were generated in the Protein Analytical Chemistry Department. The following columns were employed for analyte separation: MAbPac™ SCX-10 LC column (Thermo Fisher Scientific, South San Francisco CA USA), TSKgel® UP-SW3000 LC column (TOSOH Bioscience, South San Francisco CA USA), FcRn Affinity Column (Roche Custom Biotech, Penzberg DEU), FcγRIIIa Affinity Column (Roche Custom Biotech, Penzberg DEU), and TSKgel FcR-IIIa Column (TOSOH Bioscience, South San Francisco CA USA). The following column was used for desalting prior to mass spectrometry analysis: PLRP-S 8μm 1000Å column (Agilent Technologies, Santa Clara CA USA). In order to create an FcγRIIIa affinity column, FcγRIIIa was generated in the Travis Bainbridge lab at Genentech and the following reagents were obtained commercially: BirA biotin-protein ligase bulk reaction kit (Avidity LLC, Aurora CO USA), Streptavidin-Sepharose beads (GE Healthcare, Chicago IL USA). Tricorn 5/50 columns (GE Healthcare, Chicago IL USA), Complete Protease Inhibitor (cOmplete ULTRA Tablets, Roche Diagnostics GmbH, Basel CHE).

Methods. *Setup of the 2D-LC System.* A 2D-LC system (Figure 1-A) was set up using Agilent 1260+ Infinity modules in which the flow was re-routed to bypass the second dimension UV detector to an additional divert valve when paired to a TOF mass spectrometer. The 2D-LC capabilities of the system include comprehensive, heart-cutting, and HiRes (low sample volume, fast valve switching) sampling; for the purposes of subsequently described experiments, only the heart-cutting mode and valve topology were used (Figure 1-B), allowing for storage of up to 11

cuts at a time. The system was controlled using Agilent OpenLab Chemstation Software with the integrated 2D-LC package; the mass spectrometer, a 6200 Agilent TOF, was controlled using MassHunter Data Analysis software and was setup to be activated using an external trigger connection. When analyzing 2nd dimension intact mass spectrometry data in conjunction with 1st dimension LC data, another software, 2^D Chromatogram Creator for MassHunter, was used to combine data sets and view in MassHunter Qualitative Analysis Software.

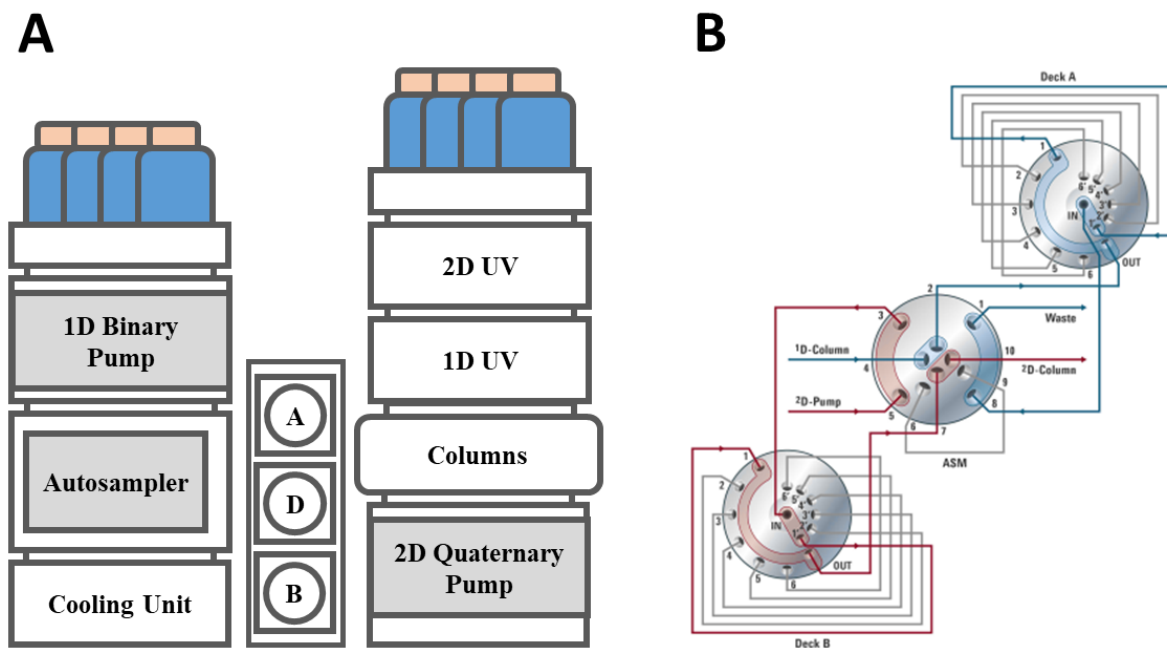


Figure 1. (A) A diagram of module positioning in the Agilent 2D-LC system setup; valves are abbreviated as A (deck A), B (deck B), and D (2D-LC ASM valve). (B) A screenshot from Chemstation software detailing the valve and sample loop configuration used during multiple-heart-cutting.

Physicochemical Characterization by Chromatography Techniques (CEX, SEC). Two primary modes of chromatographic separation were used throughout this work: cation exchange chromatography (CEX) and size exclusion chromatography (SEC). All CEX experiments

utilized a proprietary platform method developed at Genentech that was validated for each molecule the method was applied to; the method utilizes a MAbPac™ SCX-10 LC column as well as a simultaneous pH and salt gradient to resolve charge variants of monoclonal antibodies. All multi-dimensional CEX experiments were run on the Agilent 2D-LC system described in the previous section. All size exclusion experiments utilized a proprietary platform method developed at Genentech that was validated for each molecule the method was applied to; the method utilizes a TSKgel® UP-SW3000 LC column and an isocratic elution using a salt-buffered solvent to resolve size variants of monoclonal antibodies. All multi-dimensional size exclusion experiments were run on the Agilent 2D-LC system described in the previous section. For native SEC-MS analysis, the isocratic elution buffer was adjusted to a lower salt-strength buffer and the method was run on a Waters H-Class UHPLC.

Physicochemical characterization by Mass Spectrometry. Native mass spectrometry was performed on an Orbitrap Exactive Plus EMR Mass Spectrometer. The method was developed specifically for applications described in the work - in-source conditions were optimized separately for the different analyses (non-covalent higher molecular weight forms and homodimer/half Ab-ligand interactions).

Functional Characterization by Fc-Receptor Affinity Chromatography (FcRn-AC, FcγRIIIa-AC). Two primary modes of immobilized receptor affinity chromatography were used throughout this work: neonatal Fc receptor affinity chromatography (FcRn-AC) and FcγRIIIa affinity chromatography (FcγRIIIa-AC). All affinity chromatography experiments were run on the Agilent 2D-LC system described in the previous section, either as 1D or 2D-LC methods. All FcRn-AC experiments utilized a modified platform method developed at Roche that had undergone preliminary assessment (but not validation) for each molecule it was applied to; the

method utilized a pre-packed column with immobilized human FcRn receptor (Roche Custom Biotech) and a pH gradient designed to mimic the biological conditions experienced by the antibody, FcRn, and their bound complex during endosomal recycling. All Fc γ RIIIa-AC experiments utilized a modified platform method developed at Roche that had undergone preliminary assessment (but not validation) for each molecule it was applied to. The method was assessed and run using three different columns: a prototype aglycosylated Fc γ RIIIa column (TSKgel FcR-IIIa), a commercially available glycosylated Fc γ RIIIa column (Roche Custom Biotech), and a glycosylated Fc γ RIIIa column that was packed in-house at Genentech, using Fc γ RIIIa material obtained from the Protein Chemistry group (T. Bainbridge) and a column packing procedure identical to that used by Roche Custom Biotech to pack their FcRn and Fc γ RIIIa columns (described briefly in next section).

Fc γ RIIIa Column Packing. Briefly, Fc γ RIIIa material obtained from the Protein Chemistry group at Genentech was biotinylated using a BirA biotin-protein ligase bulk reaction kit and then bound to Streptavidin-Sepharose beads. The resulting slurry was used to pack Tricorn 5/50 columns and was stored in one of the Fc γ RIIIa-method solvents containing Complete Protease Inhibitor.

RESULTS and DISCUSSION

Coupling Cation Exchange Chromatography and Intact Mass Spectrometry. Charge variant characterization is a key part of MVC and is usually accomplished using either ion exchange chromatography (IEC), capillary electrophoresis (CE), or imaged capillary iso-electric focusing (iCIEF); typically, only preparatory ion exchange chromatography is used for charge variant isolation due to compatibility with fractionation. In this study, charge variant regions were previously isolated and enriched from mAb1 and bsAb1 using preparatory-scale cation

exchange chromatography (CEX). These charge variant regions were characterized on the 2D-LC system using analytical-scale CEX-UV, followed by heart-cutting of resolved variant peaks, trapping in sample loops, desalting on a reverse-phase column, and finally introduction via electrospray ionization into a time-of-flight mass spectrometer. An example analysis of the isolated basic region from bsAb1 is presented in Figure 2.

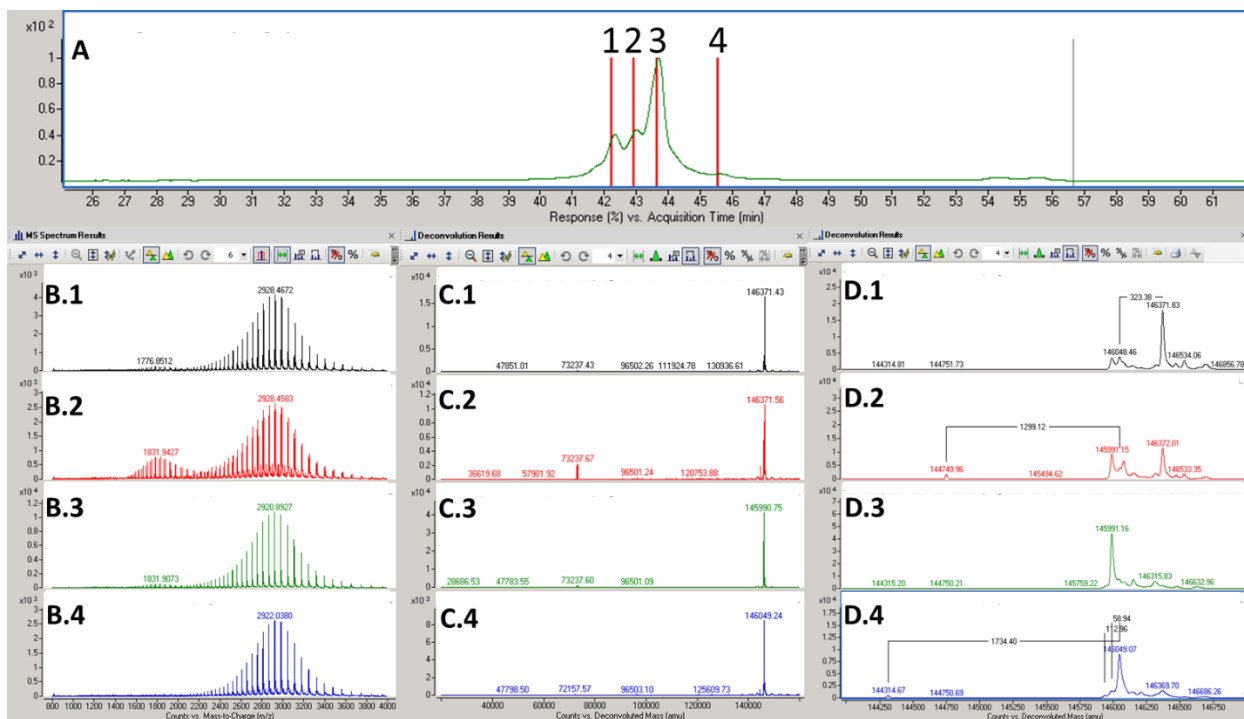


Figure 2. (A) The CEX-UV profile of the 1st dimension analysis of the isolated basic region from bsAb1 including heart-cut markers 1-4 (B) Extracted mass spectra, 800-4000 m/z, of the heart-cut TICs (not pictured) from the 2nd dimension mass spectrometer analysis (C) Deconvolution spectra, 20-160 kDa, of the extracted mass spectra. (D) Zoomed in deconvolution spectra, 144-147 kDa with mass caliper measurements of minor peaks relative to the bsAb1 mass.

