Online Electrochemical Reduction of Inter- and Intrachain Disulfide Bonds in mAbs

Jean-Pierre Chervet[1]; Martijn M. Vanduijn[2]; Theo M. Luider[2]; Hendrik-Jan Brouwer[1]; Martin Eysberg[1]
[1]Antec Scientific, Alphen a/d Rijn, The Netherlands; [2]Erasmus Medical Center, Rotterdam, The Netherlands

Objective

Use of online electrochemical (EC) reduction for S-S bond cleavage in mAbs.

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

1. Introduction

An improved electrochemical method is presented that achieves full reduction of both inter- and intrachain disulfide bonds in a set of monoclonal antibodies based on their intact mass and on MS/MS analysis.
The system uses an electrochemical flow cell positioned online between a Nano LC system and a mass spectrometer to give direct information on pairs of heavy and light chains in an antibody.
The complete reduction of the intrachain disulfide bridges is important as the redox state affects the intact mass of the antibody chain.
Disulfide bonds also hamper MS/MS fragmentation of protein chains and thus limit the confirmation of the amino acid sequence of the protein of interest if not fully reduced.

2. Disulfide Bond Reduction in mAb

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

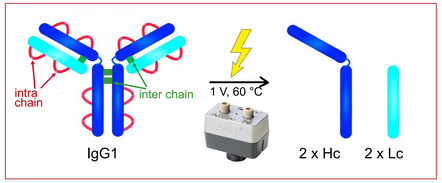

*Figure 1: Complete reduction of the 4 **inter chain** S-S bonds results in the formation of 2 Hc and 2 Lc chains with an increase of +3 Da for the Hc and +1 Da for the LC chain. Each **intra chain** S-S bond reduction results in a +2 Da mass increase on the same chain, i.e., +4 Da for Lc and +8 Da for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

3. Instrumentation

Reductions were performed in an electrochemical flow cell (µPrepCell-SS) controlled by a ROXY Exceed EC system (Antec Scientific, The Netherlands). Chromatographic separations were performed on a Waters BEH Nano LC column (100 mm x 150 µm, C4, 300 Å) on a Dionex Ultimate 3000 chromatography system.
The protein sample was trapped and desalted (Pepmap 0.3mm x 5mm, C4, 300 Å (Thermo Scientific). The gradient was 0.1% formic acid (A) and 0.08% formic acid, 80% acetonitrile in water (B), 4% to 90% (B) in 25min at 1 µL/min.
Post-column, 19 µL/min 1% formic acid, 50% acetonitrile was added as makeup flow, to a total of 20 µL/min. Mass spectra were recorded on an Orbitrap Fusion Lumos (Thermo Fisher Scientific).
A schematics of the instrumental setup is depicted in Figure 2.

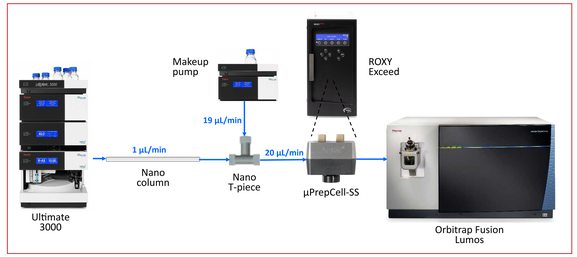

Figure 2: Schematics Nano LC-Electrochemistry-MS for (LC-EC-MS) for online reduction of mAbs. Addition 19 µL/min makeup flow for compatibility with EC flow cell.

4. Reduction of Interchain Disulfide Bridges

The electrochemical reduction of intact mAbs to the Lc and Hc was assessed on a QTOF-MS, as this gave a better signal for the intact IgG. As shown in Figure 3A, the intact mAb starts to elute form the Nano LC column after ca. 15 min.
In Figure 3 B and C, co-eluting heavy (Hc) and light (Lc) chains from the intact bevacizumab sample are shown. The reduction potential of the electrochemical cell was varied in order to assess the optimal settings for cleavage of the chains. Complete reduction was found at 1000 mV, and partial reduction was found at lower potentials, Figure 3D.

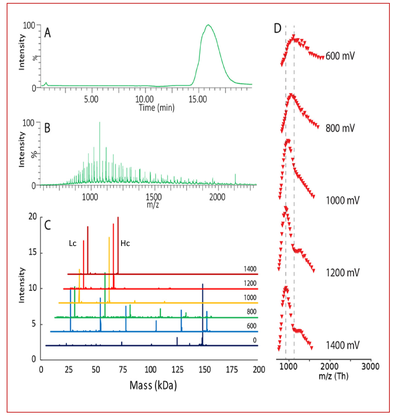

Figure 3: Electrochemical reduction of bevacizumab to cleave heavy and light chains.
***(A)** Representative chromatographic profile (TIC, 800 mV potential).*
***(B)** Q-TOF mass spectrum averaged across the chromatographic peak.*
***(C)** Waterfall plot of deconvoluted mass spectra for different electrochemical potentials*
***(D)** Peak apex from MS1 spectra, associated with the heavy chain by deconvolution analysis. Dotted lines highlight the shift in charge state distribution from 600 to 1400 mV.*

4. Reduction of Intrachain Disulfide Bridges

The detection intrachain S-S bond reduction is more challenging, as the reduction products exhibit only a small mass shift compared to the parent compound. Two reduced cysteine residues with a free −SH group contain two additional hydrogens compared to a S−S bonded cysteine pair, amounting to 2.016 Da mass increase for each reduced cysteine pair.
With two internal disulfide bridges, the maximum expected mass shift is 4.032 Da for the Lc and 8,064 for the Hc (4 bridges).
While this is easily resolved by MS, the isotopic envelopes of the protein signals are wider than that, resulting in overlapping spectra for mixtures of chains with 0, 1, or 2 reduced intramolecular disulfide bonds in case of Lc.
To obtain a more quantitative assessment, we simulated the isotopic envelope of each 19+ redox form of bevacizumab light chain (Xcalibur Qualbrowser, Thermo Fisher Scientific). The fitted spectrum in all cases approximated the experimental data indicating that the EC reduction was capable of producing the fully reduced form of the bevacizumab Lc and Hc without detectable amounts of intact intramolecular disulfide bridges.

