Application note | 000462

Mass spectrometry

Antibody subunit analysis via inline electrochemical reduction of an intact antibody using the ROXY potentiostat coupled to a Vanquish UHPLC – Q Exactive Plus MS system

Authors

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Keywords

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Application benefits

- Middle-up subunit analysis by electrochemical reduction coupled to HRAM LC-MS can be carried out from intact antibodies without the need for enzymatic digestion, specific reducing agents, or specific denaturing agents.
- The solvent switching step allows identical separation methods already used in the laboratory with only the addition of the electrochemical cell and the trap/release switching step.
- HRAM LC-MS analysis of the subunits produced from inline electrochemical reduction confirming complete reduction has occurred without the need for further analysis.

Goal

To reduce the *inter-* and *intra*chain disulfide bonds of a monoclonal antibody (mAb) with an inline electrochemical reactor cell coupled to a liquid chromatography-mass spectrometry (LC-MS) system. Addition of a reduced product trap-release column setup allowed the analytical separation and mass spectrometry analysis to be unmodified. The study demonstrates the complete reduction of an intact NISTmAb to the corresponding light and heavy chain (Lc, and Hc) subunits, as well as coupling to IdeS enzymes to produce the variable portion and crystallizable portion of the heavy chain (Fd, and Fc/2) as well as Lc.

Introduction

Heterogeneity of monoclonal antibodies (mAbs) introduced in the form of post-translational modifications (PTMs) from expression and process related stress means analytical methods are being continuously developed for their characterization. Methods for mAb analysis are commonly subdivided into native, intact, subunit, top- and middle down, and bottom-up workflows, and all aim at characterizing PTMs and other protein features, as required by The International Conference on Harmonisation (ICH) guideline Q6B, to guarantee product safety and efficacy.[1,2]

Most analytical techniques for mAbs involve the reduction and digestion of the intact protein into smaller subunits,[3,4] compared to intact mass analysis. This has the power to improve site-specific analysis of the PTMs and to overcome some technical difficulties, such as the acquisition of high-resolution MS data on the intact level. The reduction of *inter*chain disulfide bonds chemically with dithiothreitol (DTT), tris(2-carboxyethyl)phosphine produces Lc and Hc units, further coupling reduction to IdeS or IdeZ enzymes produces Lc, Fc/2, and Fd subunits. Denaturing agents such as guanidine hydrochloride are often used to relax the tertiary structure of the antibody to allow for more complete reduction. Addition of enzymes and chemical reducing agents requires time and further processing steps and removes the ability to analyze the intact sample *in situ.*

Electrochemical (EC) reduction of disulfide bonds in proteins using a flow-through reactor cell prior to MS has been successfully applied as an alternative method previously.[5-10] Disulfide bond cleavage in such an approach is achieved by inline electrochemical reduction using a titanium working electrode under acidic conditions with formic acid as an additive in the sample solution. Accessible disulfide bonds present during the reduction will be reduced to free thiol groups. Complete and partial electrochemical reduction can be achieved based on the total level of denaturation of the protein.

Full instrument control of the ROXY™ Exceed potentiostat and µ-PrepCell™ SS reactor cell (Antec Scientific, Zoeterwoude, Netherlands) in the Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) allowed for fast and easy creation of integrated methods for EC reduction within the automated LC-MS workflow for antibody subunit analysis.

In the following contribution, an inline electrochemical reduction workflow that reduces both *inter-* and *intra-* disulfide bonds of NISTmAb is presented. Full results and further analysis are presented in the paper[10] published in Analyst.

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)
- Formic acid, LC-MS grade, >99%, Pierce (P/N 28905)
- NISTmAb Reference Material 8671, acquired from The National Institute of Standards and Testing (NIST, Gaithersburg, Maryland)
- FabRICATOR™ (Genovis AB)
- Thermo Scientific™ MAbPac™ RP column, 2.1 mm × 50 mm (P/N 088648)
- Thermo Scientific™ MAbPac™ RP 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)