Comparison of the online analysis (Figure 2) to offline characterization (data not shown) resulted in identical assignments of CEX-UV peak primary compositions. Peak 1 was observed to primarily contain the VHS modification, an N-terminal signal peptide sequence comprised of

valine, histidine, and serine leading to a +323 Da mass shift. Peak 2 was observed to primarily contain enriched half-antibodies (~73 kDa). Peak 3 was observed to primarily display C-terminal proline alpha-amidation (-58 Da), and Peak 4 contained primarily the main peak mass. Routine CEX testing of stressed materials containing enriched high molecular variants (not shown) lead to the assignment of this peak as corresponding to high-molecular weight forms (primarily dimer). The offline characterization was accomplished by analyzing ~20 fractions collected during the preparatory CEX isolation and comparing the analytical CEX-UV analyses of the fractions to the results of intact mass analyses in order to correlate UV peaks to mass species. While the offline characterization process involved 2 weeks of enrichment and subsequent analysis of the individual fractions, the online 2D method yielded the same results after a few hours and involved a simpler data analysis (overall time savings: < 1 day using online characterization versus ~10 days for offline enrichment and analysis).

An identical analysis was performed for deglycosylated mAb1 isolated basic region, which has a more heterogeneous charge profile, using 11 heartcuts (Figures S1.1-1.3). For the offline characterization of mAb1, the fraction collection of the basic region in order to collect information on individual peaks was not performed due to a leaner minor variants characterization strategy. However, during validation of the CEX method for the commercial control system, a validation deviation forced the team to change the integrated region for the basic variants. The team ended up relying in part on the multidimensional characterization results in order to justify this change and prevent delays to the analytical method transfer and commercial supply campaign.

Coupling Size Exclusion Chromatography and Intact Mass Spectrometry. Size variant characterization is the second key part of MVC and is typically accomplished via

capillary electrophoresis with SDS gel (CE-SDS) for the characterization of low molecular weight forms (LMWF, eg. fragments) and size exclusion chromatography (SEC) for the characterization of high molecular weight forms (HMWF, eg. dimer, trimer, etc). Typically, only preparatory size exclusion chromatography is used for size variant isolation due to compatibility with fractionation. In this study, the online characterization of size variants using the 2D-LC system was first evaluated for the 1st dimension using SEC-UV/RP-MS: a SEC-UV analysis was performed in the first dimension followed by heart-cutting of resolved variant peaks, trapping in sample loops, desalting on a reverse-phase column, and finally introduction via electrospray ionization into a time-of-flight (ToF) mass spectrometer. An example analysis of the ion exchange-isolated basic region from mAb1 is presented in Figure S4. The 1st dimension SEC-UV profile is comparable to offline characterization (not pictured) and the 2D heart-cuts were able to sample individual SEC-resolved peaks, which contained primarily monomer and covalent dimer forms. The online intact mass spectrometry (second dimension) confirmed the levels of post-translational modifications such as different levels of glycation and VHS N-terminal modification were consistent between the monomer and dimer forms. For this study, offline isolation and characterization took approximately one week, whereas the online characterization was completed in a few hours (< 1 day using online characterization versus ~5 days offline).

Native Size Exclusion Mass Spectrometry. In order to characterize size variants which contain non-covalent and much higher molecular weight forms (eg. trimers, higher multimers), an intact mass analysis technique such as the one mentioned above may be unsuitable as it may denature the non-covalent forms or be unable to detect higher molecular weight forms due to limitations of the ToF mass spectrometer. Instead, a native SEC-MS technique was developed offline using native size exclusion conditions and an extended mass range mass spectrometer

(Thermo EMR); Xcalibur software was used to control the EMR MS and Protein Metrics Intact software was used for data analysis. A separate, mobile LC system was used to develop this method due to the practicality of moving the of the 2D-LC system and coupling to the EMR MS; however, the 2D-LC system is fully compatible with the EMR, and therefore this offline development is transferable to the single 2D-LC analytical toolkit.

A method previously developed by the Sandoval group^{(11),(12),(13)} was optimized for the detection of mAb size variants ranging from 20-300 kDa and then evaluated using various HMWF and non-covalent complexes. An example of native SEC-MS analysis of bsAb1 isolated HMWF is presented in Figure 3; a similar analysis of mAb1 isolated HMWF is presented in Figure S5. Offline characterization of the mAb1 isolated HMWF indicated that the majority of HMWF are non-covalent dimer (data not shown). In both online and offline analyses, the mass corresponding to the dimer form of the antibody is detected with high mass accuracy, demonstrating the suitability of the method for detection and mass-confirmation of antibody size variants in the 200-300 kDa mass range. Furthermore, the MS source conditions are observed to preserve the non-covalent dimer species which dominate the mAb1 HMWF.

The ability of the native SEC-MS method to preserve non-covalent interactions was further evaluated by examining a low affinity homodimer-target peptide interaction (Figures 4, S6), as well as the preservation of structure for a non-covalently associated half-antibody (half-Ab) homodimer (Figure S7). The initial Native SEC-MS analysis of the homodimer-ligand peptide interaction is presented in Figure 4, in which both bound and unbound forms were observed for the half-Ab and homodimer by mass spectrometry. The presence of unbound forms could indicate either a loss of the native interaction due to the conditions of the analysis, or the presence of a subpopulation of homodimer which is unable to bind the peptide ligand. Due to the

relative size of the peptide compared to the half-Ab and homodimer, the unbound and bound forms are unlikely to be resolved by the SEC separation. However, in the mixture containing bound homodimer and half-antibody, a retention time shift toward higher molecular weight is observed compared to the homodimer and half-Ab trace lacking any peptide, and could indicate sensitivity of the chromatographic separation to bound/unbound forms via differences in observed retention times. Interestingly, the half-Ab bound and unbound population abundances appeared in approximately a 1:1 mixture, whereas the homodimer unbound, singularly bound, and fully bound forms appeared in approximately a 1:2:1 mixture, respectively. When homodimer A was mixed with the isolated bsAb1 HMWF in the presence of the peptide, for which bsAb1 is monospecific, the bsAb1 population also appeared to contain a 1:1 mix of bound and unbound forms but no bound forms were observed for the HMWF (S6). The contributions of source conditions, product quality, and higher order structure on the apparent binding interactions of the different species are being investigated further. A non-covalently associated half-antibody half-antibody homodimer was observed to preserve its native structure entirely by this method (Figure S7).

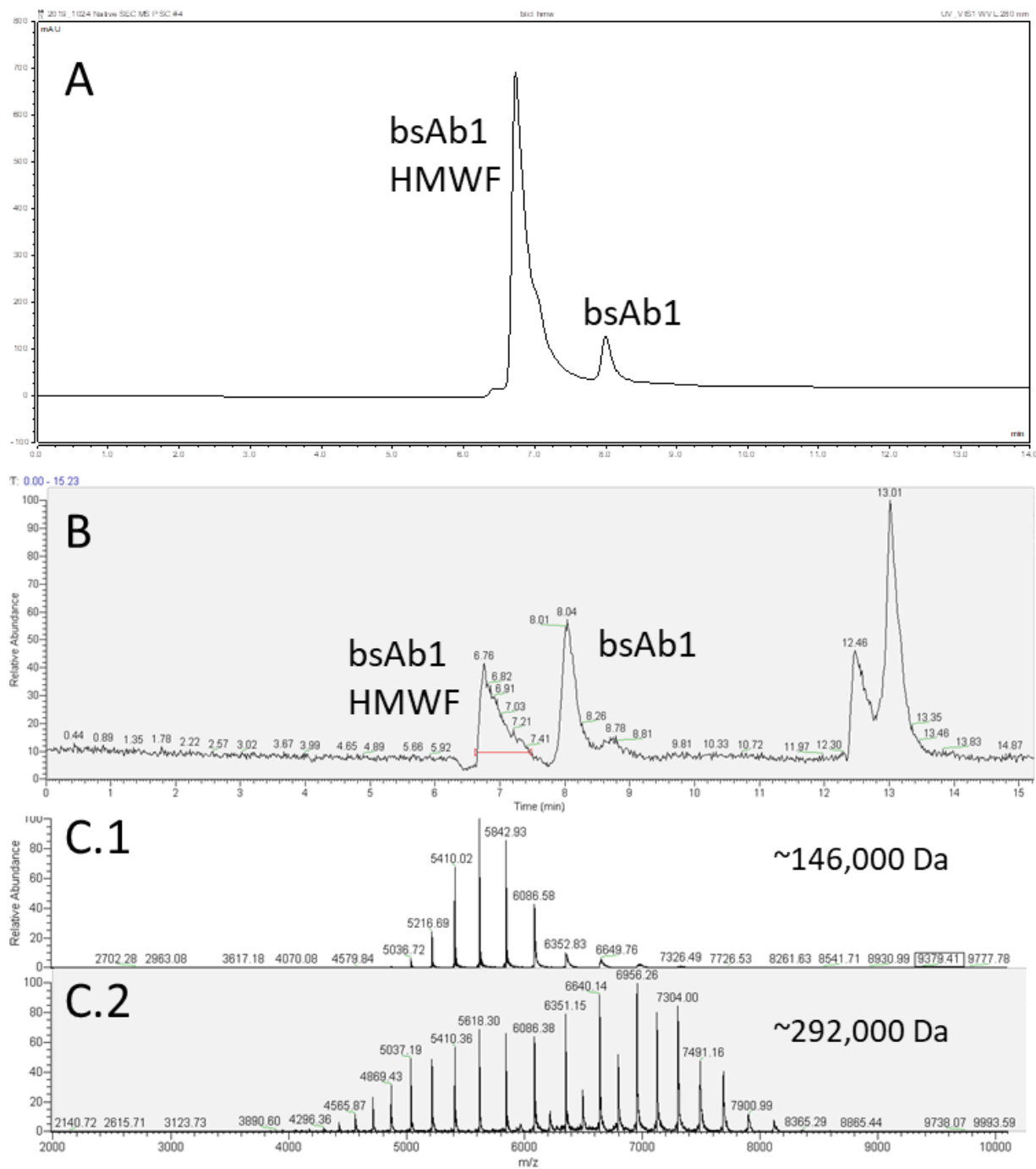


Figure 3. (A) The SEC-UV profile from the Native SEC-MS analysis of the isolated HMW region from bsAb1. (B) The total ion chromatogram from the Native SEC-MS analysis of the isolated HMW region from bsAb1. (C) Extracted mass spectra of the HMWF and bsAb1 TIC peaks, 2000-10000 m/z.

