Integration of On-column Chemical Reactions in Protein Characterization by LC/MS: Cross-Path Reactive Chromatography

Jake W. Pawlowski, Ian Carrick and Igor A. Kaltashov✉

Department of Chemistry, University of Massachusetts-Amherst, Amherst, MA 01003

✉ address correspondence to:
Igor A. Kaltashov, University of Massachusetts-Amherst, 240 Thatcher Way, Life Sciences Laboratories N369, Amherst, MA 01003; phone: (413) 545-1460; email: Kaltashov@chem.umass.edu

ABSTRACT

Profiling of complex proteins by means of mass spectrometry (MS) frequently requires that certain chemical modifications of their covalent structure (*e.g.*, reduction of disulfide bonds) be carried out prior to the MS or MS/MS analysis. Traditionally, these chemical reactions take place in the off-line mode to allow the excess reagents (the majority of which interfere with the MS measurements and degrade the analytical signal) to be removed from the protein solution prior to MS measurements. In addition to a significant increase in the analysis time, chemical reactions may result in a partial or full loss of the protein if the modifications adversely affect its stability, *e.g*. making it prone to aggregation. In this work we present a new approach to solving this problem by carrying out the chemical reaction on-line using the reactive chromatography scheme on a size-exclusion chromatography (SEC) platform with MS detection. This is achieved by using a cross-path reaction scheme, *i.e*. by delaying the protein injection onto the SEC column (with respect to the injection of the reagent plug containing a disulfide-reducing agent), which allows the chemical reactions to be carried out inside the column for a limited (and precisely controlled) period of time, while the two plugs overlap inside the column. The reduced protein elutes separately from the unconsumed reagents, allowing the signal suppression in ESI to be avoided and enabling sensitive MS detection. The new method is used to measure fucosylation levels of a plasma protein haptoglobin at the whole protein level following on-line reduction of disulfide-linked tetrameric species to monomeric units. The feasibility of top-down fragmentation of disulfide-containing proteins is also demonstrated using β_2-microglobulin and a monoclonal antibody (mAb). The new on-line technique is both robust and versatile, as the cross-path scheme can be readily expanded to include multiple reactions in a single experiment (as demonstrated in this work by oxidatively labeling mAb on the column, followed by reduction of its disulfide bonds and MS analysis of the extent of oxidation within each chain of the molecule).

INTRODUCTION

Analysis of protein covalent structure (which includes both amino acid sequence and post-translational modifications, PTMs) is now routinely carried out using LC/MS and LC/MS/MS. While comprehensive structural analyses have traditionally relied upon the so-called “bottom-up” approach, where proteolysis precedes the LC/MS step, analysis of the whole protein provides an attractive alternative as it allows valuable protein characteristics (including information on structural heterogeneity) to be obtained without requiring time-consuming proteolytic steps.[1] Furthermore, the progress made in recent years in the field of top-down MS[2] resulted in a dramatic expansion of the range of proteins amenable to analysis by this technique.[3-6]

By eliminating the need for proteolysis, top-down MS not only simplifies the sample handling step, but also greatly reduces the possibility of introducing artifacts.[7-10] One serious impediment that frequently complicates the top-down analysis of protein structure is the presence of multiple disulfide bonds. Indeed, even though dissociation of the thiol-thiol linkages can be achieved in the gas phase by using electron-based ion fragmentation techniques,[11] negative-ion CID[12] or ultra-violet photo-dissociation (UVPD),[13] these approaches typically work for small proteins[14] or peptides,[15] while larger proteins with multiple intact disulfide bonds remain out of reach of these techniques. Therefore, it is not surprising that in most cases successful top-down analysis of biopharmaceutical products relies on disulfide bond reduction prior to MS/MS measurements, which can be done using either conventional chemical reduction methods[4] or electrochemical cells interfaced with MS.[16,17] Above and beyond top-down MS/MS analysis of protein structure, reduction of disulfides prior to MS measurements may prove beneficial for other analytical tasks. For example, complexity and heterogeneity of recombinant proteins, protein/drug conjugates and endogenous macromolecules used as biomarkers can frequently be assessed by measuring their masses (or, more precisely, distribution of molecular masses in analytical and/or clinical samples).[1] While this task can be readily accomplished using modern MS tools for proteins with relatively low degree of complexity, it becomes increasingly challenging as both the size and the degree of heterogeneity increase.

One of the most significant sources of heterogeneity of many proteins is their glycosylation, the most abundant type of post-translational modifications (PTMs) found in both membrane and secreted proteins.[18] Glycosylation patterns frequently have a tremendous diagnostic value, holding enormous promise in the emerging field of personalized medicine.[18] Indeed, carbohydrate composition is known to be modulated not only by congenital disorders that affect the glycosylation machinery at the genetic level,[19] but also by a variety of other pathologies, including

Alzheimer's disease and other cognitive disorders,[20] diabetes,[20] immune disorders,[21] and cancer.[18,22]

The use of biomarkers in personalized medicine can be quite complicated, since frequently it is a panel of biomolecules, rather than a single reporter molecule, that needs to be considered. Therefore, sophisticated mathematical tools, such as multivariate statistical analysis,[23] are commonly used in order to provide meaningful results. Fortuitously, a number of serum glycoproteins offer unique opportunities for streamlined cancer diagnosis by exhibiting disease-specific glycosylation patterns.[24] Traditionally, protein glycan analysis is carried out in the bottom-up fashion, by isolating the protein of interest followed by enzymatic removal of the glycans and their analysis by MS and/or MS/MS.[22] Alternatively, composition of glycans can be established in favorable cases by the analysis of the intact protein mass,[25] bypassing the enzymatic step. Unfortunately, this approach is feasible only when applied to proteins with a relatively low extent of glycosylation (such as IgG molecules[25]), while extensive glycosylation inevitably results in high levels of heterogeneity preventing meaningful mass profiling.[26] In many cases, however, the extent of heterogeneity can be reduced to allow meaningful MS measurements without compromising the information encoded by glycans. For example, a number of plasma glycoproteins with high diagnostic value (haptoglobin and complex immunoglobulins) are multi-unit assemblies linked by disulfide bonds. Reduction of the thiol-thiol linkages may produce monomeric glycoproteins that can be readily mass-profiled by MS at the whole protein level, while their glycosylation patterns remain preserved. Unfortunately, such monomeric units are frequently only marginally stable and readily aggregate/precipitate prior to MS analysis.

Above and beyond disulfide reduction, a variety of other chemical reactions are used to probe biopolymer structure, *e.g*. selective chemical labeling and cross-linking.[27,28] Top-down MS offers an elegant way to determine the chemically modified and cross-linked sites in biopolymer complexes,[29] but it cannot be applied directly to analyze modified proteins in reaction mixtures without removing all unconsumed reagents and/or quenching agents that are incompatible with the ESI process. The clean-up step not only increases the analysis time and cost, but can also lead to the protein loss should modifications render it less stable. Clearly, there is an urgent need for an experimental scheme eliminating the clean-up step and enabling protein MS characterization in an on-line fashion immediately following the completion of the chemical transformations.

The goal of this work is to explore the possibility of carrying out chemical reactions inside a chromatographic column as a means of combining protein modification and MS analysis in a one-

step experiment. Reactions taking place inside chromatographic columns are commonly viewed as detrimental (*e.g.*, reactions between the analyte and the mobile phase components giving rise to artifacts[30]). However, there are applications where on-column reactions are carried out intentionally in order to enhance the analyte detection while maintaining the separation fidelity (*e.g.*, on-column derivatization with chromophores to enable spectrophotometric detection in LC[31,32] or with ionizable groups to enhance detection in GC/MS[33]). Integration of certain reactions with the separation process (e.g., acid/base reactions,[34] as well as a combination of metal ion complexation and redox reactions [35-37]) may also improve the LC separation selectivity. Finally, integration of chemical reactions with chromatographic separation can also be used to study organic reaction mechanisms.[38,39]

Recently, on-column chemical reactions were used to enhance the quality of biopharmaceuticals by converting trisulfide bonds to disulfides within a monoclonal antibody (mAb) captured by an affinity column.[40] Affinity capturing is a reliable way of retaining a protein while carrying out modifications that do not disrupt its native structure. However, this approach is too restrictive with regards to the types of chemical reactions. Indeed, this scheme would not allow incorporation of chemical reactions altering the higher order structure (*i.e.*, conformation and/or quaternary assembly), nor would it be tolerant to denaturing solvents. More importantly, on-column reactions are likely to target the affinity ligand in addition to captured analytes, thereby compromising the analyte retention and damaging the column. Lastly, even though affinity separation of immunoglobulins is now a routine procedure due to the availability of a wide range of antibody-specific ligands,[41,42] high-affinity ligands for other proteins may not be available as readily.

Since our goal was to devise a versatile experimental scheme allowing a variety of chemical reactions to be carried out on the column, we focused our attention on methods of separation that utilize stationary phases remaining inert towards protein-modifying agents, such as size exclusion chromatography (SEC). Since the majority of biomolecules cannot be permanently captured in SEC, we adopted a cross-path scheme: injection of the fast-moving protein molecules is delayed with respect to the loading of slow-moving chemical regents; the chemical reactions occur inside the column during the time interval when the reagent and protein plugs overlap (Figure 1). This separates the chemically modified protein upon its elution from the unconsumed reagents, enabling on-line protein analysis by MS. The feasibility of this approach is demonstrated by carrying out on-column reduction of several disulfide-containing proteins. The new method of protein structure analysis (dubbed XP-RC/MS, or cross-path reactive chromatography with MS detection) can be expanded to accommodate multiple reactions in a single experiment

(demonstrated in this work by oxidative labeling of mAb, followed by reduction of its disulfide bonds and MS analysis of each immunoglobulin chain).

