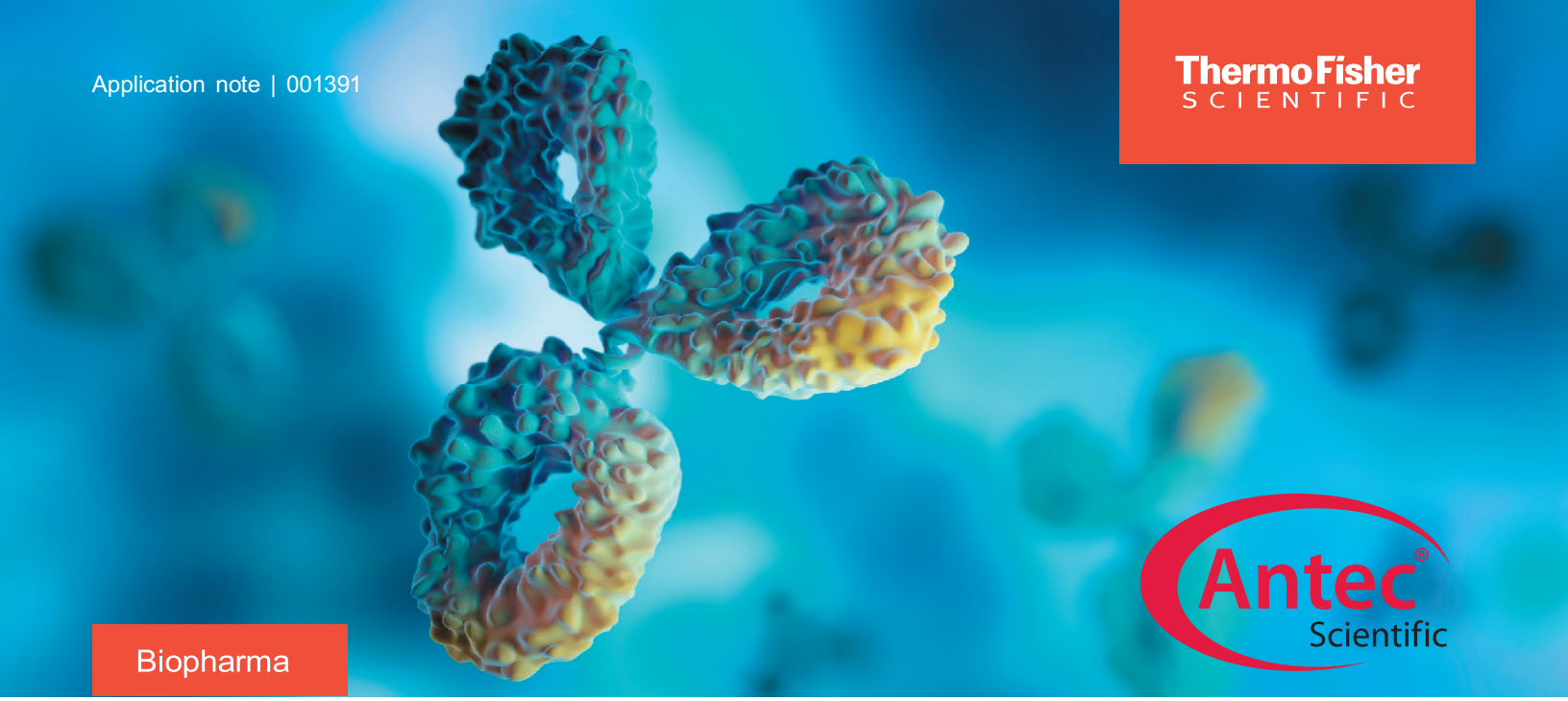
Application note | 001391



Biopharma

Improved and universal inline electrochemical reduction and subunits LC-MS analysis of multiple classes of monoclonal antibodies

Authors

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Keywords

Biopharmaceutical, biotherapeutic, monoclonal antibody (mAb), IgG, electrochemical reduction, middle-up, Vanquish Dual Pump UHPLC, Q Exactive Plus Hybrid Quadrupole-Orbitrap mass spectrometer, BioPharma Finder software, ROXY Exceed

Application benefits

- Reduced mAb and reduced subunits analysis can be carried out on a single platform without the need for reducing agents.
- An inline electrochemical cell, combined with a Thermo Scientific™ Vanquish™ Dual Pump UHPLC system allows for effortless implementation of a reduction and trapping step in the standard LC-MS workflow for subunits analysis.
- The workflow is effective on different classes of antibody, including IgG1, IgG2, and IgG4, and does not affect the quantitation of acid labile species such as sialylated *N*-glycans.