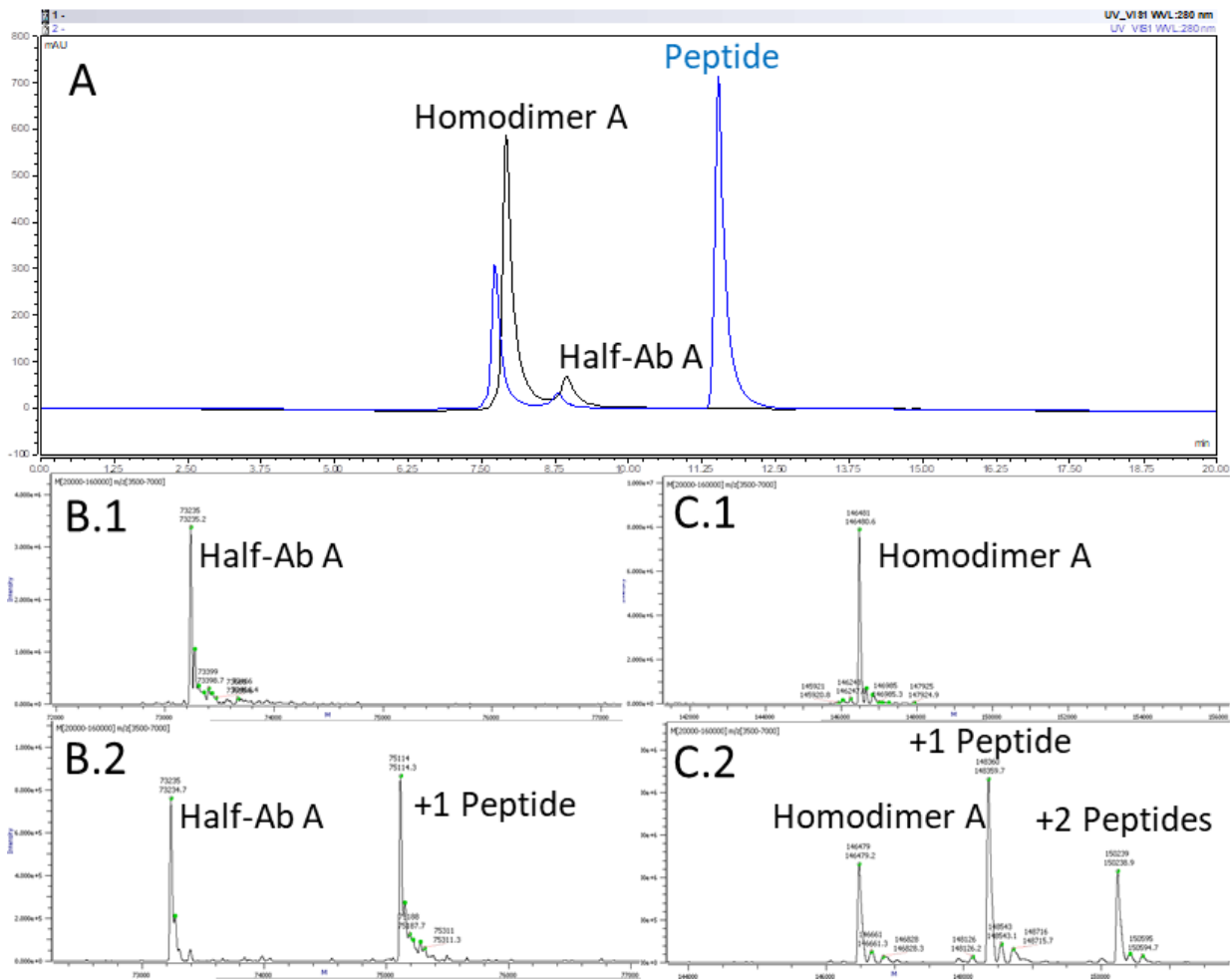


Figure 4. (A) The SEC-UV profiles from the Native SEC-MS analysis of neat homodimer A (black trace) and in the presence of peptide (blue trace). (B) Zoomed-in deconvoluted spectrum of the extracted half-Ab A TIC peak (not pictured), both neat (1) and in the presence of excess peptide (2). (C) Zoomed-in deconvoluted spectrum of the extracted homodimer A TIC peak (not pictured) both neat (1) and in the presence of excess peptide (2).

Coupling FcRn Affinity Chromatography and Intact Mass Spectrometry. The MVC study necessitates biological characterization of isolated minor forms to investigate structure-function relationships, typically via *in vitro* binding affinity and functional cell-based assays. Although cell-based assays for investigating mechanism of action and target-specific binding remain necessary for antibody therapeutic characterization, several other key biological processes have been modeled via *in vivo* – *in vitro* relationships, such as the relation of an

antibody's affinity to immobilized FcRn and its pharmacokinetic clearance via an endosomal recycling pathway⁽¹⁴⁾. In this study, the online characterization of various isolated antibody forms and impurities was accomplished by pairing FcRn affinity chromatography in the 1st dimension to reverse-phase (desalting) and intact mass spectrometry in the second dimension. Columns and the supporting methods were obtained from Roche Custom Biotech (Basel, Switzerland); analysis was done using a buffered pH gradient to imitate the FcRn endosomal recycling conditions.

As an initial evaluation of the FcRn AC-RP/MS method, mAb1 materials at different levels of 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH)-induced oxidative stress were analyzed (Figure 5). AAPH is commonly used to force oxidation of mainly tryptophan and methionine residues - some of which are located at or near key Fc-binding sites - in order to mimic process-relevant oxidation conditions. Fc-oxidation is known to impact the affinity of antibodies to the FcRn receptor⁽¹⁴⁾, and this impact was clearly observed in the 1st dimension UV profiles. In order to test the two-dimensional characterization capability of the system and method, the material with the lowest level of forced tryptophan oxidation was sampled using heart-cutting and subsequently analyzed by RP/MS. Different masses relating to different degrees of AAPH-induced tryptophan oxidation were clearly reflected in the observed deconvoluted intact mass spectra, with the highest oxidated forms (highest Da of main masses) coming from earlier FcRn AC elution times (Figure 5).

A common issue with FcRn binding techniques is the confounding effect of avidity (number of binding sites) on apparent binding affinity (strength of binding at a site). While SPR-based FcRn affinity techniques are typically insensitive to the heterogeneity of a material, affinity chromatography can display and, at times, resolve high molecular weight forms due to

their avidity effects. In an FcRn AC analysis of different isolated HMW forms of bsAb1, the HMW forms were well resolved from the bsAb1 monomer, and there was an observable retention time difference between trimer and dimer forms (Figure S8). An ongoing effort in this study is pairing FcRn AC in the first dimension to the previously described native SEC-MS technique as a way to assess materials with apparent “high” FcRn affinity for the presence of high molecular weight forms and a confounding avidity effect.

In addition to performing typical assessments of relative mAb FcRn affinities, the FcRn AC technique can also be used to study higher order structure. The aforementioned homodimer A is known to exist in two structural isoforms, which have different surface charge behavior, appear to both be covalently associated when analyzed by denaturing methods (data not shown), and are observed to be fully pH reversible - increasing pH drives formation of one structural isoform while decreasing pH drives the formation of the other structural isoform. FcRn AC was used to analyze two Homodimer A materials - one containing both structural isoforms and another in which the material was pH-treated such that only the high pH-structural isoform existed. Both materials were characterized with and without the addition of the Homodimer A target ligand peptide (Figure S9). Interestingly, the low pH-structural isoform had a very low apparent FcRn affinity, and in the presence of the ligand peptide that affinity appeared to drop even lower; the high pH-structural isoform appeared in the typical FcRn affinity retention time range for mAbs, and no additional effect was observed in the presence of peptide. Recent developments in characterization of FcRn IgG interactions suggest a two-pronged binding model, wherein the FcRn first binds to the Fc and then to the antigen-binding fragment (Fab) domain¹⁵. If the low pH-structural isomer of Homodimer A had compromised Fc binding but was still interacting with FcRn via its Fab, then these results would support that binding the

peptide (and thus occupying the Fab) induced higher order structural (HOS) changes, which then decreased the isoform's overall affinity to FcRn.

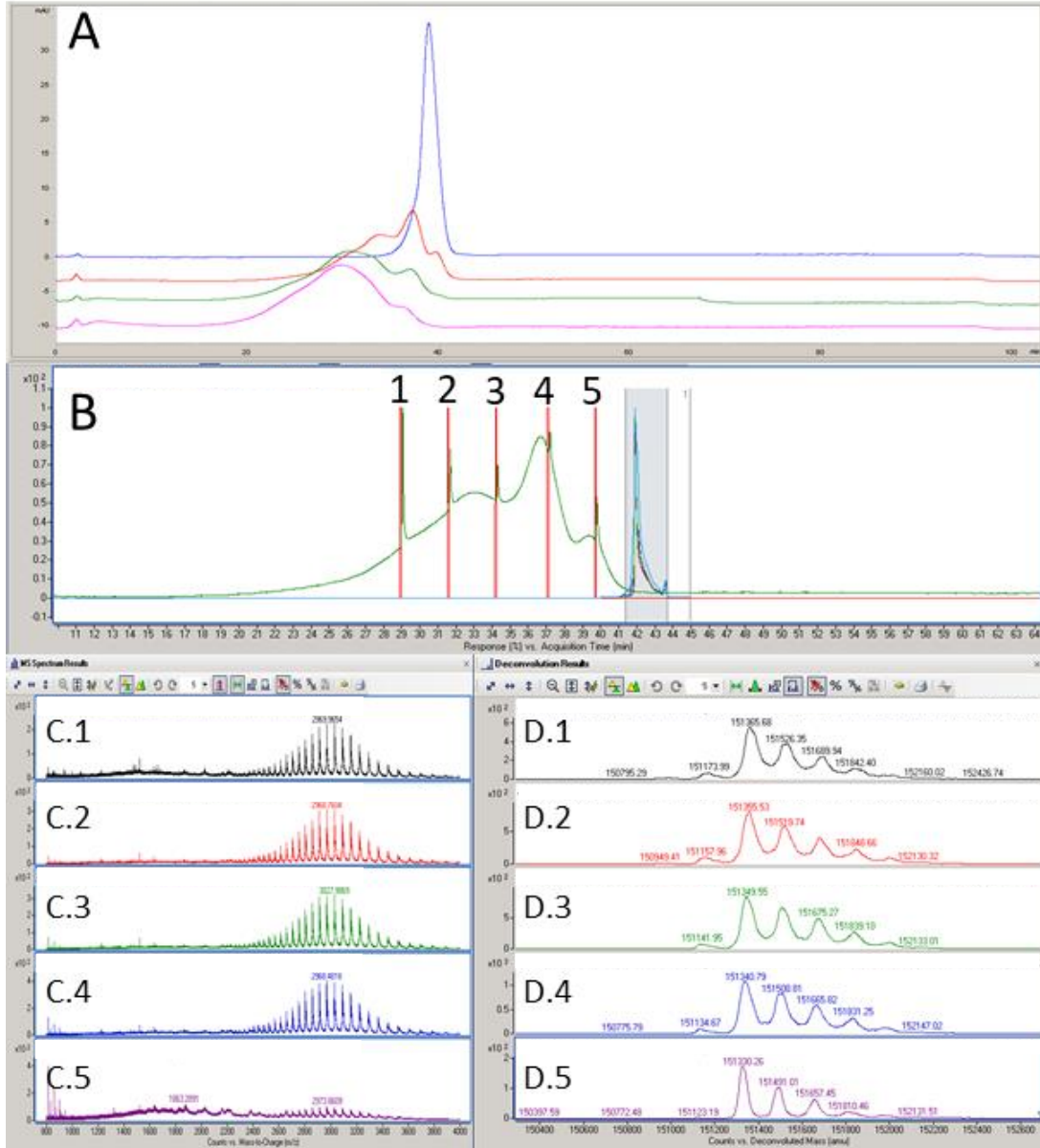


Figure 5. (A) The FcRn AC-UV profiles of unstressed mAb1 (top trace), the least AAPH-stressed material (second from the top), the second highest level of AAPH-stressed material (second from the bottom) and the most AAPH-stressed material (bottom trace) (B) The FcRn AC-UV profile of the lowest oxidative stress material with heart-cut markers and corresponding overlaid TIC profiles (shaded region). (C) Extracted mass spectra, 800-4000 m/z, of the heart-cuts 1-5 (D) Zoomed in deconvoluted mass spectra, 150-153 kDa, of heart-cuts 1-5.

Coupling FcγRIIIa Affinity Chromatography and Intact Mass Spectrometry.

