Online Electrochemical Reduction of mAbs for Rapid LC-MS Analysis

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Objective

Use of online electrochemical (EC) reduction to allow rapid LC-MS analysis of mAbs

- Complete reduction of all inter- and intrachain disulfide bonds
- Instant inline reduction to enable fast LC-MS
- Gentle reduction without reducing (TCEP, DDT) or denaturing agents (urea)
- Minimal sample handling to preserve the mAbs structure including potential heterogeneities arising from variation in the disulfide bonding

1. Introduction

The successful reduction of inter- and intrachain disulfide bonds of various monoclonal antibodies (mAbs) with an inline electrochemical flow cell coupled to an LC-MS system is presented. The addition of a trap/release column in the chromatographic set-up allowed the analytical separation and mass spectrometry analysis to be unmodified with run times of only 23 min.
The study demonstrates the complete reduction of intact mAbs to the corresponding light and heavy chain (Lc, and Hc) subunits. Middle-up subunit analysis by electrochemical reduction coupled to HRAM LC-MS can be carried out from intact antibodies without the need of modification in the chromatographic analysis [1].

2. Disulfide Bond Reduction in mAb

In Figure 1 the schematics of S-S bond reduction in a mAb (IgG1) is shown

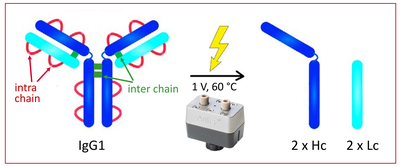

*Figure 1: Complete reduction of the 4 **inter chain** S-S bonds results in the formation of 2 Hc and 2 Lc chains with an increase of +3 Da for the Hc and +1 Da for the Lc chain. Each **intra chain** S-S bond reduction results in a +2 Da mass increase on the same chain, i.e., +4 Da for Lc and +8 Da for the Hc.*

3. Instrumentation

A µ-PrepCell SS in combination with the ROXY Exceed Potentiostat (Antec Scientific, The Netherlands), was used for the online electrochemical reduction of the mAbs, controlled by Chromeleon Enterprise CDS 7.2.10. software (Thermo Scientific).
The temperature of the EC cell was held at room temperature for partially reduced and at 60 °C for the fully reduced experiments. 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 onto a MAbPac *column (100 mm length*). Separation occurred with a linear gradient using buffer A (0.1% formic acid in water) and buffer B (0.1% formic acid in acetonitrile) see Figure 3.

LC-MS analysis was acquired on a Vanquish Flex Duo UHPLC system coupled to a Q Exactive Plus Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Scientific). A schematics of the instrumental set-up is shown in Figure 2.

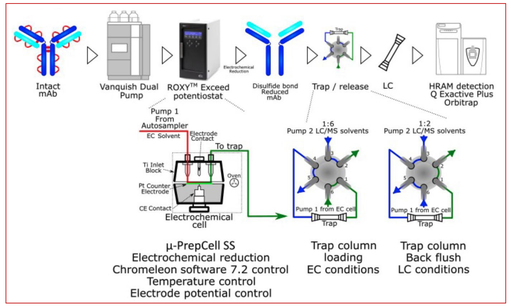

Figure 2: Schematics for inline EC reduction of Abs for LC-MS analysis. (A) Intact Ab with all disulfide bonds is introduced via a Vanquish Dual Pump system (B). The Dual Pump has two separate flows, an electrochemical cell flow and an LC analysis flow. (C) ROXY Exceed potentiostat, sample, and solvent from Pump 1 is introduced into the µ-PrepCell SS for reduction. (D) After reduction the reduced Ab is loaded onto a switching valve setup (E). The switching valve setup in the 6:1 position (F) traps the reduced Ab onto a short trapping column, before switching positions (G) to be eluted via LC pump 2 in backflush with LC-MS compatible solvents onto the HRAM LC-MS system (H)

4. LC-MS Method

In Figure 3 the gradient profile is displayed for the entire LC-MS analysis including the EC reduction, loading and backflush of the trap column and the chromatographic run. In Table 1 the applied MS conditions are listed.

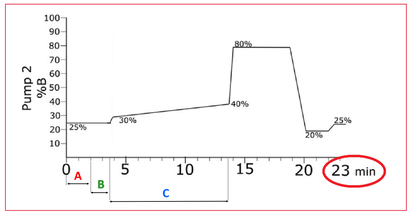

Figure 3: Gradient profile with indication of:
A) 2 min EC reduction and trap loading
B) 1.5 min backflush and
C) chromatographic run

MS source parameters	Settings
Source	Thermo Scientific™ Ion Max, 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 1: MS source and analyzer conditions

5. Reduction on Interchain S-S Bond

In Figure 4 the partial and full reduction of the NIST mAb is shown. By increasing the temperature in the µ-PrepCell to 60 °C, and by increasing the organic content to 20% acetonitrile resulted in complete intrachain disulfide bond reduction of both, heavy and light chain. This S-S bond reduction was achieved without the need for addition of chaotropes.