Goal

To demonstrate that an optimized method for inline reduction and LC-MS analysis of mAbs and mAb subunits is suitable for the analysis of several classes of monoclonal antibodies. To demonstrate that complete reduction is achieved inline on both intact mAbs and IdeS digestion generated subunits. The workflow is shown to be effective and to not alter the evaluation of labile modifications sensitive to acidic conditions, such as sialylated *N*-glycans.

Introduction

Method development for the analysis of monoclonal antibodies (mAbs) is a continuously growing sector as greater insight in their structural characterization is needed, and faster methods are required, to support biopharmaceutical production. MAbs can be characterized at different levels, including bottom-up, middle-up, and intact approaches; each of these techniques brings its own unique advantages, allowing the analyst to decipher different aspects of the molecule complexity.

A limited number of features are available at the intact level, and bottom-up approaches require longer sample preparation and analysis times. Middle-up approaches, however, bring the advantage of fast and simple sample preparation, together with the possibility to exploit mass spectrometry detection in a mass range where the use of high resolution is possible, thus providing isotopically resolved data for unequivocal and reliable proteoform identification. The two most widely used middle-up approaches are the reduction of the mAb to its light chain (Lc) and heavy chain (Hc) components, or enzymatic digestion to generate smaller subunits (scFc, Fd, and Lc) using IdeS or equivalent proteases. The first approach is achieved by hydrolysis or reduction of the disulfide bonds linking the light chain and heavy chains together through the hinge region to constitute the typical Y shaped dimeric protein. The second approach requires the use of specific enzymes able to selectively cleave conserved regions of the primary sequence around the hinge region, followed by reduction of disulfide bonds to break the remaining connections between subunits. Both methodologies include the use of reducing agents, which bring several disadvantages. First, reduction steps involve the presence of salts or additional denaturing agents to aid access of the reagent to non-exposed regions; this results in the matrix of the analyte not being MS compatible and requiring further clean-up steps prior to analysis. Moreover, reduction conditions may require prolonged exposure to heat, which may alter post-translational modifications, easily affected in these circumstances.

Recently, an automated workflow for subunits analysis using inline electrochemical reduction followed by liquid chromatography and mass spectrometry analysis (EC-LC-MS) was published and proved efficient in the generation of reduced mAb and reduced IdeS subunits, using NIST mAb standard.[1] The workflow progressed from buffer exchanged intact or IdeS digested protein and, using the µ-PrepCell™ SS on a ROXY™ Exceed potentiostat (Antec Scientific, Alphen a/d Rijn, Netherlands) and the unique capabilities of the Vanquish UHPLC system and Thermo Scientific™ Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ mass spectrometer, allowed direct MS analysis of the subunits without any further sample handling.[1]

In this study, we use an optimized workflow on the same platform reduced to 23.5 minutes. The workflow was tested on different subclassed of mAbs, which present various levels of complexity in the reduction of their disulfide bonds (Figure 1). To prove the universal suitability of the method for mAbs analysis, we also tested the platform on IdeS digested cetuximab drug product to verify the potential for site specific *N*-glycan analysis and the correct evaluation of sialic acid containing glycoforms to investigate and ensure no loss of labile sialic acid residues under the experimental conditions used.