Another *in vitro-in vivo* relationship that can be exploited for the biological characterization of antibody therapeutics is the correlation between FcγRIIIa (CD16a) affinity and antibody-dependent cell-mediated cytotoxicity (ADCC)⁽¹⁶⁾, wherein differences in glycan structure on the antibody Fc have profound effects on its binding by FcγRIIIa⁽¹⁷⁾. In order to extensively assess the ability to incorporate FcγRIIIa affinity chromatography as the first dimension in a 2D-LC system to perform biological characterization, columns with immobilized aglycosylated and glycosylated recombinant FcγRIIIa were obtained from TOSOH and Roche Custom Biotech, respectively. These AC columns were used to develop methods on the 2D system for affinity chromatography paired to reverse phase desalting and subsequent mass spectrometry. In addition to the dependence of FcγRIIIa affinity on an antibody's Fc-glycosylation, the glycosylation of the FcγRIII receptor itself is also known to impact its affinity to the Fc⁽¹⁸⁾. As part of an in-depth follow-up, another column was generated using glycosylated FcγRIIIa material with a different glycan heterogeneity profile from that of the Roche Custom Biotech material. The new material was produced at Genentech and FcγRIIIa glycan heterogeneity differences were confirmed qualitatively by mass spectrometry of released glycans; the resulting affinity column's performance was compared to the column from Roche Custom Biotech.

As an initial assessment of the immobilized aglycosylated FcγRIIIa column from TOSOH, mAb1 material was characterized using the two-dimensional workflow (Figure 6). In as little as 10 minutes, the affinity chromatography was able to resolve differences in glycan structures – mass spectrometry in the 2nd dimension confirmed the identities of early eluting peaks as primarily single-antennary glycoforms and the later eluting major bi-antennary glycoforms (G0F/G0F, G0F/G1F, G1F/G1F and G0F/G2F). Several low level minor peaks were

observed to elute between the bi- and single-antennary glycoforms; when heart-cut, these peaks (as well as the heart-cuts containing single-antennary glycoforms) appeared to contain the G0F/G0F mass despite eluting considerably earlier than the major glycoforms.

Core fucosylation of Fc N-glycans is typically classified as a CQA during biotherapeutic technical development due to its known impact on Fc γ RIIIa binding; afucosylated mAbs have considerably greater binding to Fc γ RIIIa than their fucosylated forms, which can lead to enhanced effector function¹⁹. In order to assess the Fc γ RIIIa (aglycosylated) affinity chromatography method's ability to distinguish between these forms, a 100% afucosylated mutant of mAb1 was analyzed alongside a 10% afucosylated control and standard fucosylated mAb1 material (Figure S10-A). It was observed that the current method and column were unable to fully resolve the afucosylated material profile from that of the fucosylated mAb; it is suspected that the lack of glycosylation on the TOSOH recombinant Fc γ RIIIa may account for the lower resolution due to the importance of glycans on the receptor itself in mediating its affinity to antibodies⁽²⁰⁾. It was concluded that this type of column, containing aglycosylated Fc γ RIIIa, had limited usefulness in resolving and quantifying species expected to be the primary drivers of ADCC modulation by therapeutic antibodies⁽²¹⁾.

The resolution of afucosylated species was significantly improved during the analysis of CHO-derived glycosylated mAb2 using the glycosylated Fc γ RIIIa affinity column from Roche Custom Biotech (Figure 7-A). A 2D-LC analysis of the mAb2 material Fc γ RIIIa high-affinity species (HAS) revealed that the species were comprised entirely of glycoforms lacking either single or double core fucosylation on N-glycans (Figure 7-B). By sampling across the high-affinity species (HAS) peak and its pre-peak, several observations were made about the relative Fc γ RIIIa affinities of the different core afucosylated species present. First, it was observed that

mAb2 with a single afucosylated N-glycan chain was resolved as a pre-peak to the species containing two N-glycan chains with either single or double afucosylation. Second, it was observed that higher order afucosylated glycoforms (G2 vs G1, G1 vs G0) had slightly larger relative affinities for Fc γ R11a (consistent with results observed using the aglycosylated Fc γ R11a column), although they were not resolved in the affinity chromatography UV profile. Finally, it was observed that glycoform species lacking two core fucose units had slightly larger relative affinities for Fc γ R11a compared to those lacking one core fucose, although these too were not resolved from each other in the affinity chromatography UV profile. The rank order of these glycoforms' impacts to Fc γ R11a binding is comparable to that observed in another IgG1 by a similar approach⁽²²⁾. The similar behavior of various glycoforms with different degrees of afucosylation (single vs double) suggests these forms similarly impact the Fc γ R11a binding affinity of mAb2 and can be examined cumulatively as one high-affinity species (HAS) during affinity chromatography analysis.

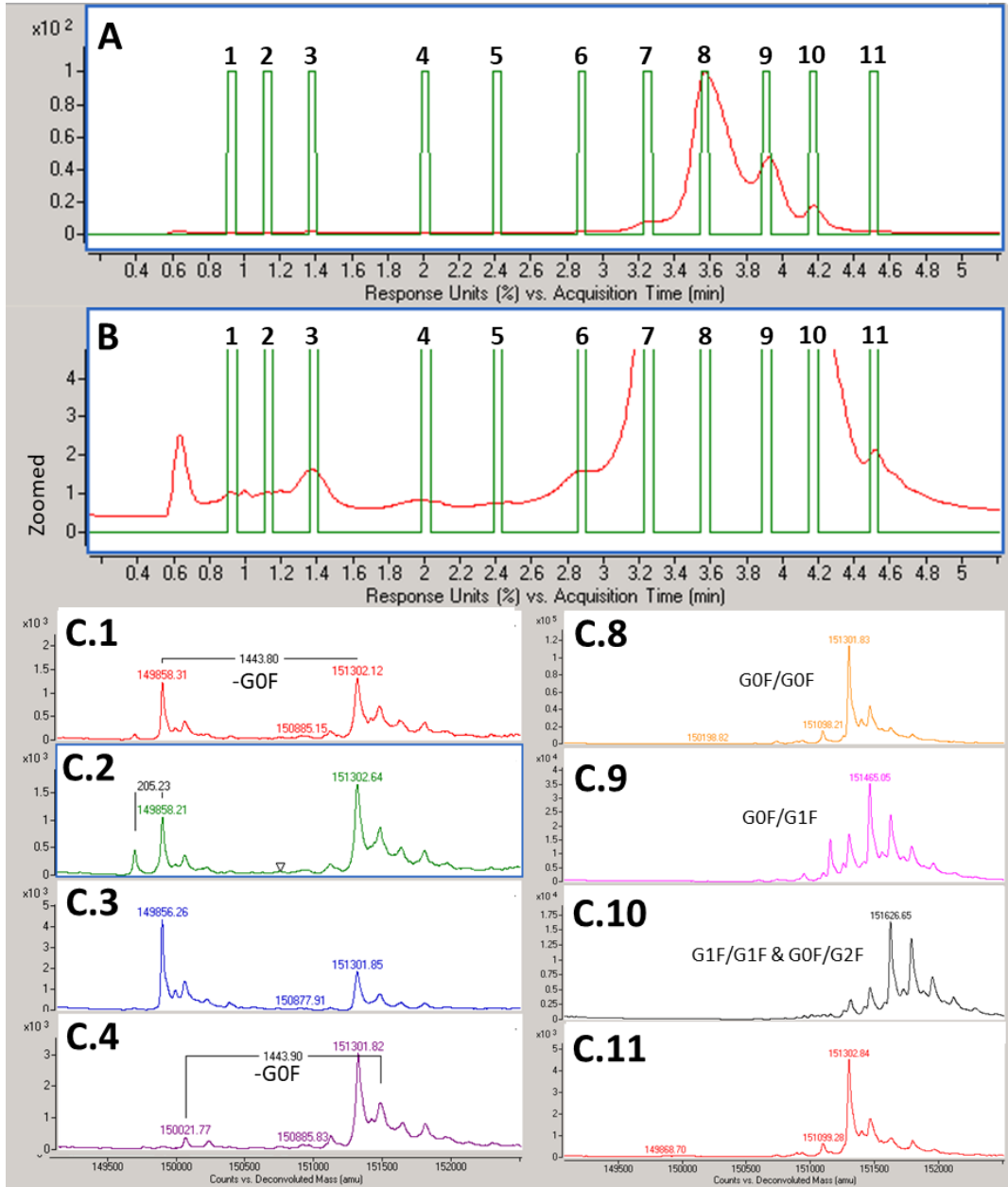


Figure 6. (A) aglycosylated Fc γ RIIIa AC-UV profile of mAb1 with heart-cut markers (B) A zoomed-in aglycosylated Fc γ RIIIa AC-UV profile of mAb1 with heart-cut markers (C) Zoomed in deconvoluted mass spectra, 149-153 kDa, of heart-cuts 1-4 and 8-11.

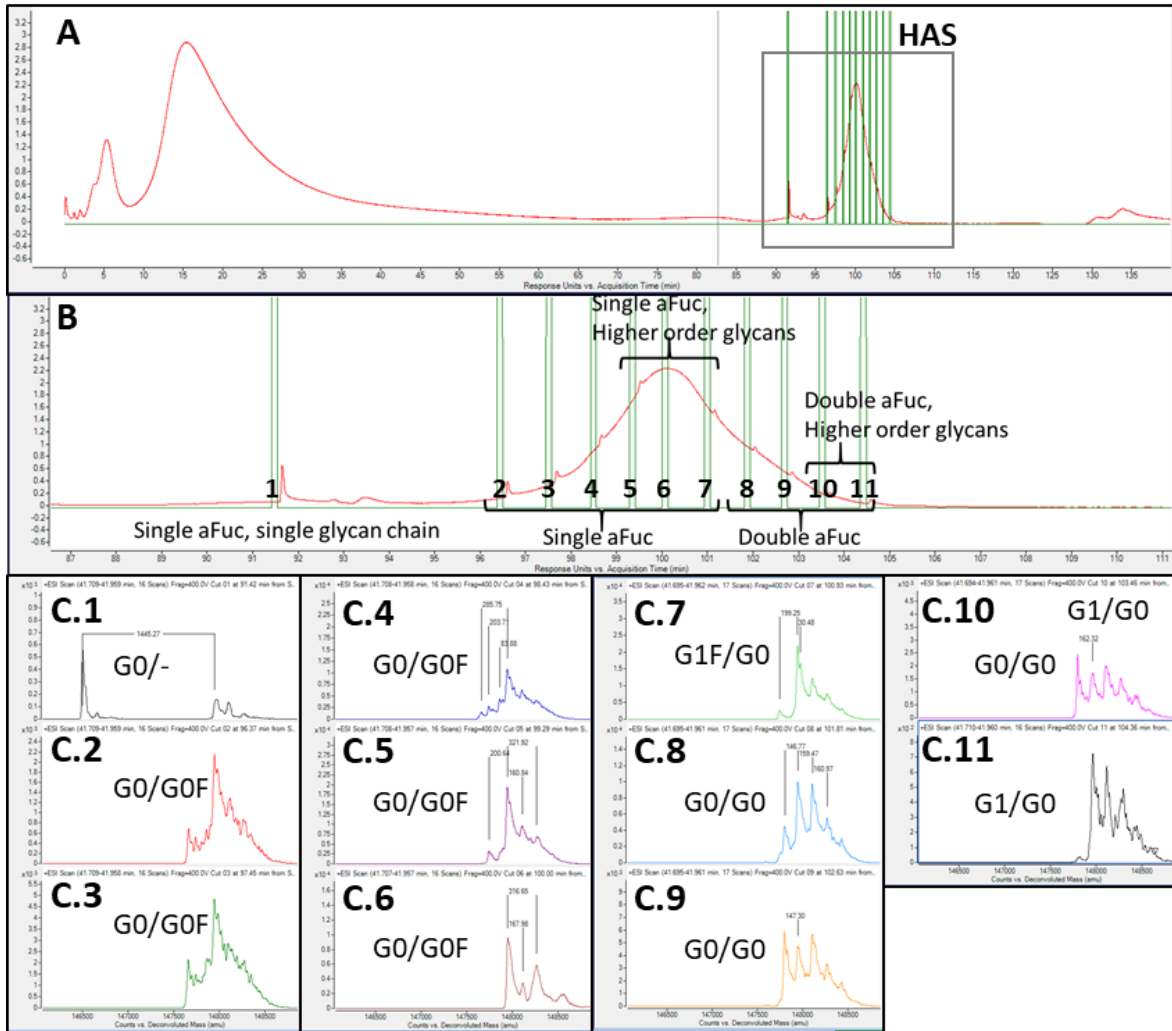


Figure 7. (A) glycosylated Fc γ RIIIa AC-UV profile of mAb2 with heart-cut markers (B) A zoomed-in Fc γ RIIIa AC-UV profile of the mAb2 HAS region with heart-cut numbers and indications of the glycoform-type localization (C) Zoomed in deconvoluted mass spectra of heart-cuts 1-11 labeled with the identities of the prevalent glycoforms.