Sample handling equipment

- Thermo Scientific™ Vanquish™ Flex Duo UHPLC system consisting of:
  - Thermo Scientific™ Vanquish™ System Base Horizon/Flex (P/N VF-P32-A-02)
  - Thermo Scientific™ Vanquish™ Dual Pump F (P/N VH-P32-A-01)
  - 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™ Viper™ MS Connection Kit for Vanquish™ LC System (P/N 6720.0405)
- Thermo Scientific™ Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer (P/N IQLAAEGAAPFALGMBDK)
- Antec Scientific ROXY Exceed SCC potentiostat (P/N 211.0035)
- Antec Scientific µ-PrepCell SS reactor cell (P/N 204.4304)

Software

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

Sample preparation

Antibody preparation: NISTmAb Reference Material 8671 was buffer exchanged into 1% formic acid (FA) and ultrapure water using centrifuge 10 kDa molecular weight cutoff filters (BioRad, Hercules, CA, USA). The final concentration of NISTmAb was adjusted to 1 mg mL^{-1} for analysis. Experiments carried out under denaturing conditions were buffer exchanged into 1% FA and concentration adjusted to 20% acetonitrile 1% FA (v/v) at a concentration of 1 mg mL^{-1} of NISTmAb. For IdeS digestion product reduction: 100 µg of NISTmAb was digested with 100 units of FabRICATOR 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 of NISTmAb. The potentiostat with integrated oven compartment was operated by instrument control in the Chromeleon Enterprise CDS 7.2.10. The µ-PrepCell SS is a dual electrode flow-through cell consisting of a replaceable platinum counter electrode (CE) and the titanium surface of the inlet block acting as the working electrode (WE) at which reduction takes place. A schematic drawing of the cell is shown in Figure 1. PEEK tubing was used to connect the electrochemical cell into the flow path. The flow rate through the electrochemical cell was held at 50 µL min^{-1} for the duration of the experiment.

The ROXY Exceed setup gives both temperature and electrode potential control within the EC cell using Chromeleon CDS. The temperature was held at room temperature for the partially reduced experiments and 60 °C for the fully reduced experiments. Solvent flow was fed directly from the autosampler switching valve through the electrochemical cell, where reduction occurred, and onto the trapping column. The electrochemical cell was operated in a pulse mode using a 2-step square-wave pulse with the following settings: E_1 = 0–1 V (reduction), E_2 = 0 V, with a t_1 = 1 s and t_2 = 0.1 s. E_2 is a short cleaning step with a duration of 100 ms. The electrode potential, (E_1) was varied between 0 V to 1 V for comparison of reduction conditions in 0.2 V increments. All the experiments comparing reduction at different temperatures and organic solvent content are performed using a potential setting of E_1 = 1 V.

LC-MS conditions

Optimized for maximum reduction LC-MS conditions: samples were dissolved in 1% formic acid, 20% acetonitrile at a concentration of 1 mg mL^{-1}. 1 µL of sample was injected onto the µ-Prepcell with products trapped with a MAbPac column (50 mm length), after column switching samples were washed in backflush onto a MAbPac column (100 mm length). Separation occurred with a linear gradient (Table 1) using buffer A (0.1% formic acid in water and buffer B 0.1% formic acid in acetonitrile.