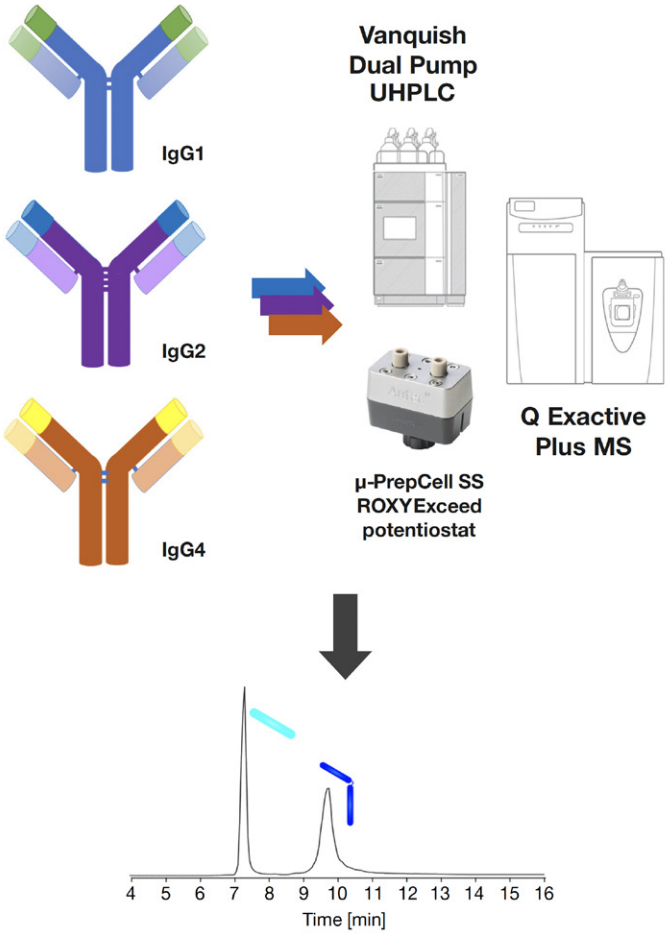


Figure 1. Experimental setup used in this study for the analysis of reduced mAbs. Different mAbs subclasses were tested and analyzed on the platform constituted by Antec Scientific ROXY Exceed potentiostat equipped with a µ-PrepCell SS, Vanquish Dual Pump UHPLC, and Q Exactive Plus MS.

Experimental

Recommended consumables

Ultrapure water, 18.2 MΩ·cm resistivity water, Optima™ LC/MS grade, Fisher Chemical™	P/N 10505904
Acetonitrile with 0.1% formic acid (v/v), Optima™ LC/MS grade, Fisher Chemical™	P/N 10118464
LC-MS grade Formic acid (>99%, Thermo Scientific™ Pierce™)	P/N 28905
Thermo Scientific™ MAbPac™ Reversed Phase HPLC column, 2.1 mm × 50 mm	P/N 088648
Thermo Scientific™ MAbPac™ Reversed Phase HPLC column, 2.1 mm × 100 mm	P/N 088647
Thermo Scientific™ Virtuoso™ vial, clear 2 mL kit with septa and cap	P/N 60180-VT405
Thermo Scientific™ Virtuoso™ vial identification system	P/N 60180-VT100

Sample handling equipment

Thermo Scientific Vanquish Dual Pump UHPLC system consisting of:

Thermo Scientific™ Vanquish™ System Base	P/N VF-S01-A-02
Thermo Scientific™ Vanquish™ Dual Pump F	P/N VH-P32-A-02
Thermo Scientific™ Vanquish™ Split Sampler HT	P/N VH-A10-A-02
Thermo Scientific™ Vanquish™ Column Compartment	P/N VH-C10-A-02
Thermo Scientific™ Vanquish™ Switching Valve 2-P/6-P	P/N 6036.2520
Thermo Scientific™ Viper™ MS Connection Kit for Vanquish LC Systems	P/N 6720.0405
Q Exactive Plus Hybrid Quadrupole-Orbitrap Mass Spectrometer	P/N IQLAAEGAA-PFALGMBDK
Antec Scientific ROXY Exceed SCC Potentiostat	P/N 211.0035
Antec Scientific µ-PrepCell SS reactor cell	P/N 204.4304

Software packages

- Thermo Scientific™ BioPharma Finder™ 4.1 software
- Thermo Scientific™ Chromeleon™ Enterprise CDS 7.2.10
- ROXY Exceed driver for Chromeleon CDS

Sample preparation

Antibody preparation: Denosumab (IgG2), rituximab (IgG1), and nivolumab (IgG4) were commercially sourced. Drug products were buffer exchanged into 1% formic acid (FA) using centrifuge 10 kDa molecular weight cut-off filters (Merck-Millipore). The final concentration was adjusted to contain 20% acetonitrile 1% FA (v/v) at a mAb concentration of 1 mg/mL for analysis.