CONCLUSIONS

Minor variant characterization of biotherapeutics can be a lengthy late stage analytical development activity requiring a variety of resources and instrumentation. Often its completion is gated by the material, instrumentation, and resource requirements for performing variant enrichment and running a full suite of structural and functional assays. In this report, a novel proof of concept is detailed wherein physicochemical chromatography modes are combined with functionally relevant Fc receptor affinity chromatography and mass spectrometry on a single, modular multi-dimensional system. As a concept, such a system should be capable of performing both the structural and functional components of minor variant characterization of recombinant monoclonal antibody product variants in an online, multi-dimensional fashion. In this work, this concept is proven via the system's application to the structural characterization of size and charge variants of recombinant monoclonal antibodies, as well as characterization of the impacts of those variants on *in vivo*-relevant functionalities such as endosomal recycling and antibody-dependent cellular cytotoxicity. Online multi-dimensional characterization is able to identify individual peaks and species from platform ion exchange and size exclusion assays in a much more rapid and resource-friendly manner than offline characterization. A native SEC-MS mode is observed to be capable of preserving non-covalent interactions in multimeric complexes, as well as in antibody-ligand and half antibody-ligand interactions. FcRn affinity chromatography is demonstrated to be sensitive to oxidation of the Fc binding region, avidity binding impacts from high molecular weight forms, and potentially charge interactions with the Fab. Investigation of glycosylated antibodies using Fc γ RIIIa affinity chromatography by the online 2D-LC/MS system yields a rank order of glycosylated variants' impacts to Fc γ RIIIa binding, particularly that of afucosylation. Overall, this single-system physicochemical and biological analytical toolkit

represents a significant advance and novel direction towards the development of more rapid and efficient minor variant characterization workflows.

To the best of my knowledge, this is the first work wherein both structural and functional minor variant characterization of biotherapeutics is performed on a single analytical system. This direction and its proof of concept, of incorporating physicochemical and biological characterization in an online fashion without the use of a variety of extensively custom and complicated instrumentation, are a valuable addition to the biotherapeutic technical development field where resource efficiency and rapid quality characterization are paramount. Although the concept was demonstrated for late stage analytical characterization of recombinant monoclonal antibodies, it may be customized and applied to other common and novel biotherapeutic formats (eg. fusion proteins and Fabs) and in early phase analytical development activities such as during clone selection and purification process development. In order to transition this work from a concept to implementation, a thorough understanding of the functional modes' (eg. FcRn, FcγRIIIa affinity chromatography) representativeness of their corresponding *in vivo* activities would need to be developed and assessed by bridging studies. Coverage of structural and functional characterization gaps, such as characterization of site-specific modifications in variants (eg. deamidation, isomerization) and affinity columns for mechanism of action-relevant target binding would need to be addressed on a molecule-by-molecule basis. Nevertheless, application of this proof of concept to future analytical workflows will greatly enhance the versatility and efficiency of biotherapeutic analytical systems.

FUTURE DIRECTIONS

For future studies, I would like to focus on three areas critical to realize implementation of systems such as the one described in this work: developing and accessing more affinity chromatography-friendly functional modes (using receptors that can be immobilized and remain stable and active after immobilization), demonstrating the biological relevance of such modes in a quantitative manner, and developing a preparatory component for the front-end of the multi-dimensional characterization system. The gap in the current proof of concept, that of demonstrating multi-dimensional characterization of site-specific modifications of size and charge variants, is already a well-established space in the multi-dimensional LC-MS development field.

The functionally-relevant affinity chromatography repertoire will be expanded via the generation and assessment of novel receptor affinity columns such as other members of the Fc γ R family (I, IIa/b, IIIb) and target affinity columns (such as immobilized targets of mAb1, mAb2 and bsAb1). Another interesting direction for the research will be to generate mixed functional-mode columns, containing endogenously relevant mixtures of receptors such as Fc γ RIIIa/Fc γ RIIIb, and each of their high and low affinity forms.

In addition the generation and assessment of these columns, their biological relevance must be tested by comparing their sensitivities and specificities to the impacts of product variants within cell-based bioassays (eg. comparing Fc γ RIIIa affinity chromatography vs. ADCC cell-based potency assay). It will be important to recognize and address what impacts of product variants cell-based methods may be sensitive to that are downstream of the binding interaction, as the immobilized receptor or target-affinity chromatography methods may be blind to these.

Finally, in the case that safety, pharmacokinetic, and efficacy-relevant biology for a particular biotherapeutic cannot be represented by a binding interaction alone, the development

of an online preparatory enrichment dimension for the front-end of the multi-dimensional charge variant characterization system would serve to solve the issue of enriched variant mass requirements for offline biological characterization.

REFERENCES

- (1) Harris R. J. Heterogeneity of recombinant antibodies: linking structure to function. *Dev Biol (Basel)* 2005, 122, 117–127.
- (2) Khawli, L.; Goswami, S.; Hutchinson, R.; Kwong, Z.; Yang, J.; Wang, X.; Yao, Z.; Sreedhara, A.; Cano, T.; Tesar, D. et al. Charge Variants In IgG1. *mAbs* 2010, 2, 613-624.
- (3) Lu, C.; Liu, D.; Liu, H.; Motchnik, P. Characterization Of Monoclonal Antibody Size Variants Containing Extra Light Chains. *mAbs* 2013, 5, 102-113.
- (4) Zhou, Q.; Shapiro, M. Regulatory Considerations For The Development Of Novel Antibody-Related Products. *American Pharmaceutical Review* 2016.
- (5) Pirok, B.; Stoll, D.; Schoenmakers, P. Recent Developments In Two-Dimensional Liquid Chromatography: Fundamental Improvements For Practical Applications. *Analytical Chemistry* 2018.
- (6) Gstöttner, C.; Klemm, D.; Habegger, M.; Bathke, A.; Wegele, H.; Bell, C.; Kopf, R. Fast And Automated Characterization Of Antibody Variants With 4D HPLC/MS. *Analytical Chemistry* 2018, 90, 2119-2125.
- (7) Datta-Mannan A, Chow CK, Dickinson C, Driver D, Lu J, Witcher DR, et al. FcRn affinity-pharmacokinetic relationship of five human IgG4 antibodies engineered for improved in vitro FcRn binding properties in cynomolgus monkeys. *Drug Metab Dispos* 2012; 40:1545-55
- (8) Bruggeman, C.; Dekkers, G.; Bentlage, A.; Treffers, L.; Nagelkerke, S.; Lissenberg-Thunnissen, S.; Koeleman, C.; Wuhrer, M.; van den Berg, T.; Rispens, T.; Vidarsson, G.; Kuijpers, T. Enhanced Effector Functions Due To Antibody Defucosylation Depend On The Effector Cell Fcγ Receptor Profile. *J. Immunology* 2017, 199 (1), 204-211.
- (9) Schlothauer, T.; Rueger, P.; Stracke, J.; Hertenberger, H.; Fingas, F.; Kling, L.; Emrich, T.; Drabner, G.; Seeber, S.; Auer, J.; Koch, S.; Papadimitriou, A. Analytical FcRn Affinity Chromatography For Functional Characterization Of Monoclonal Antibodies. *mAbs* 2013, 5 (4), 576-586.
- (10) Thomann, M.; Schlothauer, T.; Dashivets, T.; Malik, S.; Avenal, C.; Bulau, P.; Rüger, P.; Reusch, D. In Vitro Glycoengineering Of Igg1 And Its Effect On Fc Receptor Binding And ADCC Activity. *PLOS ONE* 2015, 10 (8), e0134949.
- (11) Scalco, F. A new method for the determination of CQAs in therapeutic products using 2D-affinity chromatography compatible with MS. Pharmaceutical Sciences, Masters Thesis, ETH Zurich: Zurich, CH, 2019.
- (12) Schachner, L.; Han, G.; Dillon, M.; Zhou, J.; McCarty, L.; Ellerman, D.; Yin, Y.; Spiess, C.; Lill, J.; Carter, P.; Sandoval, W. Characterization Of Chain Pairing Variants Of Bispecific Igg Expressed In A Single Host Cell By High-Resolution Native And Denaturing Mass Spectrometry. *Analytical Chemistry* 2016, 88 (24), 12122-12127.
- (13) Ren, C.; Bailey, A.; VanderPorten, E.; Oh, A.; Phung, W.; Mulvihill, M.; Harris, S.; Liu, Y.; Han, G.; Sandoval, W. Quantitative Determination Of Protein–Ligand

- Affinity By Size Exclusion Chromatography Directly Coupled To High-Resolution Native Mass Spectrometry. *Analytical Chemistry* 2018, *91* (1), 903-911.
- (14) Roopenian, D.; Akilesh, S. Fc γ : The Neonatal Fc Receptor Comes Of Age. *Nature Reviews Immunology* 2007, *7* (9), 715-725.
- (15) Jensen, P.; Schoch, A.; Larraillet, V.; Hilger, M.; Schlothauer, T.; Emrich, T.; Rand, K. A Two-Pronged Binding Mechanism Of IgG To The Neonatal Fc Receptor Controls Complex Stability And IgG Serum Half-Life. *Molecular & Cellular Proteomics* 2017, *16* (3), 451-456.
- (16) Yeap, W.; Wong, K.; Shimasaki, N.; Teo, E.; Quek, J.; Yong, H.; Diong, C.; Bertoletti, A.; Linn, Y.; Wong, S. CD16 Is Indispensable For Antibody-Dependent Cellular Cytotoxicity By Human Monocytes. *Nature Scientific Reports* 2016, *6* (1).
- (17) Chung, A.; Crispin, M.; Pritchard, L.; Robinson, H.; Gorny, M.; Yu, X.; Bailey-Kellogg, C.; Ackerman, M.; Scanlan, C.; Zolla-Pazner, S.; Alter, G. Identification Of Antibody Glycosylation Structures That Predict Monoclonal Antibody Fc-Effector Function. *AIDS* 2014, *28* (17), 2523-2530.
- (18) Drescher, B.; Witte, T.; Schmidt, R. Glycosylation Of Fc γ III In N163 As Mechanism Of Regulating Receptor Affinity. *Immunology* 2003, *110* (3), 335-340.
- (19) Bruggeman, C.; Dekkers, G.; Bentlage, A.; Treffers, L.; Nagelkerke, S.; Lissenberg-Thunnissen, S.; Koeleman, C.; Wuhler, M.; van den Berg, T.; Rispens, T.; Vidarsson, G.; Kuijpers, T. Enhanced Effector Functions Due To Antibody Defucosylation Depend On The Effector Cell Fc γ Receptor Profile. *The Journal of Immunology* 2017, *199* (1), 204-211.
- (20) Drescher, B.; Witte, T.; Schmidt, R. Glycosylation Of Fc γ III In N163 As Mechanism Of Regulating Receptor Affinity. *Immunology* 2003, *110* (3), 335-340.
- (21) Thomann, M., Reckermann, K., Reusch, D., Prasser, J. and Tejada, M., 2016. Fc-galactosylation modulates antibody-dependent cellular cytotoxicity of therapeutic antibodies. *Molecular Immunology*, *73*, pp.69-75.
- (22) Lippold, S.; Nicolardi, S.; Domínguez-Vega, E.; Heidenreich, A.; Vidarsson, G.; Reusch, D.; Habegger, M.; Wuhler, M.; Falck, D. Glycoform-Resolved Fc γ III Affinity Chromatography–Mass Spectrometry. *mAbs* 2019, *11* (7), 1191-1196.