EXPERIMENTAL

Materials. Haptoglobin 1-1 (Hp) was purchased from Athens Research (Athens, GA); β_2-microglobulin (β2m) was purchased from Lee Biosolutions (Maryland Heights, MO), and the mAb sample was generously provided by Biogen (Cambridge, MA). PNGase F (500,000 U/mL) was purchased from New England Biolabs (Ipswich, MA). Hydrogen peroxide (30%), ammonium acetate (HPLC grade), Tris and TCEP were purchased from Thermo-Fisher Scientific (Hampton, NH). All solvents and chemicals were of analytical grade or higher.

***Deglycosylation of mAb*.** The mAb sample was buffer exchanged into 100 mM Tris buffer (pH 7.5) followed by pipetting solution containing 25 mg of mAb (by dry weight) into a vial and diluted up to 990 µL with 100 mM Tris buffer (pH 7.5). A 10 µL aliquot of PNGase F was added to the vial, followed by incubation in a water bath at 37 ^{O}C. After incubation, deglycosylated IgG1 was buffer exchanged into a 150 mM ammonium acetate solution and stored at 2-8 ^{O}C.

***On-column protein modification*.** On-column chemical reactions were carried out on a TSKgel SuperSW mAb HTP (Tosoh, Tokyo, Japan) SEC column used with an HP 1100 (Agilent, Santa Clara, CA) HPLC system. A 75 mM ammonium acetate solution (pH 5.5) at a flow rate of 0.15 mL/min was used as a mobile phase for the analysis of Hp glycosylation patterns; a 150 mM ammonium acetate solution (pH 3.0) at a flow rate of 0.1 mL/min was used as a mobile phase for the top-down β2m analysis; and a 9:1 (v:v) mixture of aqueous 75 mM ammonium acetate solution (pH 3.0) with acetonitrile at a flow rate of 0.15 mL/min was used as a mobile phase for the structural analyses of deglycosylated mAb. The reduction plugs were composed of 100 mM TCEP (β2m analysis), 50 mM TCEP and 4M guanidinium chloride (Hp analysis); and 0.5 M TCEP and 5M guanidinium chloride (mAb analyses) in their respective mobile phases. The plugs were introduced using a manual injector with a loop volume of 100 µL (β2m) and 150 µL (mAb and Hp), which was placed between the sample injector and the SEC column. The protein samples were injected with a delay time of 10 sec (β2m) or 1 min (mAb and Hp) following the reagent plug injection.

Sequential on-column reactions (oxidation followed by disulfide reduction) were carried out with a TSKgel 3000SW xl (Tosoh, Tokyo, Japan) SEC column used with an HP 1100 HPLC system. A 75 mM ammonium acetate solution (pH adjusted to 3.0) with 10% methanol at a flow rate of 0.5mL/min was used as a mobile phase for the analysis of deglycosylated mAb. A flow splitter

was used to send ~10% of the flow to the mass spectrometer and the rest to waste. The oxidation plug was composed of 2% hydrogen peroxide, 10% methanol, and 75 mM ammonium acetate (pH 3.0). The reduction plug was composed of 100mM TCEP, 10% methanol, and 75 mM ammonium acetate (pH 3.0). Each plug was introduced using a manual injector with a loop volume of 250 μL. The reduction and oxidation plugs were injected three and one minute prior to deglycosylated mAb injection, respectively.

***MS Measurements and data analysis*.** All MS and MS/MS measurements were performed with a SolariX 7 (Bruker Daltonics, Billerica, MA) Fourier transform ion cyclotron resonance (FT ICR) mass spectrometer equipped with a 7.0 T superconducting magnet and a standard ESI source. Protein ions at successive charge states +11 through +14 (β2m) and +12 through +16 (mAb light chain) were isolated in the front-end quadrupole for MS/MS measurements; collision-induced dissociation (CID) in the hexapole region was used to induce ion fragmentation. The excitation voltage was set for at 22V and 30V for β2m and mAb light chain ions, respectively. MS/MS data were analyzed with DataAnalysis™ and BioTools™ software packages (Bruker Daltonics, Billerica, MA); all assignments made by BioTools™ were manually inspected to eliminate a possibility of false positives.

RESULTS AND DISCUSSION

Feasibility of using on-column reactions in LC/MS: XP-RC MS profiling of haptoglobin. Haptoglobin 1-1 (Hp) is a plasma glycoprotein composed of four subunits (two heavy chains, H, and two light chains, L) connected by disulfide bonds as H-L-L-H.[43] There are eight glycosylation sites within this protein (residing exclusively within the H chains), making the carbohydrate content of this protein nearly 20% of the total mass (92 kDa). Such a significant extent of glycosylation gives rise to a high level of structural heterogeneity making it nearly impossible to obtain reliable MS measurements.[26] Indeed, even though an SEC/MS spectrum of intact Hp contains abundant ionic signal (Figure 2A, B), the peaks representing different charge states are broad and do not show distinct contributions from individual glycoforms. This makes it impossible to deduce any meaningful information on the composition of Hp glycans. Reduction of disulfides would be an obvious approach to glycoform profiling at the intact polypeptide level (it should produce monomeric H-chains with a mass of only 37 kDa) and lower the extent of glycosylation (four glycans per each H-chain). However, these monomeric species become unstable upon disulfide reduction, and aggregate readily during a buffer exchange step preceding MS analysis. The new approach to disulfide reduction explored in this work (on-column chemistry followed by on-line MS detection) minimizes the time between the protein reduction and the MS measurement and

provides an opportunity to vary it from tens of seconds to several minutes by selecting an appropriate delay for protein injection (Figure 1). Increasing this delay decreases the time period spent by the metastable chemically modified species inside the column, dramatically reducing the specter of on-column aggregation.

On-column reduction (with TCEP used as a reducing reagent) gives rise to an abundant ionic signal of L-chains (12 min elution) and H-chains (11 min) in SEC/MS (Figure 2C). The reduction is incomplete, as evident by the presence of covalent dimers L_2. We also note that limited aggregation of metastable polypeptides does occur, as evidenced by the SEC peak at 8 min. elution (no interpretable ionic signal could be obtained for these high molecular weight species). This highlights the intrinsic instability and aggregation propensity of monomeric H-chains. Nevertheless, the abundance of both L- and H-chains ions in the mass spectra collected at longer elution times (11-12 min) is high, allowing the assignment of all eluting species to be readily made based on their masses. In a stark contrast to intact Hp, ionic signal of monomeric H-chains displays a number of baseline-resolved peaks representing different glycoforms (Figure 2D). Measuring the mass differences between adjacent peaks allows three major clusters to be identified (as labeled in Figure 2D). The mass difference between the clusters corresponds to a segment comprising a GlcNAcGalNeuAc trisaccharide (N-acetyl-glucosamine, galactose, and N-acetylneuraminic acid). The spacing between adjacent peaks within each cluster corresponds to a fucose residue mass (142.1 Da), with the total level of fucosylation ranging from zero to four (as indicated in Figure 2D). Fucosylation patterns are highly reproducible, allowing the extent of fucosylation to be calculated with an error not exceeding 9% RSD (see ***Supporting Information***). Therefore, XP-RC MS provides a means of exploiting the high diagnostic value of Hp fucosylation patterns without the need to remove/isolate carbohydrate chains from the protein. Sufficient amounts of monomeric species are produced during Hp transient exposure to the reducing agent (TCEP) inside the column, while the cross-path scheme eliminates any interference from TCEP during the on-line MS analysis of these monomeric polypeptides: the reagent plug does not emerge from the column until after all Hp components have eluted (Figure 2A).

Top-down sequencing of small disulfide-containing proteins: XP-RC MS/MS analysis of β2-microglobulin. Despite its modest size (11.7 kDa), β2m presents a challenge for top-down MS/MS analysis. Its single disulfide bridge (Cys^{25}-Cys^{80}) exerts a two-fold negative effect on the diagnostic value of the top-down MS/MS data. First, collision-induced dissociation (CID) of the peptide bonds within the [Cys^{25}-Cys^{80}] segment does not give rise to observable fragment ions (the two fragments are still physically connected by the thiol-thiol linkage, and the mass of this

dimer is indistinguishable from that of intact protein ions). Indeed, all CID-generated *b*- and *y*-fragments of β2m with the intact disulfide are confined to the short terminal segments of the polypeptide, [Ile^{1}-Cys^{25}] and [Cys^{80}-Met^{99}] (see ***Supporting Information***). Second, the presence of the disulfide cross-link within the polypeptide chain results in a significant reduction of the conformational space it can sample in solution even under denaturing conditions. Since the physical size of the protein is the major determinant of the extent of its multiple charging in ESI,[44,45] the number of charges accommodated by β2m ions with the intact disulfide bridge will remain modest, limiting the collision energy. The highest charge state observed for β2m ions in conventional SEC/MS is +10; and the efficiency of cumulative CID of four precursor ions (from +7 to +10) is rather modest (see ***Supporting Information***).