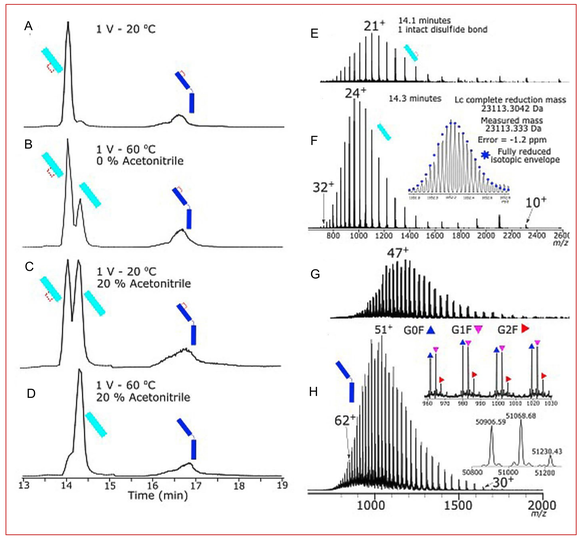

Figure 4:
(A) Partially reduced experiment at room temperature in 1% formic acid and 100% H2O.
(B) By increasing the temperature of the electrochemical cell to 60 °C, the formation of fully reduced Lc was observed.
(C) Presence of fully reduced Lc at 20 °C and 20% acetonitrile increased the fully reduced product.
(D) By combining both (60 °C and 20% ACN in 1.0 FA) fully reduced Lc and Hc are obtained.
(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.
(G) Shows the partially reduced Hc distribution.
(H) Fully reduced Hc showing mass agreement with the fully reduced species of the three main glycoforms, as well as significant charge state shift of the fully reduced Hc.
The heavy chain has shifted to a higher charge state and masses align to that of fully reduced species.

6. Reduction of Different mAbs

To validate the method, different mAbs were analyzed using the described instrumental set-up and conditions. Each antibodies was fully reduced within an total LC-MS run time of 23 min.

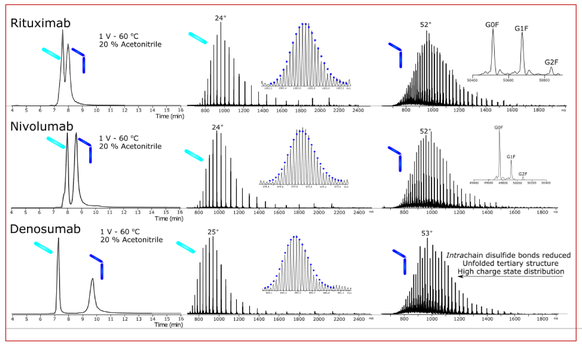

Figure 2: Complete electrochemical reduction of inter- and intrachain disulfide bonds in Rituximab, Nivolumab, and Denosumab by in-line EC-LC-MS

7. Conclusions

Electrochemical reduction of both intra- and interchain disulfide bonds in different mAbs could be performed using electrochemical reduction inline with an HRAM LC-MS system. At a potential of 1 V, at 60°C, complete reduction is achieved.

- The LC-MS system required no modification of the chromatographic or MS methods other than the introduction of the electrochemical cell and the trap/release switching step.
- The developed workflow reduces an antibody down to light and heavy chain subunits without the need of enzymes, denaturing or reducing agents.
- It can be used for the analysis of previously digested fabricator samples as well as intact antibodies.
- Selectivity of the electrochemical S-S bond reduction by tandem MS could yield useful information about the formation of non-uniform disulfide bonding structures within mAbs

References

[1] T.E. Morgan, C. Jakes, H-J. Brouwer, S. Millán-Martín, J-P. Chervet, K. Cook, S. Carillo, J. Bones. Inline electrochemical reduction of NISTmAb for middle-up subunit liquid chromatography-mass spectrometry analysis. *Analyst* **2021**,146, 6547-6555, DOI: 10.1039/d1an01184g
[2] M.M. Vanduijn, H-J. Brouwer, P.S. de la Torre, J-P. Chervet, T.M. Luider. Online Electrochemical Reduction of Both Inter- and Intramolecular Disulfide Bridges in Immunoglobulins. *Analytical Chemistry* **2022**, 94 (7), 3120-3125, DOI: 10.1021/acs.analchem.1c04261