IdeS digestion product reduction: 100 µg of cetuximab was digested with 100 units of FabRICATOR™ (Genovis AB) for 2 hours at 37 °C. Following digestion, samples were reduced to dryness via vacuum centrifugation before being resuspended in 1% FA, 20% ACN in water.

Electrochemical cell

A µ-PrepCell SS reactor cell in combination with the ROXY Exceed potentiostat was used for the online electrochemical reduction. The potentiostat with integrated oven compartment was operated by instrument control in Chromeleon CDS 7.2.10. The temperature was held at 60 °C. The ROXY EC was connected to a Vanquish Dual Pump UHPLC with the pump module constituted by two independent pumps (Pump 1 and Pump 2). Solvent flow was fed directly from the autosampler switching valve through the electrochemical cell, where reduction occurred, and onto the trapping column, a MAbPac RP column (2.1 × 50 mm). For this step, Pump 1 was kept at 0.05 mL/min and used 100% buffer A1 (20% acetonitrile, 1% FA). The electrochemical cell was operated in a pulse mode using a 2-step square-wave pulse with the following settings: E1 = 1 V (reduction), E2 = 0 V, with a t1 = 1 s and t2 = 0.1 s. E2 was a short cleaning step with a duration of 100 ms. For each injection 1 µL of sample was analyzed.

LC-MS conditions

At 2 minutes, valve switching brings sample on the analytical column. For mAb subunits separation, a MAbPac RP column (2.1 × 100 mm) was employed. Separation occurred with a linear gradient (Table 1) using buffer A2 (0.1% formic acid in water) and buffer B2 (0.1% formic acid in acetonitrile).

MS detection and analysis of the separated subunits was performed on a Q Exactive Plus Hybrid Quadrupole-Orbitrap mass spectrometer. The mass spectrometer was operated in positive ion mode at a spray voltage of 3.8 kV and capillary temperature of 320 °C. MS spectra were collected in the range of *m/z* 600–5,000.

Table 1. Analytical gradient conditions for pump 2. During the first 2 minutes before the valve switches to position 1:2, pump 1 delivers sample through the µ-PrepCell SS and into the trap column.

Time (min)	Flow (mL min^{-1})	% Mobile phase B2 (100% ACN, 0.1% FA)
0	0.3	25.0
2	Switching valve setup from 1:6 to 1:2 position	
3.5	0.3	25.0
3.6	0.3	30.0
13.5	0.3	40.0
13.6	0.3	80.0
18.6	0.3	80.0
19.6	0.3	80.0
21.5	0.3	25.0
23.5	0.3	25.0

Table 2. MS source and analyzer conditions

MS source parameters	Setting
Source	Thermo Scientific™ Ion Max™ source with HESI II probe
Sheath gas pressure	25 arbitrary units
Auxiliary gas flow	10 arbitrary units
Probe heater temperature	150 °C
Source voltage	3.8 kV
Capillary temperature	320 °C
S-lens RF level	60

Table 3. MS method parameters utilized for middle up subunit analysis

General setting		
Runtime	0 to 30 min	
Polarity	Positive	
	Full MS parameters (Lc, IdeS subunits)	Full MS parameters (Hc)
Mass range	600–5,000 *m/z*	600–5,000 *m/z*
Resolution	140,000	35,000
ACG target value	3.0×10^6	3.0×10^6
Max. injection time	100 ms	100 ms
In-source CID	0 eV	0 eV
Microscans	5	5
Intact protein mode	On	On

MS data processing

BioPharma Finder 4.1 software was used for all data processing. The isotopically resolved Lc, Fd, and scFc were analyzed using the Xtract algorithm with a mass range between 20 and 30 kDa. The isotopically unresolved Hc was analyzed using the ReSpect™ algorithm with a mass range between 40 and 60 kDa, charge state range between 10 and 100, and minimum adjacent charge between 6 and 10. Average over selected time range tool was used throughout.