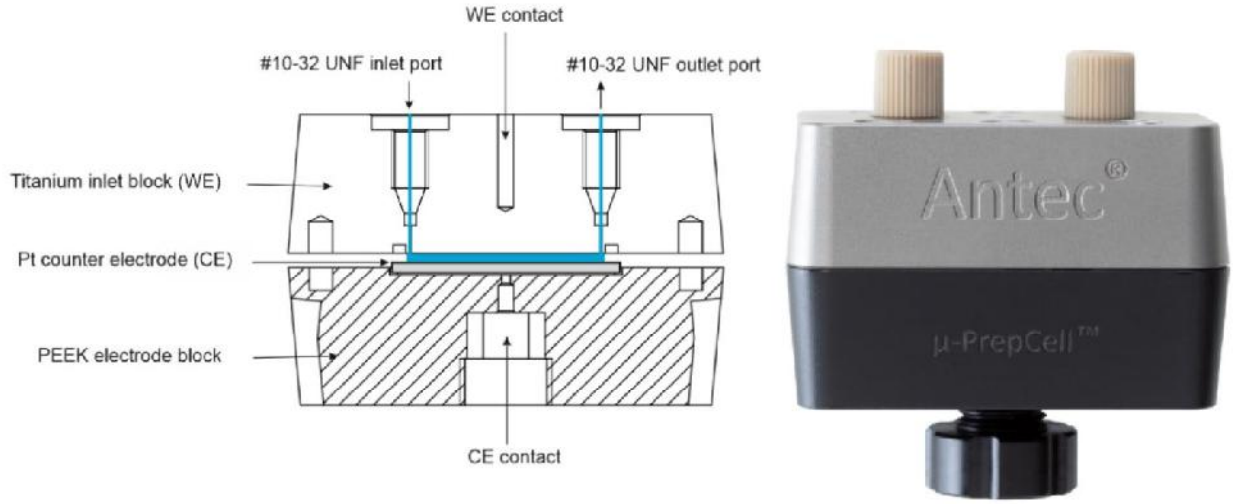

Figure 1. Antec Scientific dual-electrode µ-PrepCell-SS consisting of a titanium inlet block acting as a working electrode and platinum counter electrode. 1/16˝ inlet and outlet ports allow the flow of mobile phase and sample solution along the electrodes of the thin layer flow cell (area marked in blue).

Table 1. Chromatographic separation - LC gradient conditions – optimized switching and backflush times

Time (min)	Flow (mL/min)	% Mobile phase B (100% ACN, 0.1% FA)
0	0.3	25.0
5	Switching valve setup from 6:1 to 1:2 position	
10	0.3	25.0
10.1	0.3	30.0
20	0.3	40.0
20.1	0.3	80.0
25.1	0.3	80.0
26.1	0.3	80.0
28.0	0.3	20.0
30	0.3	25.0

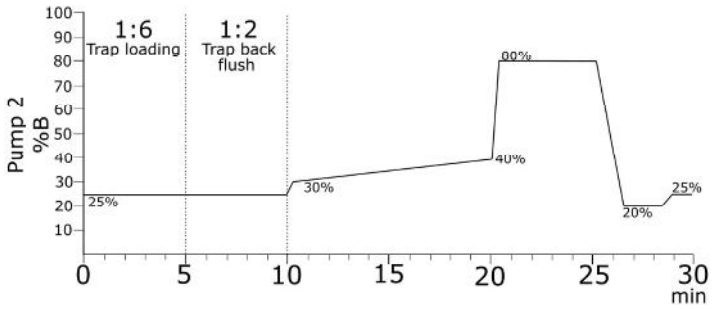

Figure 2. %B on the analytical pump (Pump 2) with switching times of solvent switching valve

LC-MS analysis of the reduced sample was acquired on a Vanquish Flex Duo UHPLC system coupled to 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. MS1 spectra were collected with the mass range set to *m/z* 600–5,000.

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

MS data processing

BioPharma Finder software 4.1 was used for all data processing. The isotopically resolved MS spectrum obtained from the Lc was analyzed using the Xtract algorithm, and the isotopically unresolved MS spectrum obtained from the Hc was analyzed using the ReSpect™ algorithm.

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

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

Results and discussion

The ROXY Exceed potentiostat with µ-PrepCell SS was operated in pulse mode. A dual potential wave form with the following settings was applied on the cell: E_1 = 0 – 1 V, E_2 = 0 V, with a t_1 = 1.0 s and t_2 = 0.1 s. Cell potential E_1 facilitates the electrochemical reduction of the *inter-* and *intra*chain disulfide bonds at the titanium electrode. E_2 is a short cleaning step with a duration of 100 ms. Full discussion of the reduction of antibodies via electrochemistry is presented in the publication.[10]

Reducing *inter*chain disulfide bonds fragments Abs into smaller light chain (Lc) and heavy chain (Hc) subunits. Separate analysis of Lc and Hc provided accurate glycoform and potential modification information. *Intra*chain disulfide bond reduction relaxes the 3D structure of proteins, increasing their overall charge state via electrospray analysis as well as addition of hydrogen with the reduction of each disulfide bond.

An inline electrochemical reduction system (Figure 4) was applied to NISTmAb, allowing electrochemical reduction of the antibody (Figure 4A–D) followed by trapping (Figure 4E–G) to adjust the solvent from electrochemical compatible system to LC compatible solvent constituents. Analysis was then carried out under standard LC-MS conditions (Figure 4H).