SUPPLEMENTARY MATERIALS

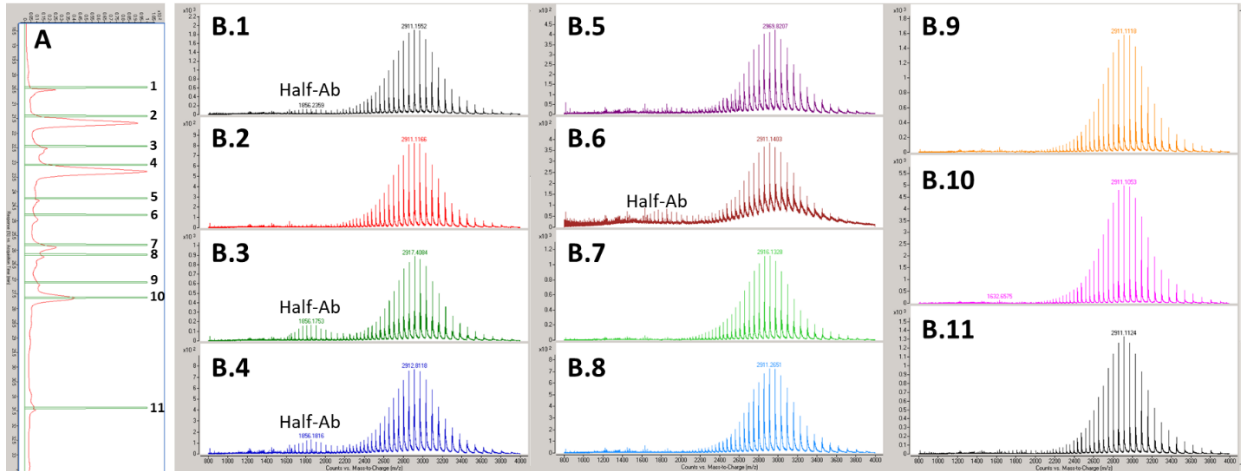


Figure S1.1 (A) The CEX-UV profile of the 1st dimension analysis of the isolated Basic region from mAb1 including heart-cut markers 1-11 (B) Extracted mass spectra, 800-4000 m/z, of the heart-cut TICs (not pictured) from the 2nd dimension mass spectrometer analysis.

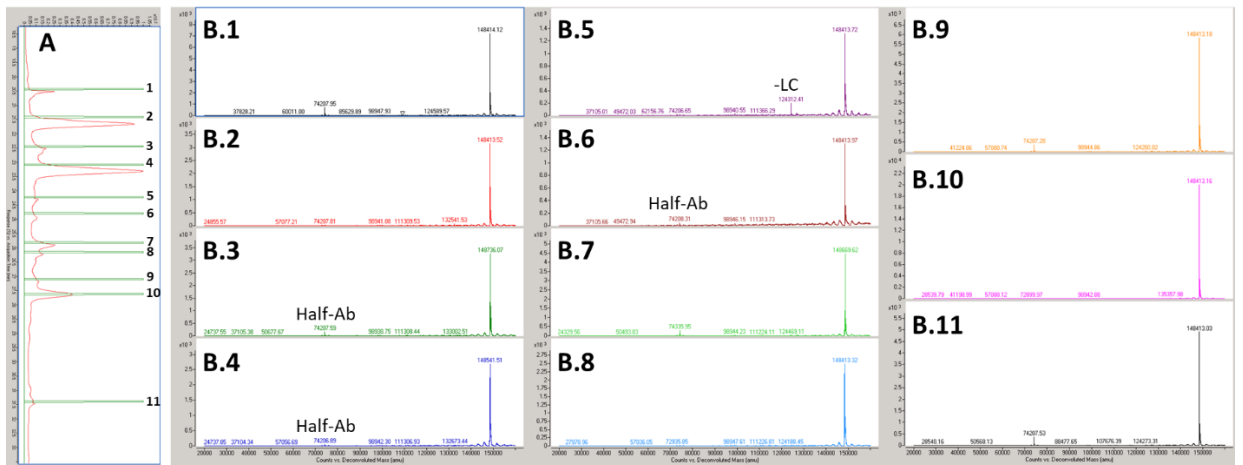


Figure S1.2 (A) The CEX-UV profile of the 1st dimension analysis of the isolated Basic region from mAb1 including heart-cut markers 1-11. (B) Deconvolution spectra, 20-160 kDa, of the extracted mass spectra.

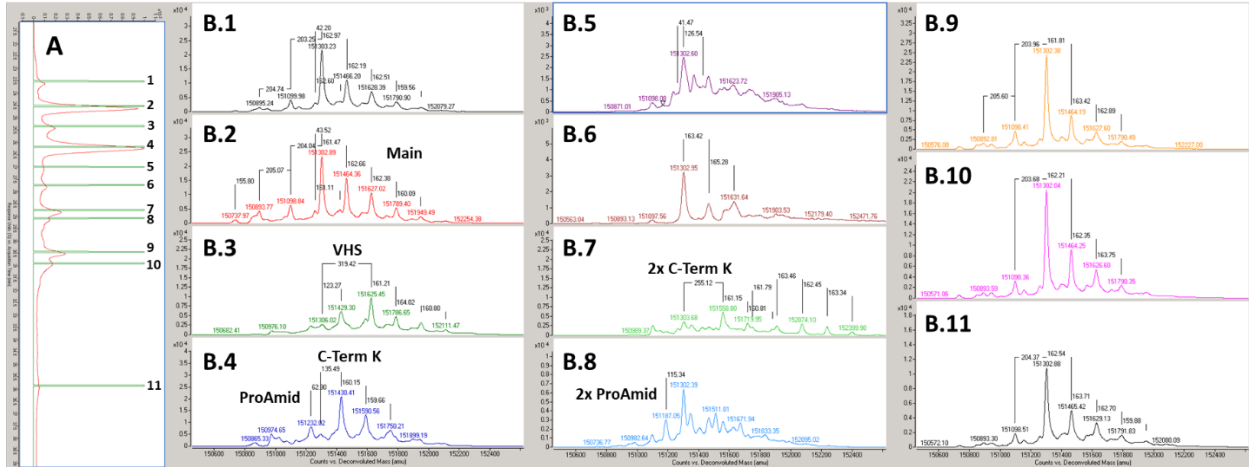


Figure S1.3 (A) The CEX-UV profile of the 1st dimension analysis of the isolated Basic region from mAb1 including heart-cut markers 1-11. (B) Zoomed in deconvolution spectra, 148-150 kDa with mass caliper measurements of minor peaks relative to the mAb1 mass.

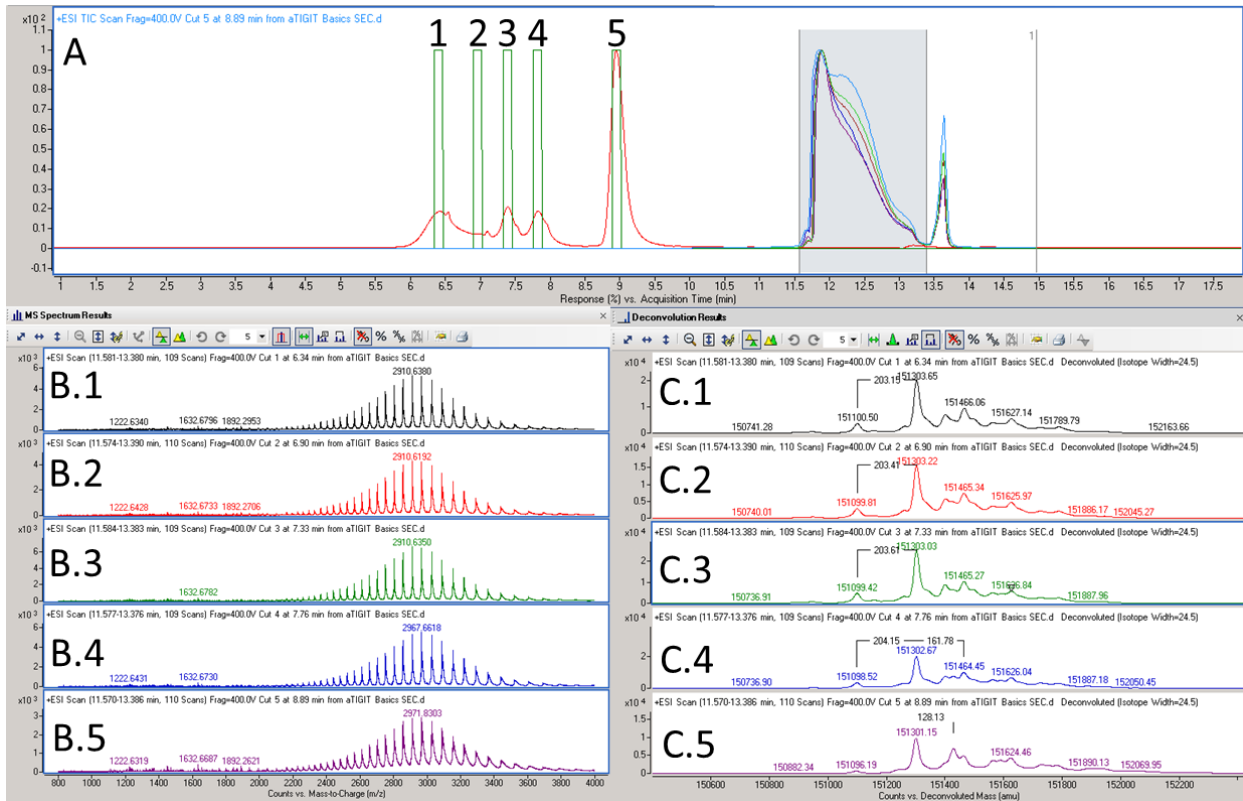


Figure S4 (A) The SEC-UV profile of the 1st dimension analysis of the isolated Basic region from mAb1 including heart-cut markers 1-5. (B) Extracted mass spectra, 800-4000 m/z, of the heart-cut TICs (overlaid, pictured in shaded region in A) from the 2nd dimension RP-MS analysis (C) Zoomed in deconvolution spectra for heart-cuts 1-5, 150-153 kDa with mass caliper measurements of minor peaks relative to the mAb1 mass.