Results and discussion

Optimized workflow for the analysis of different mAb classes

Inline electrochemical reduction and subunit EC-LC-MS analysis has been successfully applied using a ROXY EC system coupled with a Vanquish Dual Pump UHPLC and a Q Exactive Plus MS system (Figure 2). The workflow required a total run time of 30 minutes and was tested on NIST mAb.[1]

For the reduction step, at 1 V, 60 °C, 20% acetonitrile, and 1% FA content, products are mainly converted to fully reduced Lc and Hc. Compared to the previous setup, the analytical run was further optimized by shortening the column loading and backflush times. The column loading time was decreased to 2 min and the back flush time was decreased to 90 s. The following TIC (total ion chromatogram) was produced for NIST mAb sample using a 3 min, 30 s trap column loading/elution time followed by standard chromatography separation (Figure 3). The optimized workflow was also tested on mAbs belonging to different subclasses, such as rituximab (IgG1), denosumab (IgG2), and nivolumab (IgG4) (Figure 4). In all cases, complete reduction was observed, confirming the suitability of the workflow also for samples presenting different disulfide bond organization, including IgG2, which contains an extra bond in the hinge region and IgG4 where the hinge region characteristics allow extra freedom to the tertiary structure.

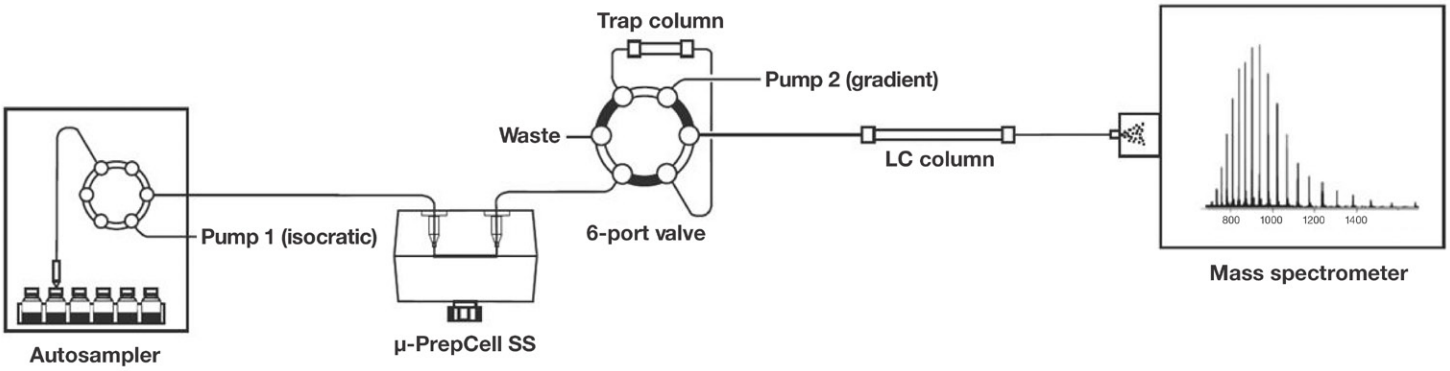


Figure 2. Schematic of the system used for the EC-LC-MS workflow. Sample is injected from the autosampler and introduced into the µ-PrepCell SS electrochemical flow cell using Pump 1 of a Vanquish Dual Pump UHPLC; samples are trapped prior reversed phase separation and MS analysis. After trapping, Pump 2 moves reduced samples through the analytical column hyphenated with a Q Exactive Plus mass spectrometer.