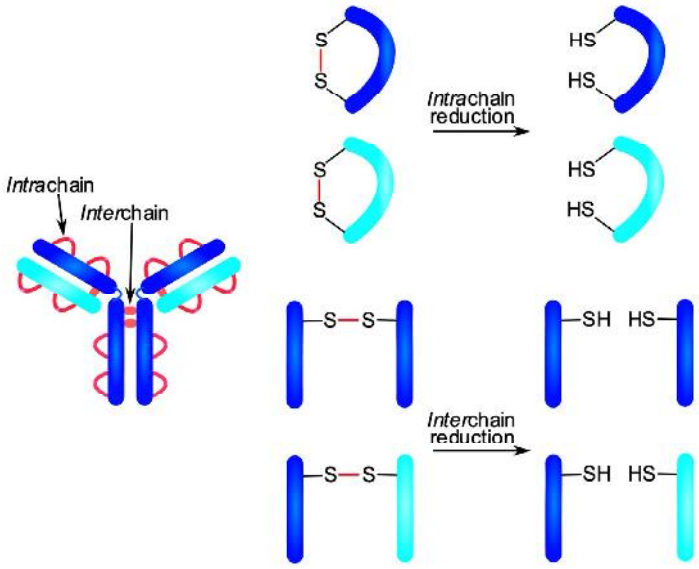

Figure 3. *Inter*chain disulfide bond reduction resulting in a mass increase of 1 Da on each chain, and *intra*chain disulfide bond reduction resulting in a 2 Da mass increase on the same chain. Full analysis and explanation are carried out in the corresponding paper.[10]

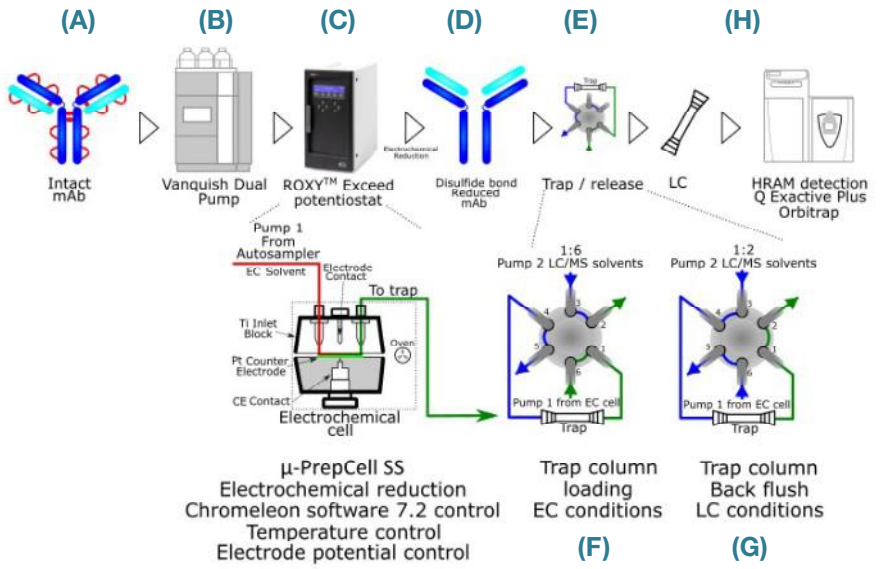

Figure 4. Overview of experimental set up for inline electrochemical reduction of Abs for integrated LC-MS analysis. (A) Intact Ab with all disulfide bonds intact is introduced via a Vanquish Dual Pump system (B). The Vanquish Dual Pump system has two separate flows, an electrochemical cell flow and an LC analysis flow. (C) ROXY Exceed potentiostat, sample, and solvent from Pump 1 of the dual pump system is introduced into the µ-PrepCell SS where reduction occurs. (D) After reduction a reduced Ab is produced, which is loaded onto a switching valve setup (E). The switching valve setup in the 6:1 position (F) traps the now reduced Ab onto a short trapping column, before switching positions (G) to be fed from the second LC pump, backflushing the reduced Ab off the trapping column with LC-MS compatible solvents onto the HRAM LC-MS system (H).

Inline integration of the electrochemical cell was controlled through Chromeleon software 7.2. The cell is operated in "Pulse" mode, inducing analyte reduction with an oxidative cleaning step. The cell can be controlled through the ROXY Exceed cell ePanel, where temperature control as well as on/off and mode commands can be selected.