In contrast, the extent of multiple charging of polypeptide ions produced by ESI following the on-column disulfide reduction is relatively high (extending up to a charge state +17, see ***Supporting Information***). In addition to the dramatic change in the protein ion charge state distribution, disulfide reduction also manifested itself by a mass increase of 2 Da for ions at lower *m/z*. Mass increase for ions at lower charge states (<+9) was also evident, although the overall shift was less than 2 Da, indicating the presence of both disulfide-reduced and surviving disulfide-intact proteins. CID of ions corresponding to the disulfide-reduced β2m (charge states +11 through +15 were selected as precursors) gives rise to a large number of fragments (see ***Supporting Information***). In addition to a significant gain in the overall intensity of fragment ions, the fragmentation pattern also changes dramatically, with half of the observed fragment ions resulting from amide bond cleavages within the [Cys^{25}-Cys^{80}] segment, which failed to generate distinguishable fragment ions without on-column protein reduction. Clearly, on-line reduction of β2m results in a dramatic increase of the quality of information that can be extracted from the top-down MS/MS measurements while minimizing both sample preparation and analysis time.

Top-down analysis of a disulfide-connected protein assembly's subunit: on-line mAb's light chain analysis. While the XP-RC MS/MS analysis of β2m yields sequence information not accessible via CID of the disulfide-intact protein, it should be remembered that β2m is a rather modest protein whose single disulfide bond can be cleaved in the gas phase using electron capture dissociation.[14] The vast majority of biopharmaceuticals are significantly larger and contain multiple disulfide bonds (which could both reinforce the conformation of a single polypeptide chain by providing intra-chain cross-links, and connect several monomeric units in a multi-unit assembly). These features are epitomized by mAbs, recombinant proteins based on the IgG1 structural template.

There are sixteen disulfide bonds in the mAb used in our work. This includes twelve internal thiol-thiol connections, two in each of the light chains (L) and four in each of the heavy chains (H), and four inter-chain linkages (with each L/H pair being connected by a single disulfide bond, and the remaining two thiol-thiol linkages connecting the two H-chains). The inter-chain bonds are more labile, as they can be reduced under native conditions, when the intra-chain bonds remain intact.[46] Since our goal was to explore the utility of top-down MS for structural characterization of mAbs, we used low-pH conditions to maximize the reduction of all disulfide bonds. The chromatogram of mAb that underwent on-column reduction has a convoluted shape; on-line MS analysis reveals the presence of both monomers (L and H) and incompletely reduced assemblies (HL, H_2 and H_2L), see ***Supplementary Material*** for more detail.

While the effective reduction of external disulfide bonds in XP-RC is evident due to the presence of L- and H-chain ions, mass spectra acquired on-line do not produce direct evidence that the on-column reduction also succeeded in eliminating the internal disulfide bonds. Some indirect evidence is provided by the charge state distributions of the L- and H-chain ions. Indeed, the bimodal character of the charge state distributions, as well as the presence of ionic species with high charge density (in the low *m/z* regions of the spectra) suggest that at least some internal disulfides have been reduced. In order to determine if any internal disulfide bonds were indeed eliminated as a result of the on-column reduction, on-line MS/MS analysis of the L-chain was carried out. Five charge states (+16 through +12) were mass-selected as precursors for CID. The presence of 5 M guanidinium chloride in the reagent plug results in the most facile fragmentation (as judged by both overall intensity of the fragment ions and the number of amide bonds undergoing dissociation, see Figure 3 and ***Supplementary Material***). The detected high-abundance fragment ions (both *b*- and *y*-type) correspond to cleavages of nearly half of the amide bonds within the constant region of the L-chain (fifty-two out of one hundred and thirteen).

Guanidinium chloride is a very effective chaotrope frequently used as a protein unfolding agent. Its presence in the reagent plug likely results in more efficient unfolding of mAb chains, exposing the disulfide bonds to the reducing agent. Guanidinium chloride cannot be used in ESI MS measurements; however, in our scheme this interference is eliminated by separating polypeptide chains from the chaotrope prior to MS analysis. Importantly, twenty fragment ions detected in the XP-RC/MS/MS analysis of the L-chain correspond to the region of the polypeptide chain flanked by two cysteines (Cys^{134} and Cys^{194}) forming an internal disulfide bond (Figure 3). This provides unequivocal evidence that this internal disulfide had been successfully eliminated during protein exposure to the reagent plug inside the column. A comparable sequence coverage was obtained

in XP-RC MS/MS measurements in the absence of guanidinium chloride in the reagent plug, but the overall abundance of the fragment ions was noticeably lower (see ***Supplementary Material***). As an alternative approach to MS/MS, in-source fragmentation of ions without mass selection was carried out within the time window corresponding to the elution of L-chains (14-20 min, see ***Supplementary Material*** for more detail). Although the total fragment ion abundance was lower compared to the on-line MS/MS experiments, the extent of the sequence coverage was comparable, suggesting that XP-RC MS can be implemented on inexpensive MS platforms lacking tandem capabilities.

Since the presence of guanidinium chloride appears to favor dissociation of disulfide bonds, it seems reasonable to assume that other chaotropic agents may also prove beneficial as far as breaking thiol/thiol linkages. One particularly attractive possibility lies with the use of co-solvents that do not have to be confined to the reagent plug, but instead can be used as a part of the mobile phase. For example, addition of alcohols to the mobile phase is likely to destabilize the tertiary structure of the proteins, while keeping the secondary structure largely intact. This should increase the solvent exposure of disulfide bridges (and, therefore, reduction efficiency) without raising the specter of protein aggregation. Indeed, addition of even relatively modest amount of methanol to the mobile phase (10% by volume) results in a notable decrease of the relative abundance of all partially reduced species (H_2L, H_2, and HL), and near-complete elimination of the ionic signal of the surviving intact assembly H_2L_2 (see ***Supplementary Material*** for more detail).

An important question that should be addressed in connection with the on-column disulfide reduction is the possibility of recombination of free thiol groups outside of the reagent plug. Should this process occur, it would lead to (re)formation of disulfides prior to MS/MS detection. Above and beyond its obvious negative effect on the overall efficiency of the XP-RC process, thiol/thiol recombination can give rise to artifacts (*e.g.*, formation of disulfide bonds that were not present in the original protein). We note, however, that all multimeric species observed in the XP-RC MS of the mAb sample appear to be “legitimate” products of partial disulfide reduction (*e.g.*, H_2L, H_2, and HL), while any signs of *de novo* disulfide formation are absent (*e.g.*, HL_2, L_2, *etc.*). This provides a reasonable assurance that no disulfide recombination occurs under the conditions employed in XP-RC measurements following the on-column disulfide reduction.

It is interesting to compare the results of XP-RC/MS/MS analysis of mAb in this work with the top-down characterization of IgG molecules carried out using common approaches. Due to their large size, structural analyses of antibodies by MS until recently were almost exclusively carried out

using the bottom-up approaches, where gas-phase fragmentation is preceded by proteolysis in solution. This is now beginning to change mainly due to the rapidly increasing demands for the high-throughput analysis of mAbs and mAb-related products in the biopharmaceutical sector, with several groups actively exploring the feasibility of the top-down approach.[6,16,47] Not surprisingly, intact disulfide bonds present a formidable problem for the top-down analysis of mAb: while electron-based ion dissociation techniques allow some thiol-thiol linkages to be cleaved in the gas phase, the large number of disulfides typically present in mAbs limits the number of fragment ions derived from polypeptide segments flanked by disulfide-connected cysteine residues.[47] Chemical reduction of disulfides prior to antibody analysis by top-down MS results in a dramatic increase of the number of structurally diagnostic fragments and the extent of sequence coverage. Interestingly, sequence coverage of the variable regions is highly antibody-specific. For example, Marshall and co-workers observed that despite the 88% sequence identity between the variable domains of the light chains of Adalimumab and Efalizumab, the sequence coverage in this region differed by nearly six-fold, while the coverage of the constant regions was nearly identical between the two antobodies.[6] Therefore, a meaningful comparison of two different techniques *vis-à-vis* the extent of mAb sequence coverage should focus on the constant (κ) region, rather than compare the overall sequence coverage across the entire polypeptide chain. The number of the amide bonds within the constant region of mAb light chains that dissociate under ETD/CID combination giving rise to structurally diagnostic fragment ions reported by Marshall and co-workers for disulfide-reduced proteins is 61-62.[6] This number far exceeds the extent of sequence coverage that can be obtained without the reduction step prior to dissociation (up to 23 for the same segment[47]), but is comparable with that obtained in XP-RC MS/MS experiments (50 in the constant region of the light chain, as shown in Figure 3).

Interestingly, the fragmentation efficiency of the light chain of a mAb molecule subjected to the top-down MS/MS analysis following the on-line reduction in an electrochemical cell was relatively modest in comparison: even though the intra-chain disulfides were successfully reduced, the number of structurally diagnostic fragments derived from the light chain was relatively low, and their localization within the sequence was consistent with the notion of the internal thiol-thiol linkages remaining intact.[16] Clearly, XP-RC MS appears to be a more robust method for on-line reduction coupled to top-down MS analysis of monoclonal antibodies. Another important advantage offered by this technique is its versatility, as it allows various types of chemical modifications to be carried out prior to MS analysis (*vide infra*). Furthermore, multi-step modification procedures can be implemented in a single experiment, as outlined in the following section.