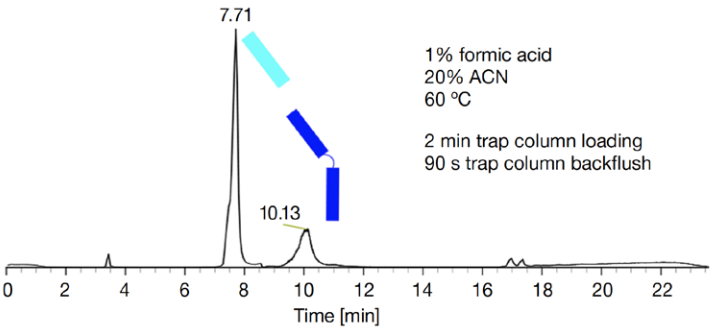


Figure 3. Optimized experimental conditions for EC-LC-MS analysis on NIST mAb

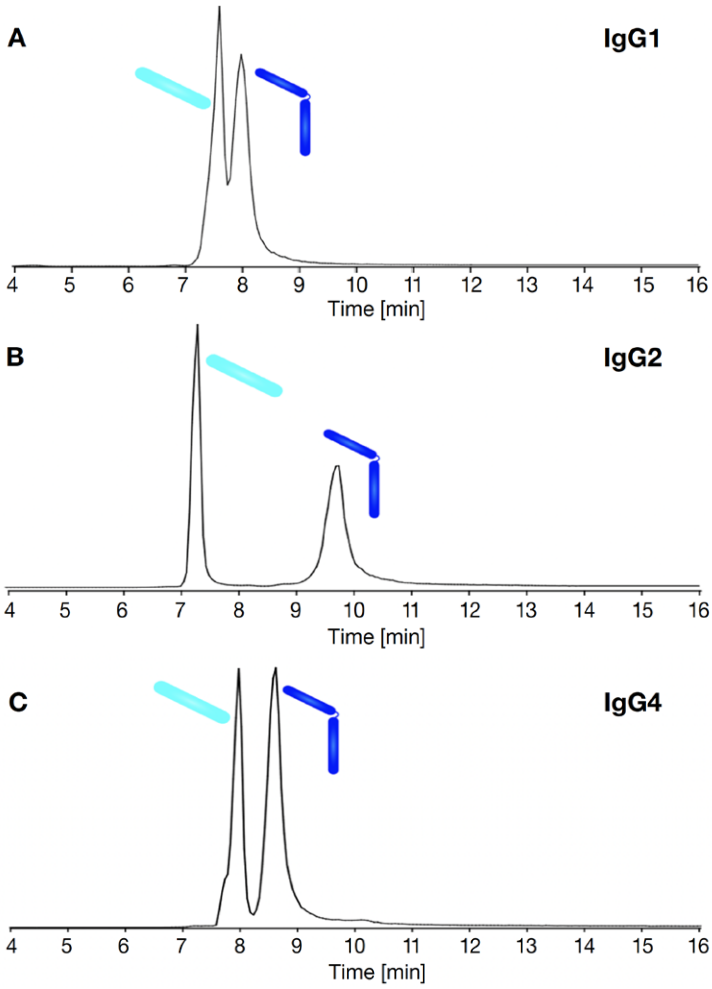


Figure 4. EC-LC-MS analysis of drug products from different classes. (A) Rituximab DP, (B) denosumab DP, and (C) nivolumab DP were chosen to represent monoclonal antibodies belonging to IgG1, IgG2, and IgG4 subclasses, respectively.

EC reduction coupled to IdeS digestion

IdeS digestion is an easy tool to obtain mAb subunits. As the enzyme is cleaving below the mAb's hinge region, the subunits resulting from the enzymatic treatment are 2×scFc and the Fab region constituted by 2×Fd and 2×LC held together by disulfide bonds. A reduction step is necessary to obtain the three separate subunits, which can be analyzed using similar settings as their masses fall in similar range. Reduction is usually performed with the aid of reducing agents, which are not compatible with MS analysis and require diversion of the upfront unretained peak to waste, or their clearance before analysis, adding multiple steps to the entire workflow. Inline EC reduction allows direct reduction and reversed phase (RP)-MS analysis after enzymatic treatment. Once digestion occurred, the Fab and scFc units were introduced to the same electrochemical conditions of the intact NISTmAb resulting in the formation of completely reduced Fc/2 and Fd as well as majority fully reduced Lc. Figure 5A reports the results obtained for NISTmAb: the glycan structures of Fc/2 were isotopically resolved and closely aligned to their theoretical values (Figure 5B), while the measured isotopic envelope of Fd and the theoretical isotopic envelope for fully reduced Fd were in good agreement (Figure 5C), showing that all the subunits were now present as fully reduced species.