Figure 5. Roxy Exceed cell ePanel highlight showing pulse and on/off commands

Multiple cell on/off commands may be programmed in the script method editor, as well as temperature and potential changes at multiple steps within the run. The cell can be run until the column switching occurs and can then be turned off. Adjustment of the cell temperature is not recommended during analysis.

Table 4. Three script lines which are the three most adjusted experimental parameters for the functioning of the ROXY potentiostat

Script line	Parameter
Decade.Oven.Nominal	60 [°C]
Decade.Decade_Cell1.Potential	1.00 [V]
Decade.Decade_Cell1.Cell_On	Off

The data acquired from the ROXY potentiostat is the current induced through the reactor cell during the electrochemical reduction process. A higher cell potential (E1) caused a larger current flowing through the electrode, indicating a greater number of reactions taking place due to the supply of a larger amount of activation energy. The cell current measured with the potentiostat can be recorded in Chromeleon CDS. The current signals can be monitored in Chromeleon CDS and used for confirmation of current flow and sample electrochemical reduction through the analysis.

The electrochemical reduction has both a thermodynamic and kinetic component to the reaction. The thermodynamic component of the reaction is driven by the instability of the disulfide bond under reducing conditions. The kinetic component is the ability for the disulfide bonds to interact with the electrode surface and charge carriers to induce reduction. Increasing the electrode potential increases the energetic favorability of reduction occurring until a kinetic limit may be reached. From previous studies, the reduction of *inter*chain disulfide bonds more easily occurs due their relative exposed position compared to *intra*chain disulfide bonds. The effect of applied cell potential on reduction is further discussed in the paper.[10] The chosen E_1 of 1 V was the optimum setting that produced the greatest reduction effect. Higher cell potentials led to electrolysis of the water (gas formation) in the acidic aqueous mobile phase, which was detrimental for the efficiency of the EC reduction.

Two key metrics were used to assess total levels of reduction by mass spectrometry:

- Exact mass measurement and isotopic envelope comparisons: The exact mass of the formed products deviated by 1 Da for every *inter*chain disulfide bond reduced and 2 Da for every *intra*chain disulfide bond reduced. The isotopic envelope shifted with the increase in mass and allowed direct comparison between theoretical isotopic envelope spectra.

- Shift of charge state distribution: The charge state distribution of a protein will shift to higher charge state, and therefore lower *m/z*, if the 3D structure of the protein has been relaxed. The relaxation of 3D structure increases the surface area of the protein, increasing the number of charges per protein during ionization.

Reduction of the NISTmAb could be increased by increasing the denaturing ability of the electrochemical system. Increasing the temperature inside the electrochemical cell resulted in the formation of both a partially reduced Lc as well as completely reduced Lc species (Figure 6B). A similar result was achieved by increasing the organic content of the EC reaction solvent (Figure 6C). At 1% FA and 20% acetonitrile content, the reduction products were successfully trapped for the column switching steps but also increased the total reduction achieved. Figure 6D presents the majority product being fully reduced Lc and Hc by combining a higher organic content and temperature together.

Figure 6F is a fully reduced Lc with a unimodal charge state distribution with a charge state maximum at +24, and an isotopic envelope that closely aligns with the theoretical isotope distribution for a fully reduced Lc. The Hc mass spectrum is also unimodal with a small increase in charge state maximum from +47 to +51 and the masses aligning with a fully reduced Hc species. The increase in total reduction by increasing the organic content and temperature of the electrochemical cell is evidence for a kinetic barrier being overcome and the reduction process now being able to take place.

Further analysis of the disulfide bond reduction was carried out with tandem mass spectrometry with results included in the final paper.[10]