Feasibility of using multiple reactions in XP-RC MS: sequential on-column oxidative labeling of mAb and reduction of disulfide bonds. All examples of protein analysis with XP-RC MS considered so far utilize a single reagent plug. However, one can envision using multiple plugs containing different reagents in a single experiment. As long as all reagents fall under the permeation limit, the plugs will travel inside the SEC column along parallel trajectories, and the protein injected with a delay will be exposed to these reagents in a sequential manner (Figure 4). This would provide an opportunity to expand the use of XP-RC MS to probing higher order structure of proteins and protein assemblies, *e.g*. by employing chemical labeling as a probe of solvent accessibility.[48] The feasibility of this approach was evaluated using a scheme depicted in Figure 4, where oxidative labeling of mAb was carried out by using a plug of a 2% H_2O_2 solution, followed by exposure of the labeled protein to the TCEP plug. The ensuing disulfide dissociation generates L- and H-chains along with partially reduced species (H_2L, H_2, and HL), as previously observed in a "single-reaction" XP-RC MS/MS analysis of mAb. On-line MS detection provides clear evidence for the three oxidation events occurring within the H-chain (manifested by a mass shift of 50±2 Da), but not in the L-chain (see the top panels in Figure 4). The mass shifts observed within the partially reduced species and the intact assembly are also consistent with the notion of the H-chain undergoing oxidation at three sites, while the L-chain does not suffer any oxidative damage (48±3 Da shift for HL, 97±2 for H_2, and 98±3 for H_2L).

While H_2O_2 is hardly the best choice as a labeling reagent in terms of its efficiency with respect to protein labeling and the effect on the column longevity, the multiple reaction XP-RC scheme can be used for probing higher order protein structure with a variety of labeling reagents in the first plug, including amino-acid specific labeling reagents.[27] Another application where the multiple-reaction feature of XP-RC MS will be advantageous is the ranking of disulfide susceptibility to reduction using isotopically labeled thiol-capping reagents.[49]

CONCLUSIONS

Top-down MS analysis of proteins is a powerful tool for elucidation of various aspects of both covalent[50-52] and higher order structure.[53-56] Many applications of top-down MS require chemical treatment of proteins prior to MS analysis, which inevitably introduces ESI-incompatible low-molecular weight components (unconsumed reagents, quenchers, *etc*.) that must be removed prior to the MS analysis. This creates problems for proteins where the chemically modified forms are metastable and undergo aggregation/precipitation during the sample clean-up step. Furthermore, even for proteins that remain stable throughout the clean-up step, the latter results in a significant increase of the sample handling/analysis time. The cross-path reactive

chromatography (XP-RC) presented in this work as a means of facilitating top-down MS protein analysis solves this problem by initiating the chemical transformations inside the chromatographic column, and separating the high-molecular weight products (modified proteins) from the low-molecular weight reagents prior to the on-line MS analysis.

In this initial report we focus primarily on disulfide reduction as a means of increasing the value of information provided by on-line MS and MS/MS measurements for proteins that have traditionally been challenging for the top-down MS analysis. This new approach offers a straightforward way to control the extent of chemical modifications by varying either the width of the reagent plug or the reagent concentration (or both). It also provides a means of controlling the undesirable post-reaction processes (*e.g.*, aggregation of metastable chemically modified species) by allowing the time interval between the analyte's exposure to the regent plug and its elution from the column to be minimized by selecting an appropriate injection delay. An additional benefit offered by this technique is the (partial) separation of the reaction products, which allows the spectral crowding to be reduced and the quality of the MS data to be enhanced.

Above and beyond disulfide reduction, XP-RC allows other reactions to be implemented, including those that can be used to probe protein higher order structure. In some ways, the cross-path scheme presented in this work resembles the "catch-me-if-you-can" approach introduced by Krylov and co-workers as a means to measure kinetics of non-covalent interactions of proteins with small ligands.[57] A unique advantage of the cross-path scheme demonstrated in our work is the possibility of carrying out multiple reactions in sequence during a single experiment (*e.g.*, oxidative labeling followed by disulfide reduction to assist on-line MS characterization). Lastly, even though all experiments presented in this work had been carried out using SEC, the XP-RC methodology can be implemented on a variety of other LC platforms, provided the chemically treated protein(s) can be separated from the unconsumed reagents prior to the on-line MS analysis (we are currently exploring the utility of ion exchange chromatography for this purpose).

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ASSOCIATED CONTENT

Supporting Information. The following Supporting Information is available free of charge on the ACS Publications website: (*i*) distribution and reproducibility of Hp fucosylation patterns obtained with XP-RC MS; (*ii*) isotopic distributions of β2m ions produced by XP-RC MS with TCEP in the reagent plug; (*iii*) comparison of charge state distributions and top-down fragmentation patterns of β2m produced by conventional MS/MS and XP-RC MS/MS; (*iv*) extracted ion chromatograms for fully-, partially- and non-reduced species of mAb detected in conventional SEC MS and XP-RC MS; and (*v*) fragment ion spectra of the light chain of mAb generated by top-down XP-RC MS/MS with and without the precursor ion selection (the latter referring to the “in-source” CAD).

FIGURE LEGENDS

Figure 1. Schematic representation of the XP-RC using a 2-D depiction of the chromatographic process. The numerals on the diagram indicate injection of the low-molecular weight reagent plug (0), injection of the protein (1), chemical reaction between the protein and the reagent (2), and elution of the unreacted protein species (3) and the products of the chemical reaction (4 and 5).

Figure 2. XP-RC MS analysis of haptoglobin 1-1. **A**: A UV chromatogram of a control Hp injection without the on-column disulfide reduction (black trace) and the XP-RC chromatogram (magenta). **B**: On-line mass spectrum of the control Hp injection (averaged across the 9-10 min elution window). **C**: On-line mass spectra acquired in XP-RC of Hp (the colored arrows in panel **A** show where the two mass spectra were acquired). **D**: a zoomed view of the on-line mass spectrum of the H-chain of Hp produced by on-column reduction. Three clusters of peaks represent the following glycoforms (based on the measured masses): $[NeuAc_2Gal_2Man_3GlcNac_4]_4/Fuc_x$ (black labels), $[NeuAc_2Gal_2Man_3GlcNac_4]_3$/NeuAc3Gal3Man3GlcNac5/$Fuc_x$ (purple) and $[NeuAc_2Gal_2Man_3GlcNac_4]_2/[NeuAc_3Gal_3Man_3GlcNac_5]_2/Fuc_x$ (gold); the numerals indicate the number of fucose units (*x*) within each species.

Figure 3. XP-RC MS/MS of mAb showing CID mass spectrum and fragmentation pattern of the L-chain produced upon the on-column reduction of the intact protein. Amino acid sequence is shown only for the constant region of the L-chain; the vertical lines indicate amide bonds whose cleavage gives rise to the detected *b*- and *y*-ions (red lines correspond to this data set; blue line correspond to XP-RC MS/MS measurements carried out without using guanidinium chloride in the reagent plug; and black lines correspond to fragments generated in XP-RC MS using in-source collisional activation). The inset shows selected extracted ion chromatograms for several ionic species in XP-RC chromatogram (reference XICs obtained in the absence of the reducing agent in the reagent plug are shown as color-filled curves). The complete set of XICs with representative mass spectra (MS1) is shown in the **Supplementary Material** section.

Figure 4. A schematic diagram of an XP-RC experiment employing two reagent plugs and the mass spectra of the constituents of mAb produced by the on-column reduction with TCEP (reagent plug 2) following the on-column oxidative labeling with hydrogen peroxide (reagent plug 1). The numerals on the diagram indicate injection of the protein (1), chemical reaction between the protein and the reagent 1, *e.g*. oxidation with H_2O_2 (2), chemical reaction between the protein and the reagent 2, *e.g*. reduction of disulfide bonds (3), and elution of the unreduced (disulfide-intact) protein species (4) and the products of the external disulfide reduction (5 and 6).