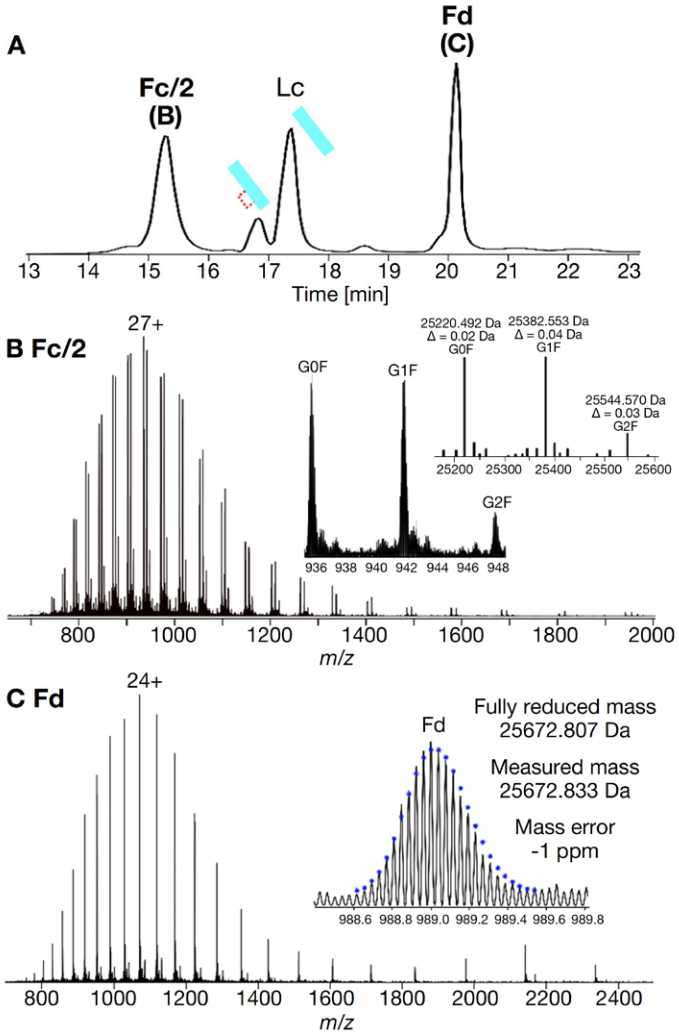


Figure 5. IdeS digested NISTmAb run under electrochemical reduction conditions. (A) TIC of the fully reduced IdeS sample resulting in formation of Fc/2, Lc, and Fd subunits. (B) Mass spectrum of the Fc/2 subunit showing glycan profiles and deconvoluted monoisotopic masses (inset) acquired using the ReSpect algorithm. (C) Mass spectrum of the Fd species; the Fd has been completely reduced with a unimodal charge distribution with a maximum of +24 and an isotopic envelope that closely aligns with the theoretical isotopic envelope.

This workflow was used on cetuximab drug product to obtain site specific *N*-glycan profiles for the Fc and Fd region (Figure 6). Moreover, it is important to demonstrate that the conditions employed by the EC cell do not affect modifications that may be sensitive to acidic conditions and high temperature, such as sialic acid quantitation.