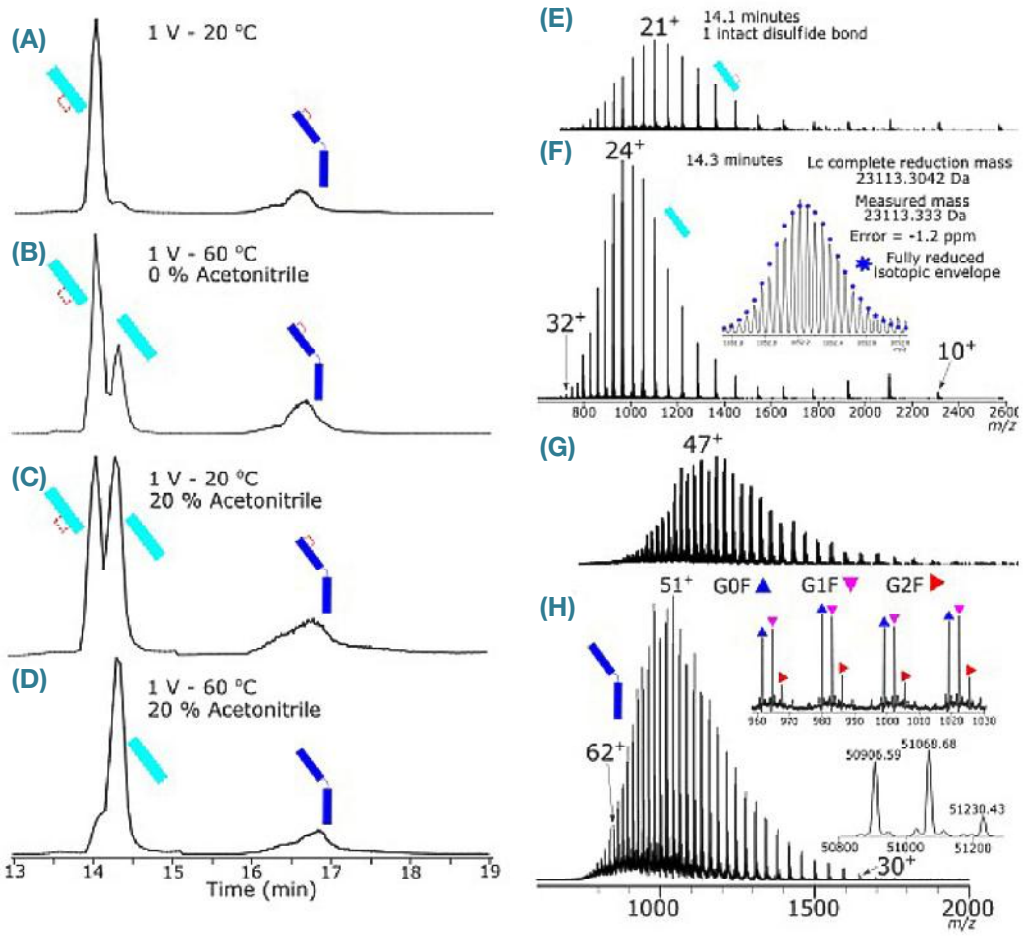

Figure 6. (A) The original partially reduced experiment carried out at room temperature in 1% formic acid and 100% H_2O. (B) By increasing the temperature of the electrochemical cell to 60 °C, the formation of fully reduced Lc was observed. (C) Presence of fully reduced Lc at 20 °C and 20% acetonitrile increased the fully reduced product. By combining both (D), the two major products are fully reduced Lc and Hc. (E) and (F) show the comparison of partially and fully reduced Lc, respectively. The isotope distribution aligns closely to that of a fully reduced theoretical distribution. The heavy chain has shifted to a higher charge state and masses align to that of fully reduced species.

Conclusions

Electrochemical reduction of both the *intra*- and *inter*chain disulfide bonds of NISTmAb could be carried out using electrochemical reduction inline with an LC-MS system. Increasing the electrochemical potential of the electrochemical cell resulted in more complete disulfide bond reduction. Tertiary structure of the NISTmAb was shown to reduce electrochemical efficiency, but denaturing the antibody increased the total reduction. The LC-MS system required no modification to the separation and mass spectrometry methods other than the introduction of the electrochemical cell.

- Reduction of an antibody can be carried out inline with the addition of an electrochemical cell into the path reducing the intact mAb.
- The developed workflow reduces an antibody down to light chain and heavy chain subunits without the need for addition of enzymes or specific denaturing agents.
- The electrochemical reduction workflow can be used for the analysis of previously digested fabricator samples as well as intact antibody species.
- Selectivity of the disulfide bond electrochemical reduction by tandem MS could yield useful information about the formation of non-uniform disulfide bonding structures within antibodies.[10]

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