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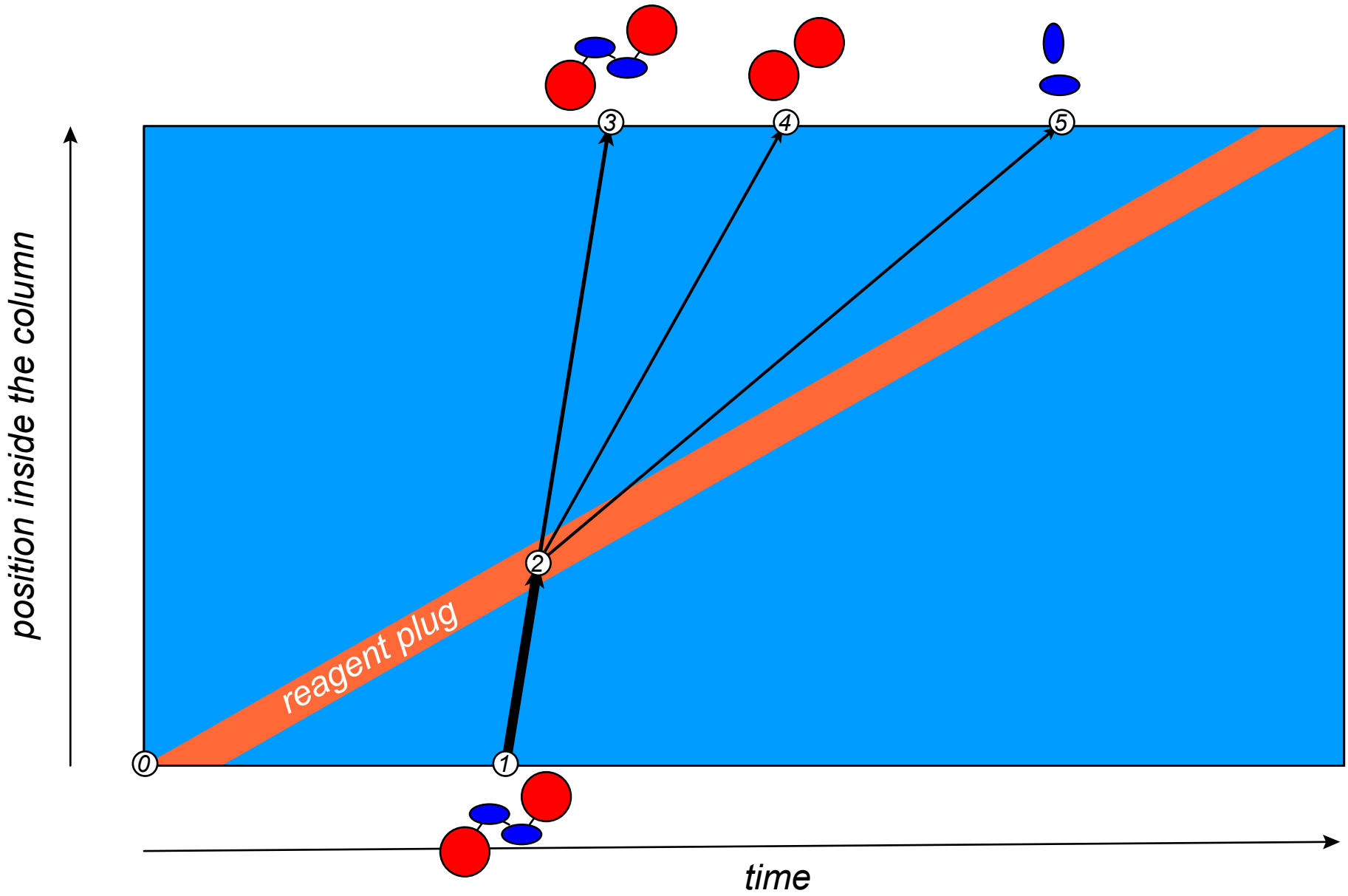
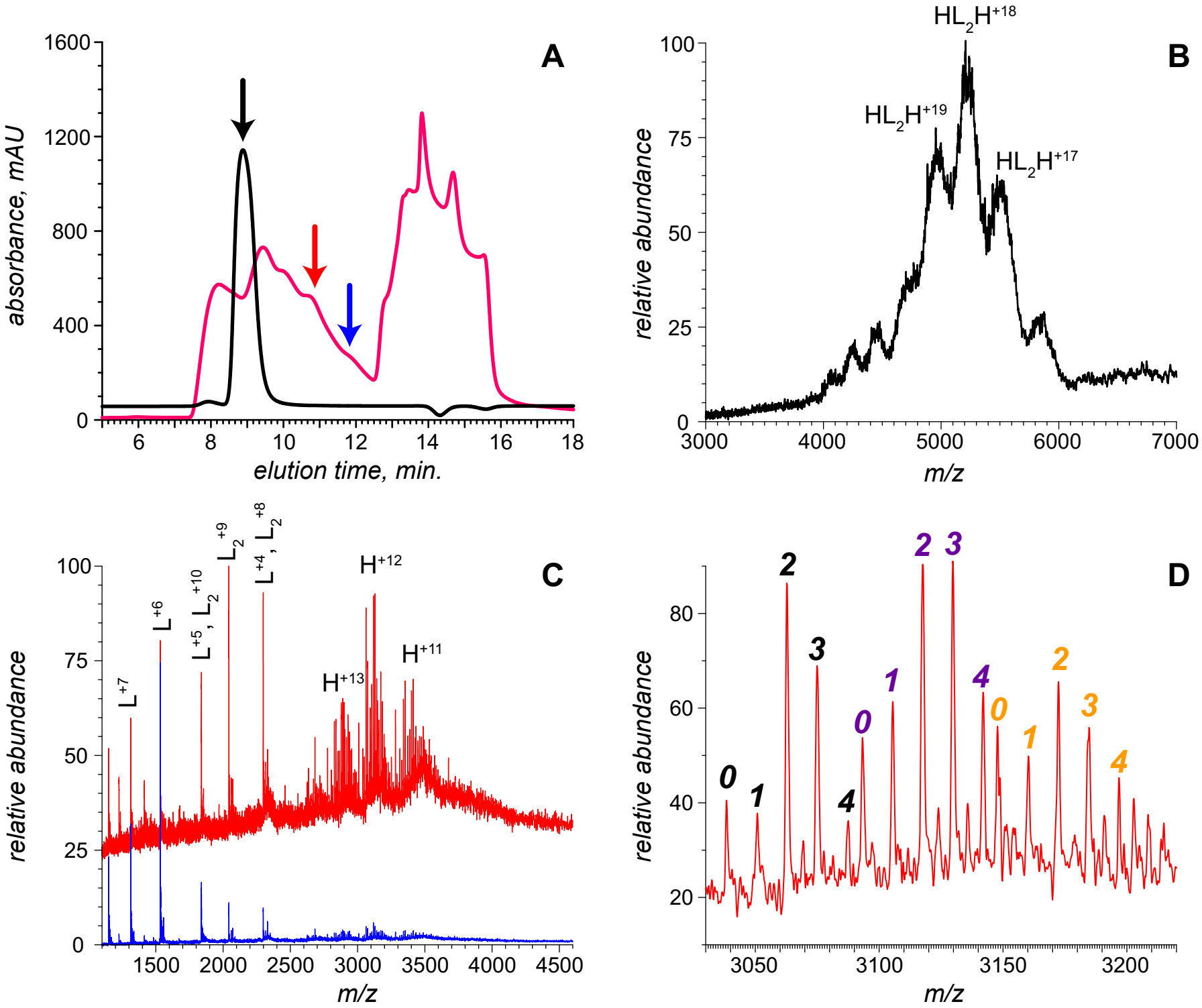