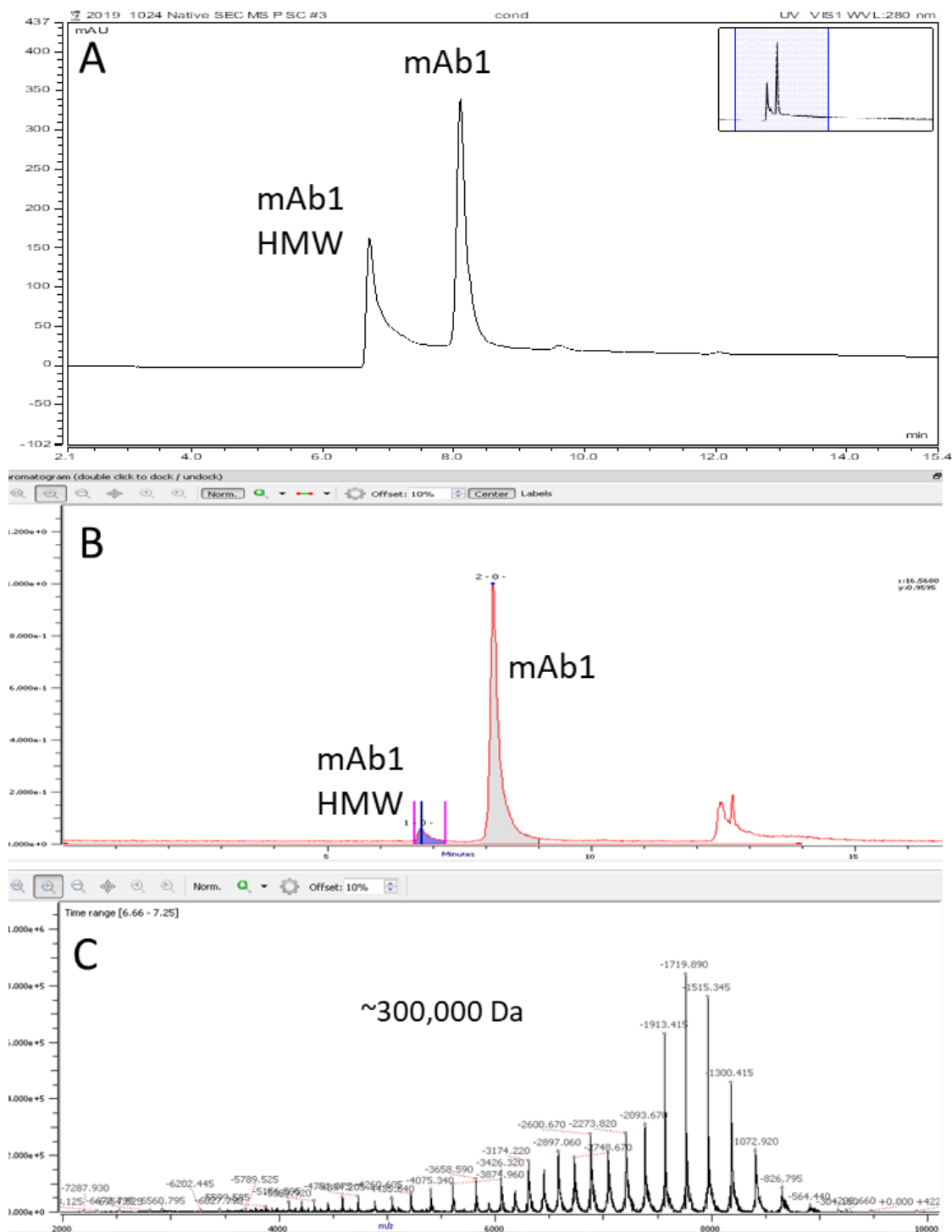


Figure S5. (A) The SEC-UV profile from the Native SEC-MS analysis of the isolated HMW region from mAb1. (B) The total ion chromatogram from the Native SEC-MS analysis of the isolated HMW region from mAb1. (C) Extracted mass spectra of the HMW TIC peak, 2000-10000 m/z.

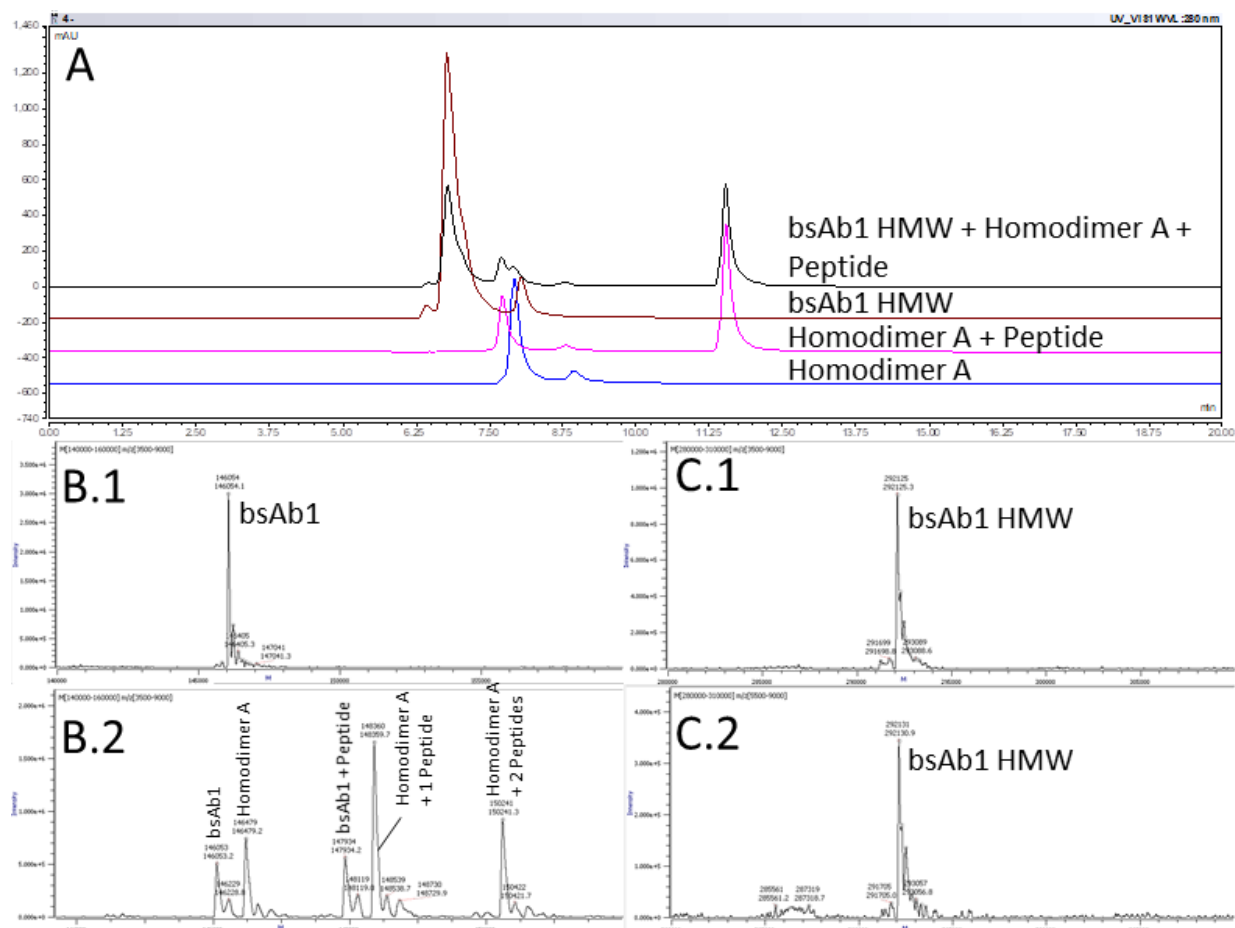


Figure S6. (A) The SEC-UV profiles from the Native SEC-MS analysis of a bsAb1 HMW both neat (second trace from the top) and mixed with Homodimer A in the presence of peptide (top trace); included also are the SEC-UV profiles from the Native SEC-MS analysis of neat homodimer (bottom trace) and in the presence of peptide (second from the bottom). (B) Zoomed-in deconvoluted spectrum of the extracted bsAb1/homodimer TIC peak (not pictured) both neat (1) and in the presence of excess peptide (2). (C) Zoomed-in deconvoluted spectrum of the extracted bsAb1 HMW TIC peak (not pictured) both neat (1) and in the presence of excess peptide (2).

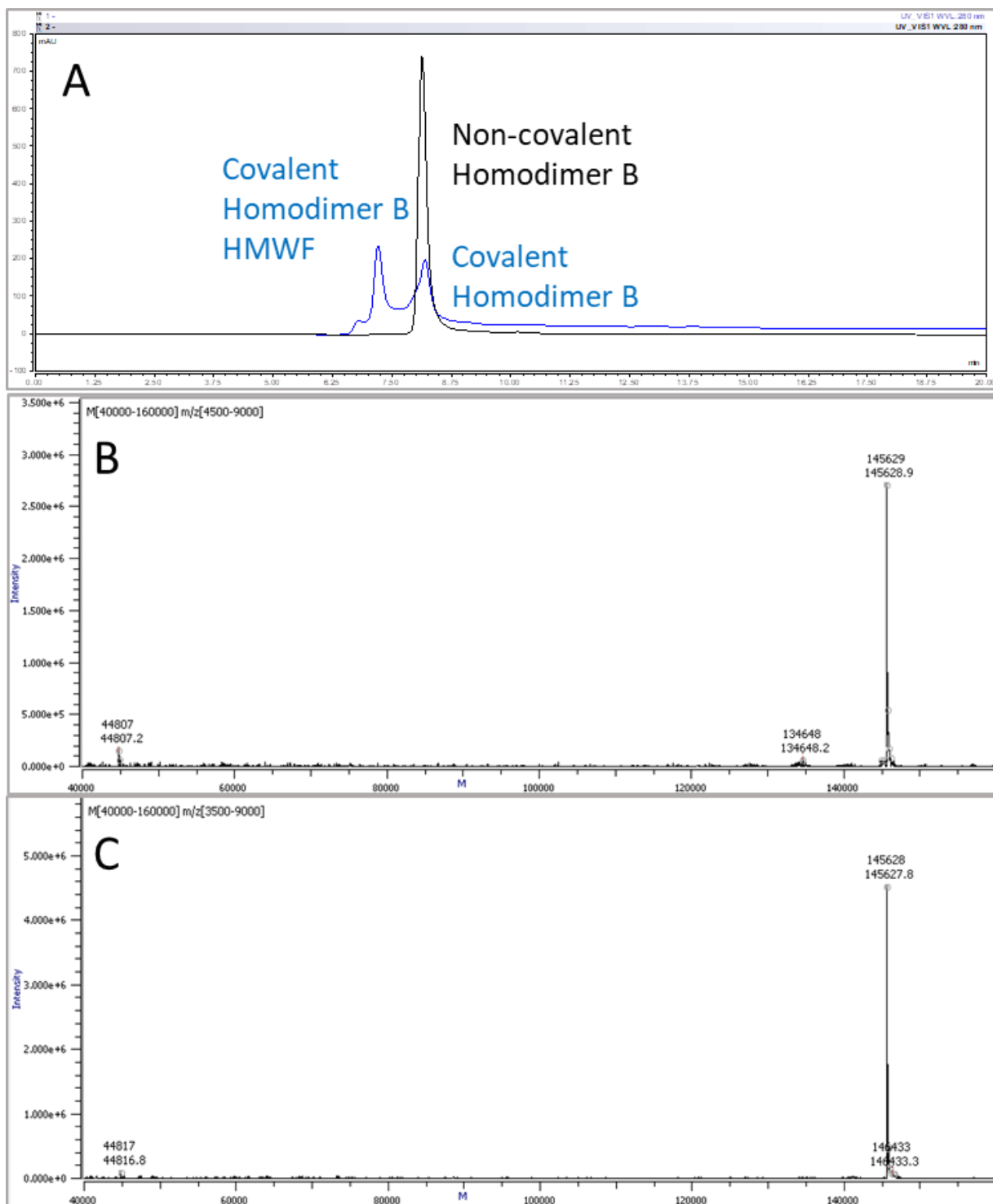


Figure S7. (A) The SEC-UV profiles from the Native SEC-MS analysis of the covalent (black trace) and non-covalent (blue trace) homodimer B materials. (B) The deconvoluted mass spectrum, 40-160 kDa, of the non-covalent homodimer B. (C) The deconvoluted mass spectrum, 40-160 kDa, of the covalent homodimer B.