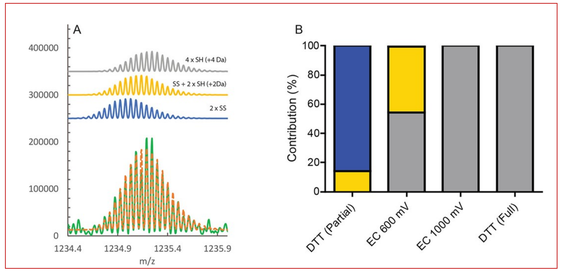

Figure 4: Assessment of intramolecular disulfide bond reduction from MS1 spectra.
*(A) Top panel: simulated spectra for the redox forms of bevacizumab light chain 19+ that were used to fit to the experimental data. **Blue**: both SS intact. **Yellow**: one SS reduced. **Gray**: both SS reduced. Bottom panel: **green**: experimental data; **dashed brown**: best fit (weighted sum of forms shown in the top panel). The analysis is for electrochemical reduction with a 600 mV potential;*
(B) Relative contributions of each redox form in the best fit to the experimental data after chemical and electrochemical reductions (EC). Colors as in panel A. Partial reduction was with DTT under native conditions, and full reduction was under denaturing conditions.

Monoclonal Antibody	Theoretical Mass (Da)	Experimental Mass (Da)	Error (Da)
Bevacizumab	23436.4	23435.5	0.89
Cetuximab	23412.5	23411.6	0.9
Alemtuzumab	23556.7	23556.7	0.03
Denosumab	23472.7	23471.7	0.97
Panitumumab	23343.4	23342.5	0.93
Adalimumab	23397.6	23397.7	-0.1
Pembrolizumab	23729.8	23728.7	1.13
Bevacizumab Fab HC	24614.1	24614.1	-0.04

Table 1: Monoisotopic mass of the Lc of different mAbs after EC reduction at 1000 mV, illustrating complete reduction.
Each closed disulfide bridge would result in a loss of 2 Da in mass. Also included data on the Hc from a Bevacizumab Fab fragment. For more data on Hc see [1].

5. Effect of EC Reduction on MS/MS Fragmentation

Another benefit of EC reduction of intramolecular bonds is the acquisition of better MS/MS data. In particular, incomplete reduction can affect the fragmentation between disulfide bonded cysteine residues. MS/MS data were assessed for a set of 7 mAbs, two are shown in Figure 5 for others see [1]. The 19+ reduced Lc of each mAb was fragmented with higher energy collisional dissociation (HCD). After deconvolution, the fragments were matched to the sequence. A tolerance of 100 ppm was applied.
Most fragments matched the predicted mass by less than 10 ppm. MS/MS analysis was also performed on a bevacizumab heavy chain, confirming that the intramolecular disulfide bridges had been electrochemically reduced.

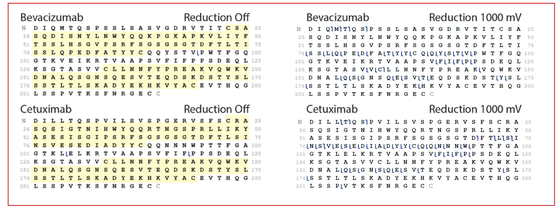

Figure 5: Assessment of intramolecular S-S bond reduction from MS/MS spectra. With EC reduction off and at 1000 mV, data were recorded from 19+ light chain precursors of mAbs fragmented by HCD (NCE 34). The resulting spectra were deconvoluted and matched to the sequence of the chains. Matching b and y ions (100 ppm) were highlighted in the sequence, and yellow boxes show the region between cysteines that form a disulfide bridge in the native mAb.

6. Conclusion

Different mAbs such as Bevacizumab (Roche), Panitumumab (Amgen), Pembrolizumab (Merck), Cetuximab (Eli Lily and Co), Adalimumab (Abbott) and Alemtuzumab (Genzyme) could be fully reduced by electrochemical reduction after chromatography of the intact molecule. The intact masses observed in deconvoluted MS spectra were consistent with reduction of both inter- and intrachain disulfide bridges. Furthermore, the analysis of MS/MS spectra of the light chains confirmed the complete reduction of the interchain disulfide bonds.

- Complete electrochemical reduction of inter- and intrachain disulfide bonds in mAbs
- Confirmed by MS and MS/MS data
- Post-column reduction with make-up flow allows for use of existing chromatographic conditions and optimal selection of reduction solvents
- Applied potential over EC cell is the only variable parameter for controlling the reduction.
- No use of harsh denaturing or reducing agents

The current work demonstrates the feasibility of on-line Nano LC-EC-MS for the complete and gentle reduction of mAbs, with minimal sample handling and risk of distortion.

Reference

[1] 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