Pawlowski, et al. **Figure 1**



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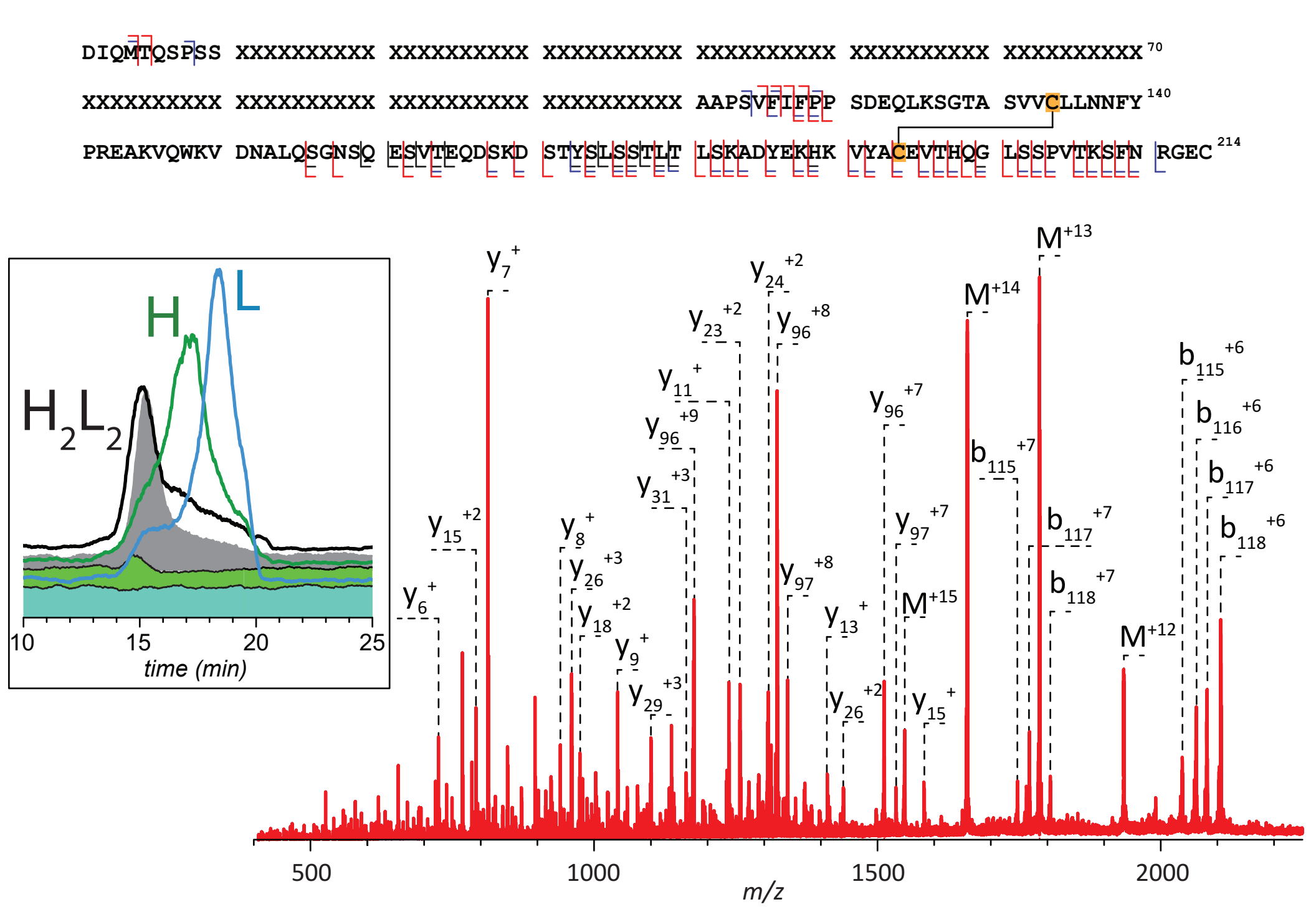
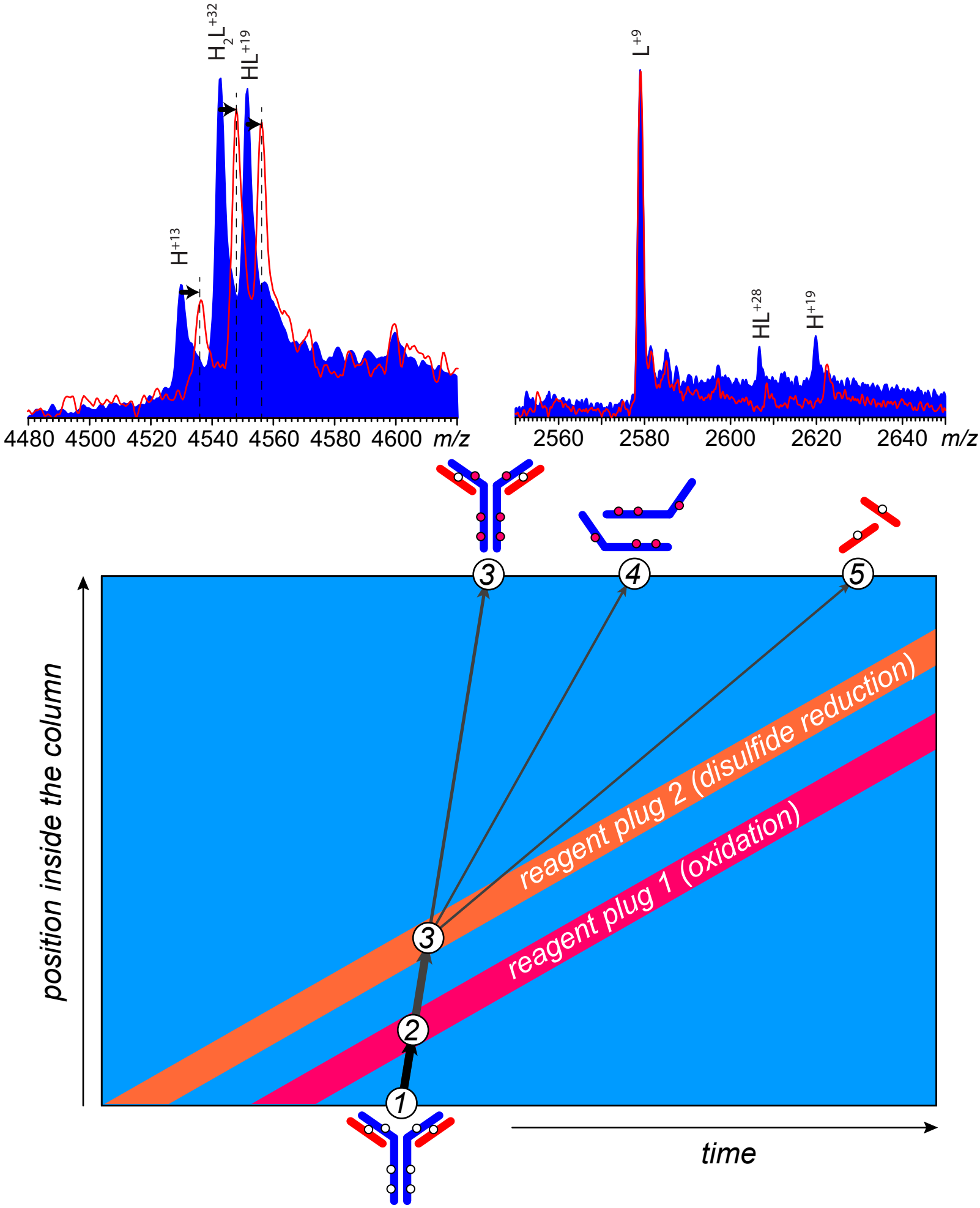


Pawlowski, et al. **Figure 3**



Pawlowski, et al. **Figure 4**

Supplementary Material for

Integration of On-column Chemical Reactions in Protein Characterization by LC/MS: Cross-Path Reactive Chromatography

Jake W. Pawlowski, Ian Carrick and Igor A. Kaltashov✉

Department of Chemistry, University of Massachusetts-Amherst, Amherst, MA 01003

✉ address correspondence to:
Igor A. Kaltashov, University of Massachusetts-Amherst, 240 Thatcher Way, Life Sciences Laboratories N369, Amherst, MA 01003; phone: (413) 545-1460; email: Kaltashov@chem.umass.edu

Table S1. Distribution of fucosylation within the $[NeuAc_2Gal_2Man_3GlcNac_4]_4/Fuc_x$ glycoforms based on the ionic peak heights in the on-line mass spectra of Hp H-chains produced upon on-column disulfide reduction

Total number of fucose residues	Relative abundance (based on the peak heights)	95% confidence interval (based on a set of three replicate measurements)
0	13%	2%
1	12%	2%
2	38%	5%
3	29%	3%
4	8%	1%

Figure S1. Reproducibility of Hp fucosylation patterns obtained with XP-RC MS (TCEP in the reagent plug). Three different data sets are shown for the monomeric H-chain at charge state +12; labeling of individual glycoforms is the same as in Figure 2D.