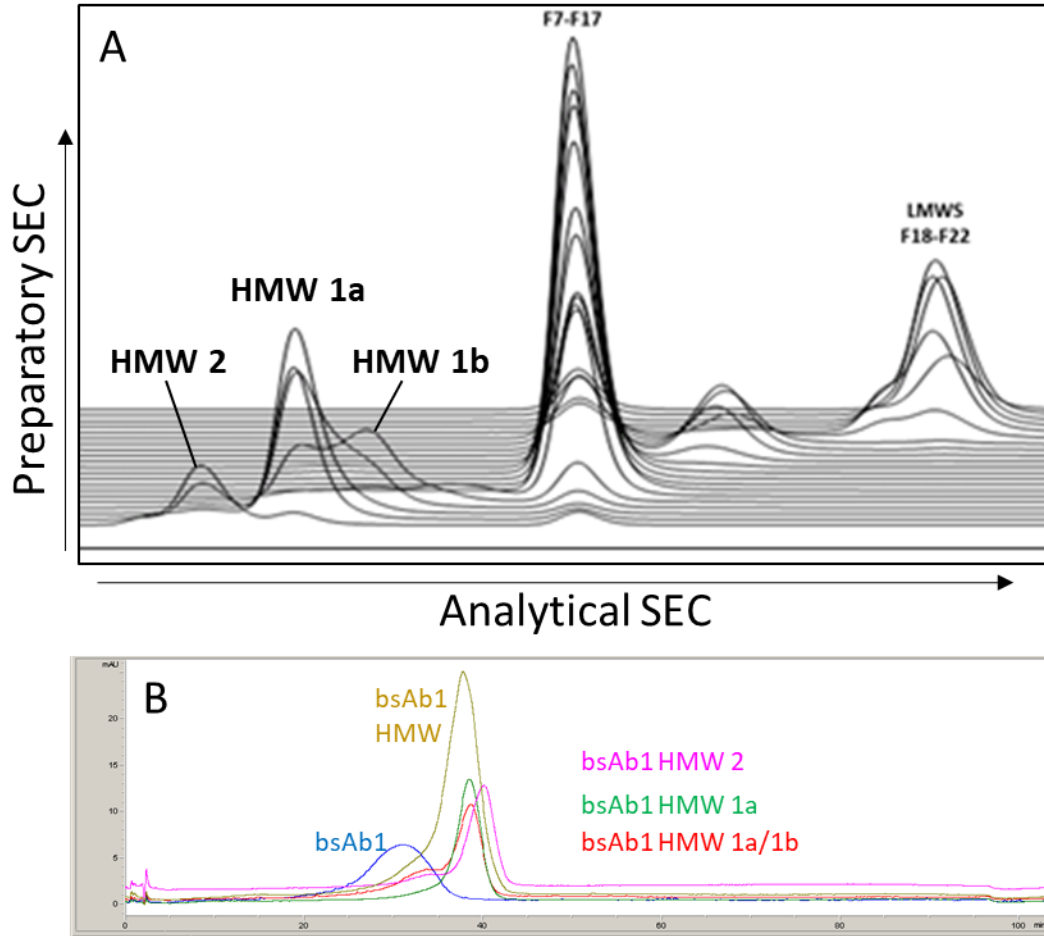


Figure S8. (A) A diagram showing the ordered SEC-UV profiles for fractions collected during a preparatory SEC isolation of bsAb1 size forms. (B) The FcRn AC-UV profiles of bsAb1 monomer (blue), bsAb1 HMWF (gold), and fractions containing enriched bsAb1 HMW 2 (pink), HMW 1a (green), and a mix of HMW 1a and 1b (red).

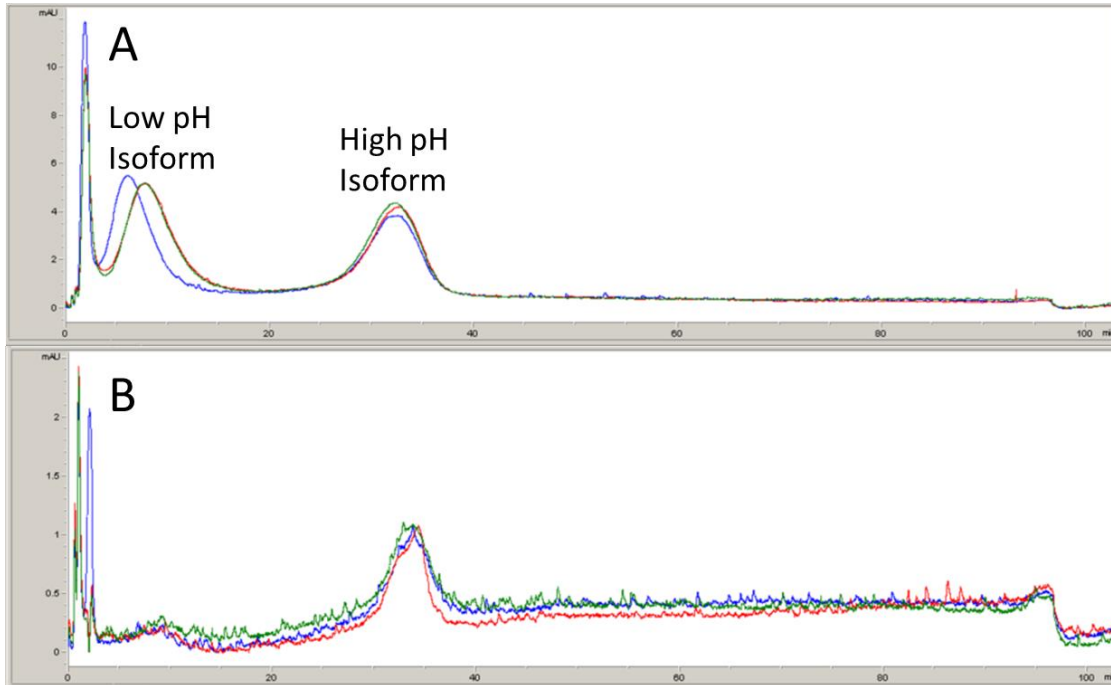


Figure S9. (A) An overlay of FcRn AC-UV profiles for neat Homodimer A (green and red) and in the presence of ligand (blue). (B) An overlay of FcRn AC-UV profiles for Homodimer A treated to just the high pH isoform, both neat (green and red) and in the presence of ligand (blue).

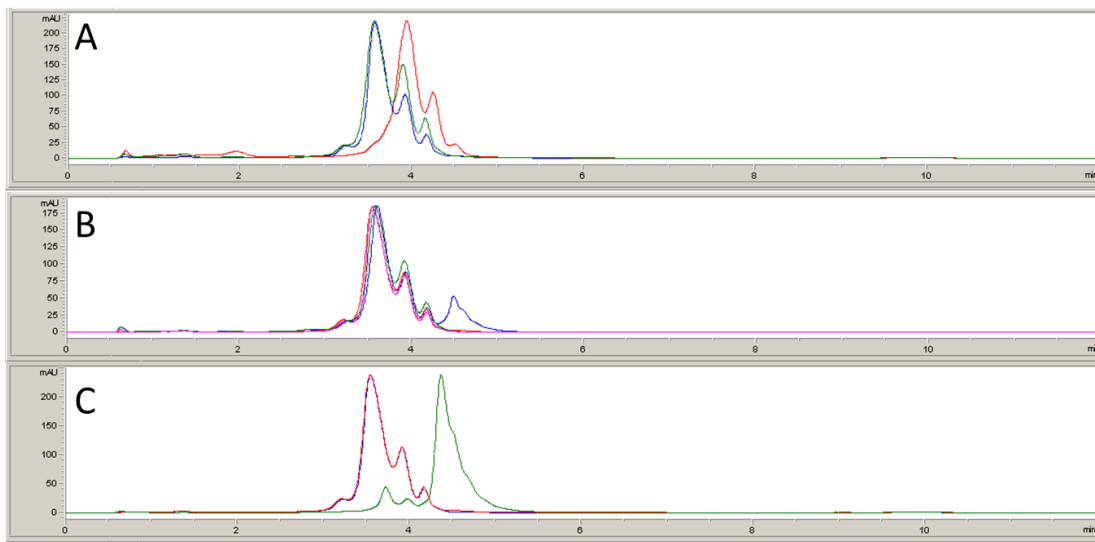


Figure S10. (A) An overlay of Fc γ RIIIa AC-UV (using column with aglycosylated Fc γ RIIIa) profiles for 100% afucosylated mAb1 (red), 10% afucosylated mAb1 control (green) and mAb1 standard (blue). (B) An overlay of Fc γ RIIIa AC-UV profiles for mAb1 standard (red), enriched acidic charge variants (green), enriched main peak (pink), and enriched basic charge variants (blue). (C) An overlay of Fc γ RIIIa AC-UV profiles for mAb1 standard (red), enriched mAb1 monomer (blue), and enriched mAb1 high molecular weight forms (green).

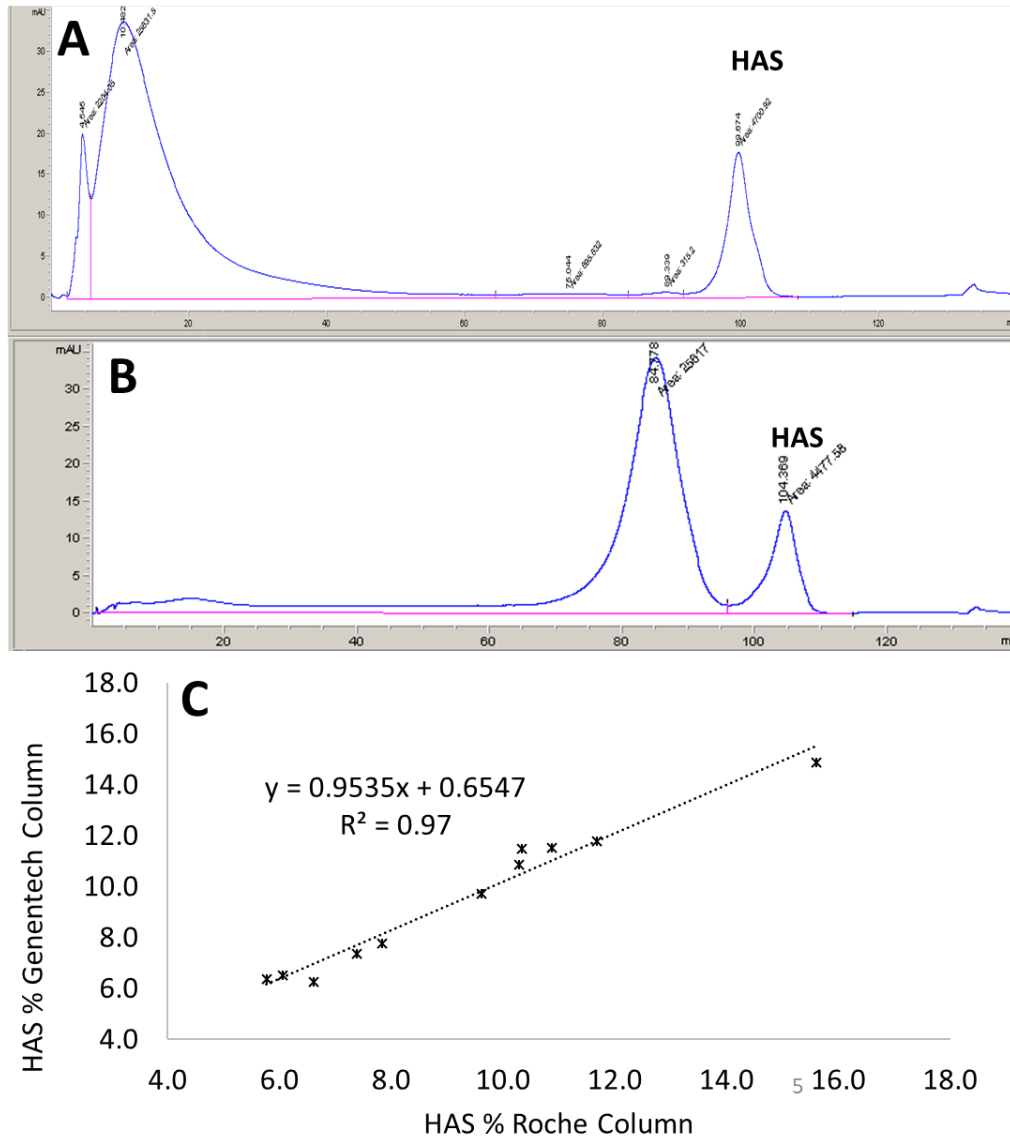


Figure S11. (A) Representative FcγRIIIa Affinity Chromatography UV profile of mAb2 using Roche Custom Biotech FcγRIIIa column. (B) Representative FcγRIIIa Affinity Chromatography UV profile of mAb2 using FcγRIIIa column made using Genentech material. (C) Quantitative comparison of percent HAS results obtained by using Roche column vs. Genentech column for select mAb2 batches.