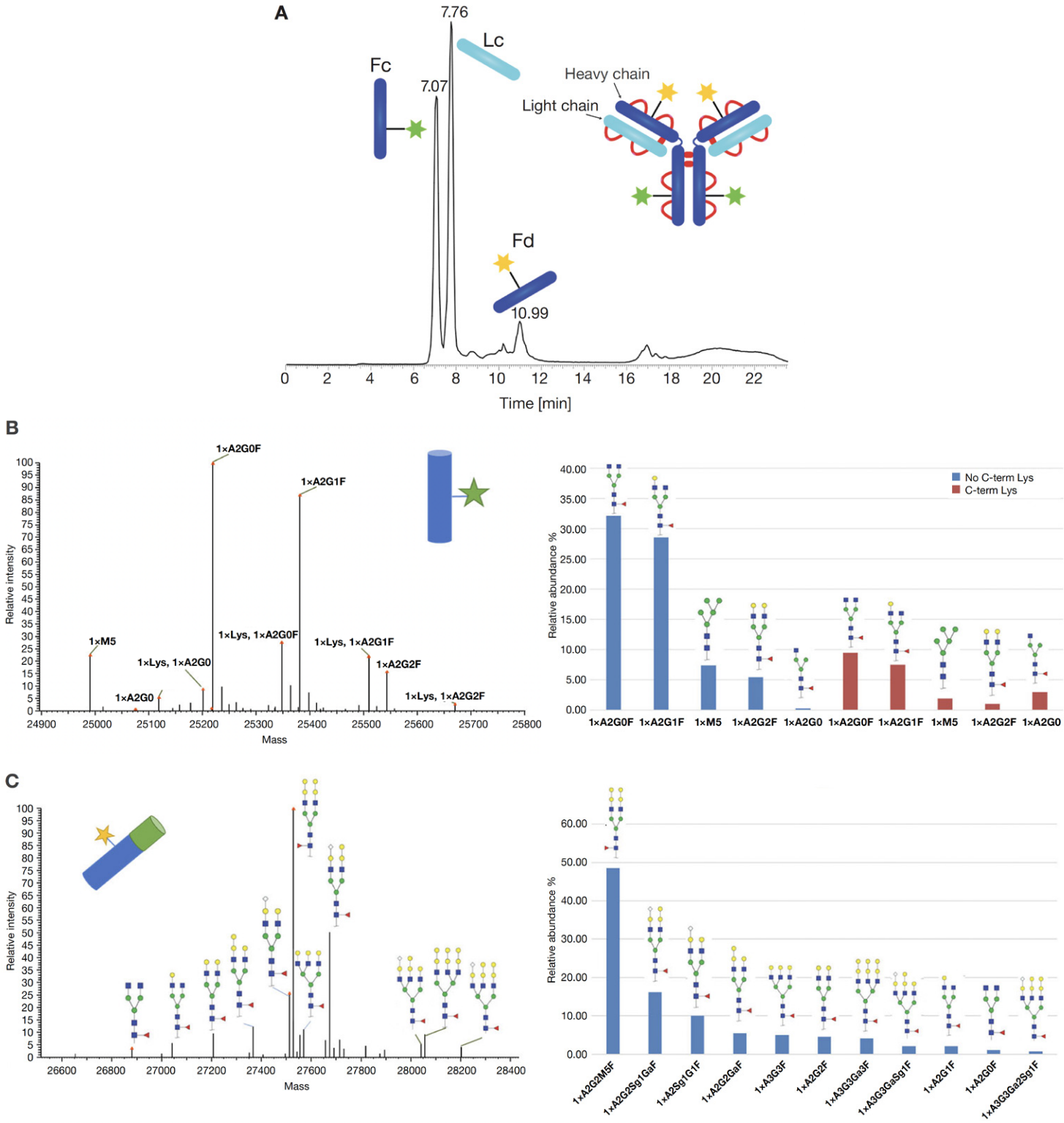


Figure 6. (A) TIC of the EC-LC-MS analysis on IdeS digested cetuximab drug product; *N*-glycosylation was evaluated separately for (B) scFc and (C) Fd regions. In both panels, it is possible to observe the deconvoluted spectrum obtained (left) and the quantitative evaluation of the glycoforms relative abundances (right).

Moreover, cetuximab drug product is known to be one of the most heterogeneous commercially available IgGs.[2] Figure 6A shows the TIC obtained for EC-LC-MS analysis of the IdeS digested cetuximab drug product, with most of the product being transformed to fully reduced scFc, Lc, and Fd portions. Spectra obtained for each individual region were deconvoluted using BioPharma Finder software. At the subunit level, it was possible to acquire data with higher resolution settings for all mAb portions, thus obtaining excellent resolved data that can simplify the identification of cetuximab variants. In Figure 6B and 6C, it is possible to observe the deconvoluted mass spectra for the two glycosylated regions. Panel B shows the heterogeneity in the Fc region derived not only by the presence of *N*-glycans modification but also the presence of *N*-terminus clipping variants. Panel C shows the results of the deconvoluted spectrum for the peak eluting at 10.99 minutes. Heterogeneity in this portion derives from an *N*-glycan modification on Asn88, characterized by high abundance of glycoforms presenting Gal-Gal motifs and *N*-glycolylneuraminic acid (Neu5G or Sg). Sialic acid is known to be labile in harsh acidic conditions, and for this reason it is important to control the suitability of our workflow for its quantitation. Plotting of the intensities obtained for each glycoform returned relative abundance values that are comparable with those present in the literature,[3] concluding the EC-LC-MS workflow does not affect sialic acid content and can be universally used for producing reduced monoclonal antibody subunits ready for LC-MS analysis.

Conclusion

- Complete reduction of an antibody can be carried out in line with the addition of an electrochemical cell into the chromatographic flow path of the intact mAb using an optimized workflow of 23.5 minutes. The method is also suitable for IdeS digested mAbs.
- The workflow presented herein was demonstrated to be effective on different subclasses of antibodies, in particular IgG1, IgG2, and IgG4 were tested in this study.
- The EC-LC-MS workflow is not affecting quantitation of critical features in monoclonal antibodies, such as *N*-terminal lysine clipping and *N*-glycan profiling, including sialylation levels.

References

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