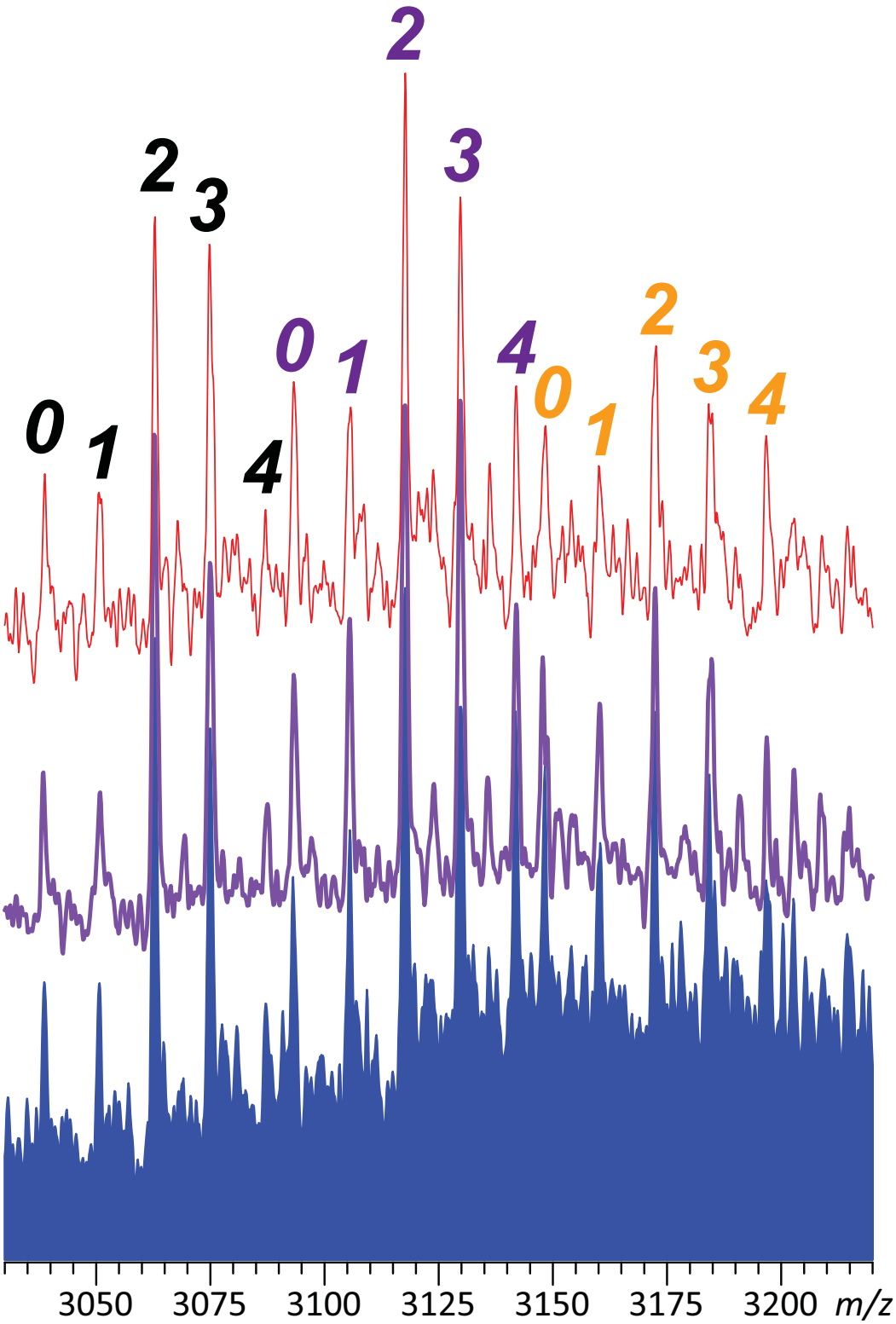


Figure S2. Isotopic distributions of β2m ions (charge states +7 and +14) produced by XP-RC MS with TCEP in the reagent plug. Black squares in the left-hand panel show the calculated isotopic distribution of a β2m ion at charge state +14 with a reduced disulfide bond. The blue trace in the right-hand side diagram shows the isotopic distribution of a β2m ion (charge state +7) produced by SEC MS (no on-column reduction).

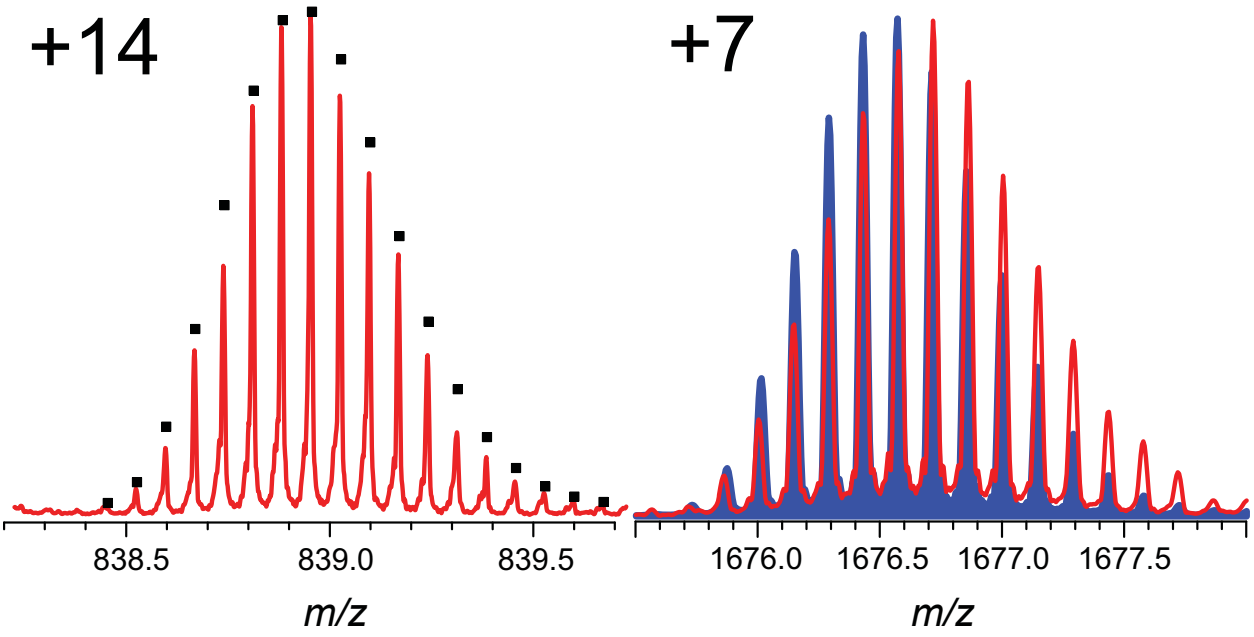


Figure S3. SEC MS/MS (top panel) and XP-RC MS/MS (bottom) analysis of β2m. The colored traces in each panel show mass spectra of intact protein (no collisional activation), and circles indicate protein ion used as precursors in CID measurements. The fragmentation patterns are shown at the top of the figure for both SEC MS/MS (blue lines) and XP-RC MS/MS (red).

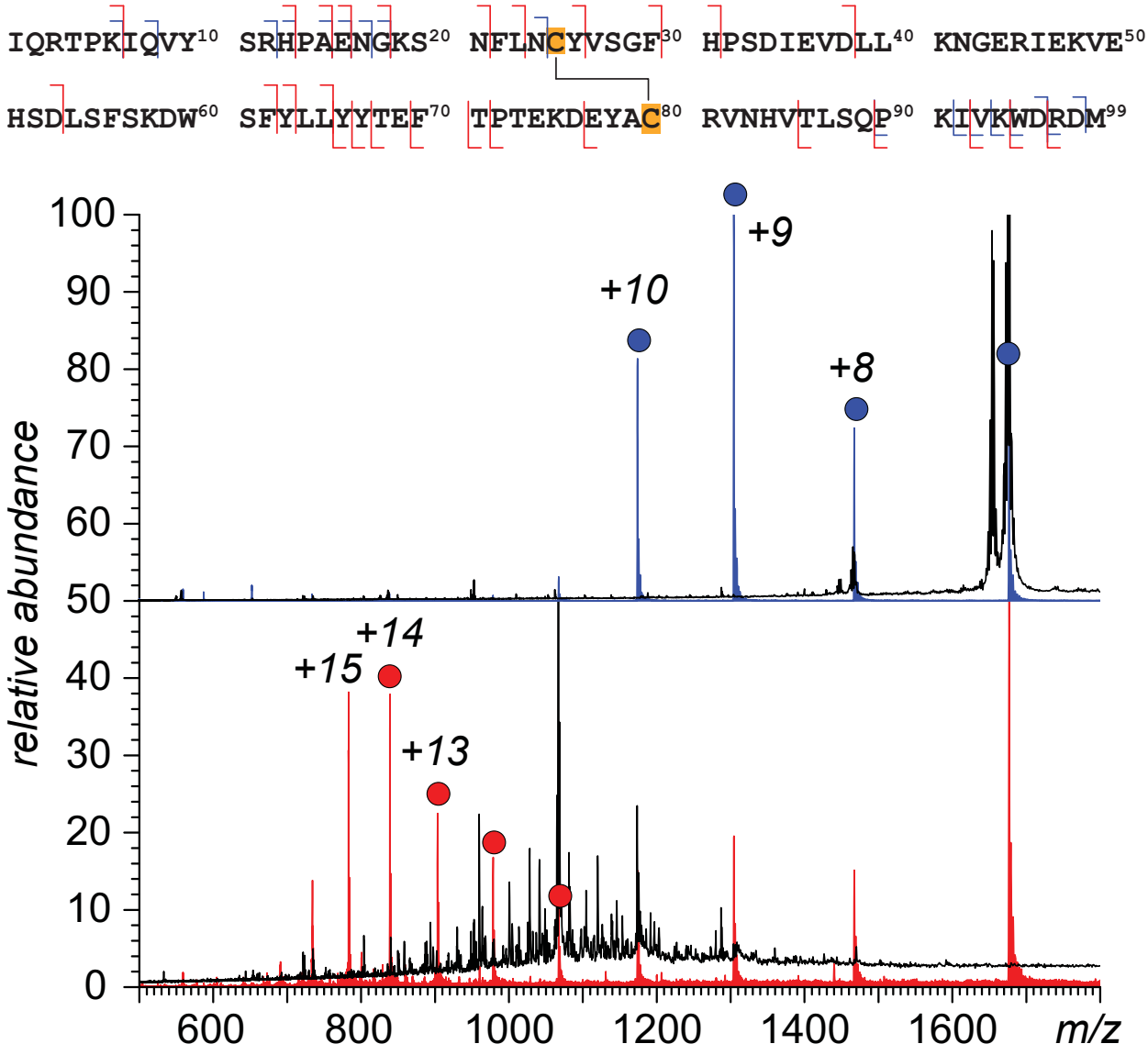


Figure S4. Extracted ion chromatograms for fully-, partially- and non-reduced species of mAb detected in SEC MS without on-column reduction (filled curves) and XP-RC MS experiments (150 mM ammonium acetate solution, pH adjusted to 3.0; TCEP in the reagent plug). The three representative on-line mass spectra shown on the right-hand side were averaged across the following elution windows: 14-16 min (top), 16-17 min (middle), and 17-19 min (bottom). The XICs were generated by plotting ionic signals for the following species: H_2L_2, charge state +33 (m/z window 4419-4423); H_2L, charge state +23 (m/z window 5332-5336); H_2, charge state +21 (m/z window 4736-4740); HL, charge state +17 (m/z window 4289-4293); H, charge state +14 (m/z window 3552-3556); and L, charge state +9 (m/z window 2576-2580). Note that the early-eluting peaks of partially- and fully-reduced species are artifacts due to the interfering signal of the intact mAb (e.g., it is impossible to distinguish the ionic signal of HL^{+17} from that of $H_2L_2^{+34}$).

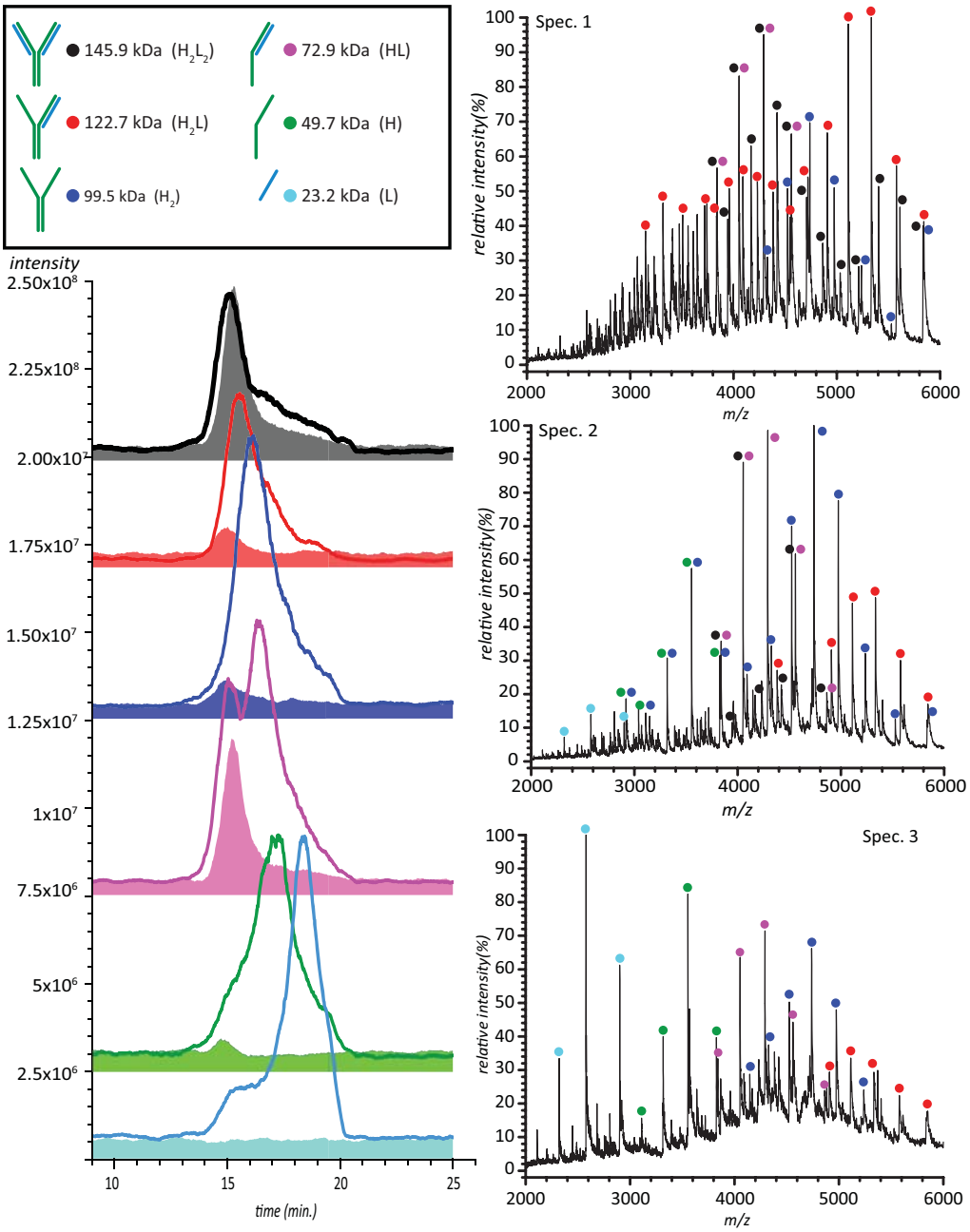


Figure S5. Extracted ion chromatograms for fully-, partially- and non-reduced species of mAb detected in SEC MS without on-column reduction (filled curves) and XP-RC MS experiments (150 mM aqueous ammonium acetate solution, pH adjusted to 3.0, with 10% methanol by volume; TCEP/10% methanol in the reagent plug). The three representative on-line mass spectra shown on the right-hand side were averaged across the following elution windows: 15-17 min (top) and 17-19 min (bottom). The XICs were generated by plotting ionic signals for the following species: H_2L_2, charge state +33 (m/z window 4419-4423); H_2L, charge state +23 (m/z window 5332-5336); H_2, charge state +21 (m/z window 4736-4740); HL, charge state +17 (m/z window 4289-4293); H, charge state +14 (m/z window 3552-3556); and L, charge state +9 (m/z window 2576-2580). Note that the early-eluting peaks of partially- and fully-reduced species are artifacts due to the interfering signal of the intact mAb (e.g., it is impossible to distinguish the ionic signal of HL^{+17} from that of $H_2L_2^{+34}$).

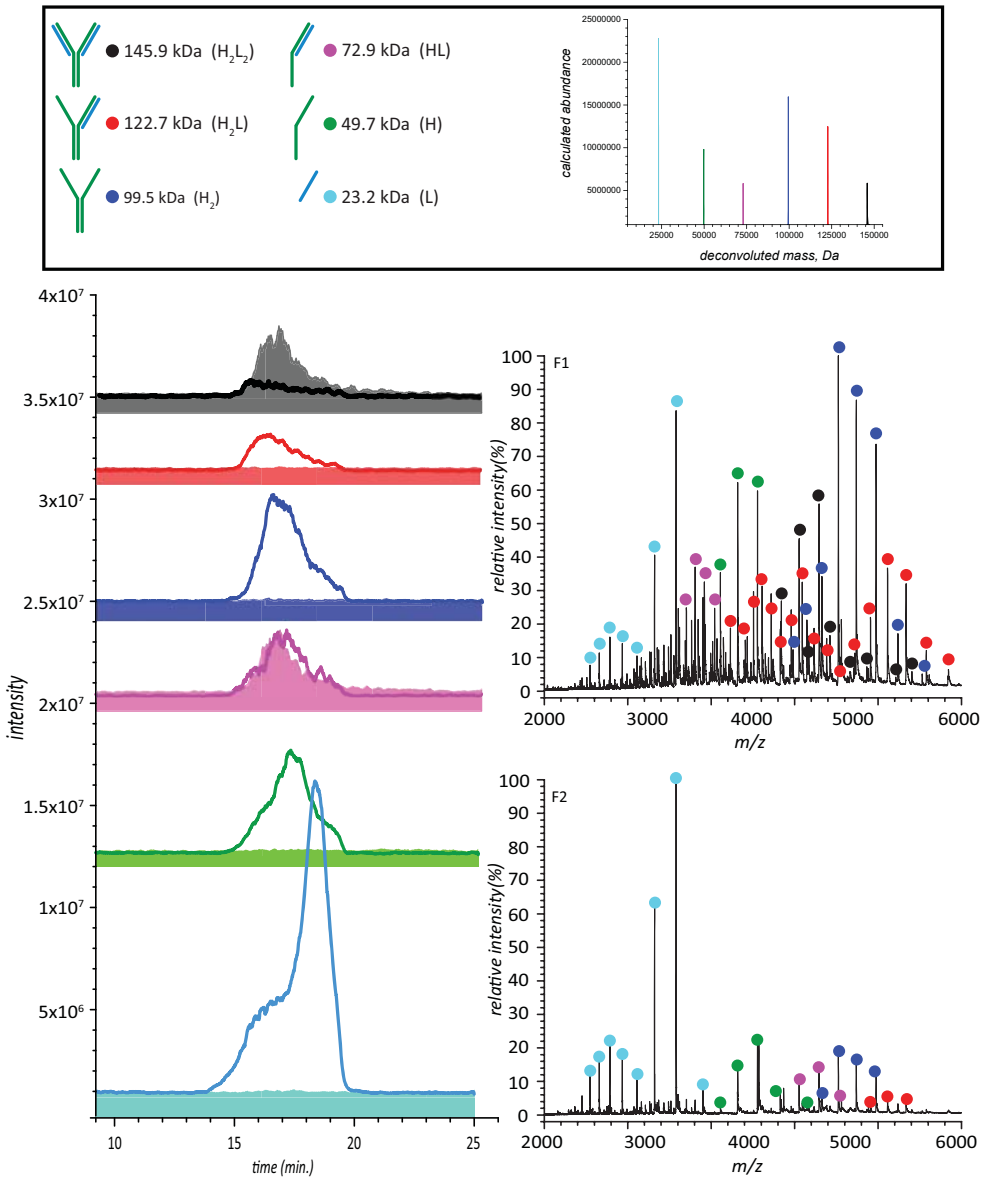


Figure S6. XP-RC MS/MS analysis of mAb: fragment ion spectra of the L-chain generated by on-column disulfide reduction of mAb (TCEP in the reagent plug). Fragmentation was induced by collisional activation of ions of monomeric L-chains at charge states +12 through +16 (blue-filled curve) and by collisional activation of all ions in the ESI interface without precursor ion selection (in-source fragmentation, black trace). The two fragmentation patterns are overlaid in the top diagram (the amino acid sequence is shown only for the constant region of the L-chain).