New Electrochemical Cell for Reduction of Disulfide Bonds in Proteins & Biopharmaceuticals

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Introduction

Disulfide bonds are one of the most important post-translational modifications of proteins. They are stabilizing the protein's 3D structure and are crucial for their biological function. The reduction of intra- and intermolecular disulfide bonds is necessary for successful characterization and assignment of the bonding sites by MS. For the reduction of disulfide bonds in proteins prior to MS analysis, concentrated reducing agents such as dithiothreitol (DTT) or TCEP (tris (2-carboxyethyl)phosphine) are often used. Both can interfere with the LC-MS analysis and sample preparation remains laborious and often difficult.

The working conditions of the new cell were established by using infusion- and FIA-EC-MS. It was found that Titanium as working electrode and Platinum as a counter electrode with potentials of -1.0 to -1.5 V in pulse mode were optimal for the reduction of most proteins including monoclonal antibodies.

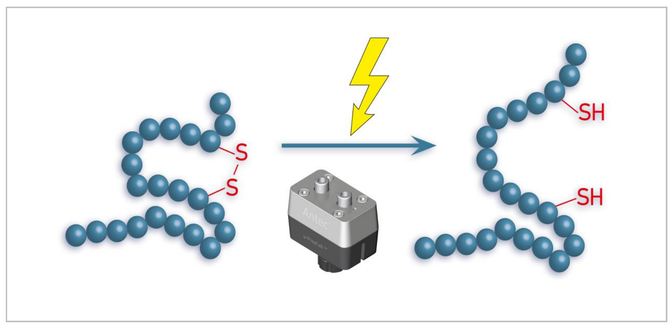

Figure 1: Schematics of electrochemical reduction of disulfide bond.

Objective

Development of a new Electrochemical Cell for reduction of disulfide bonds in proteins/peptides with the focus on:

- High reduction efficiency
- Excellent long-term reproducibility and stability
- Minimal contamination/fouling of the cell and/or the electrode
- High-pressure stability

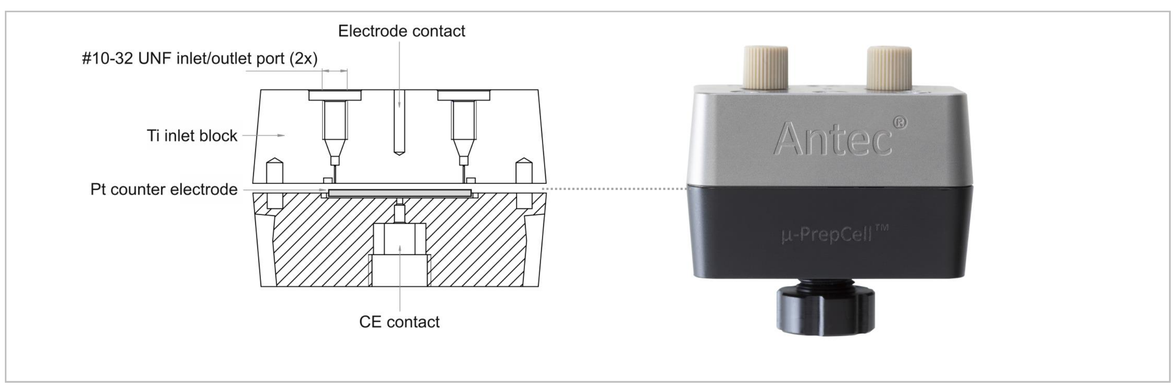

Figure 2: Dual electrode µ-PrepCell SS consisting of a Titanium inlet block and a Platinum counter electrode.

Instrumental Setup

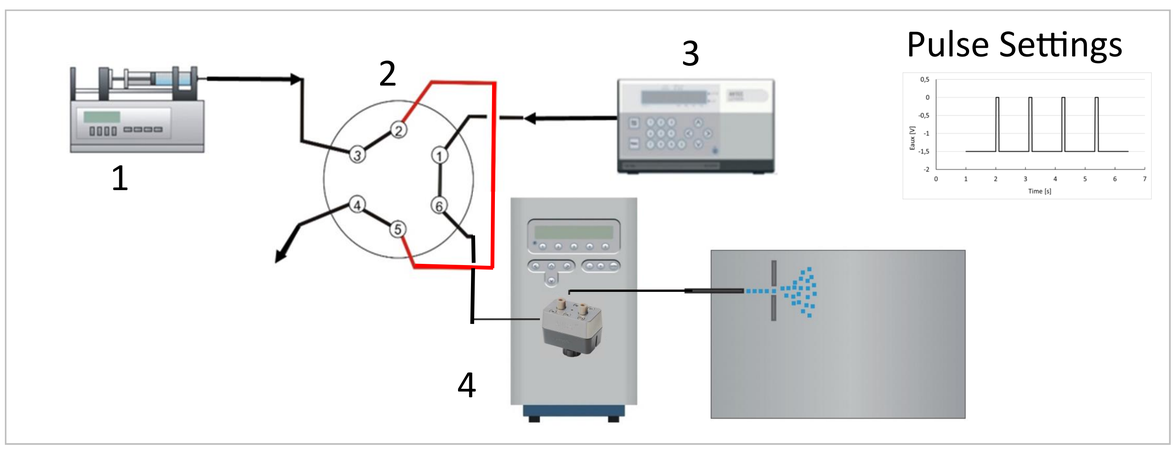

Figure 3: Schematics of Flow Injection Analysis EC-MS : 1) Sample infusion pump, 2) injection valve, 3) HPLC loading pump , 4) ROXY Potentiostat equipped with dual electrode µ-PrepCell, 5) MS.
Pulse settings: E1 = 1.5V, t1 = 1s, E2 = 0V, t2 = 0.1s, ts = 40 ms

Long-term Reproducibility and Stability

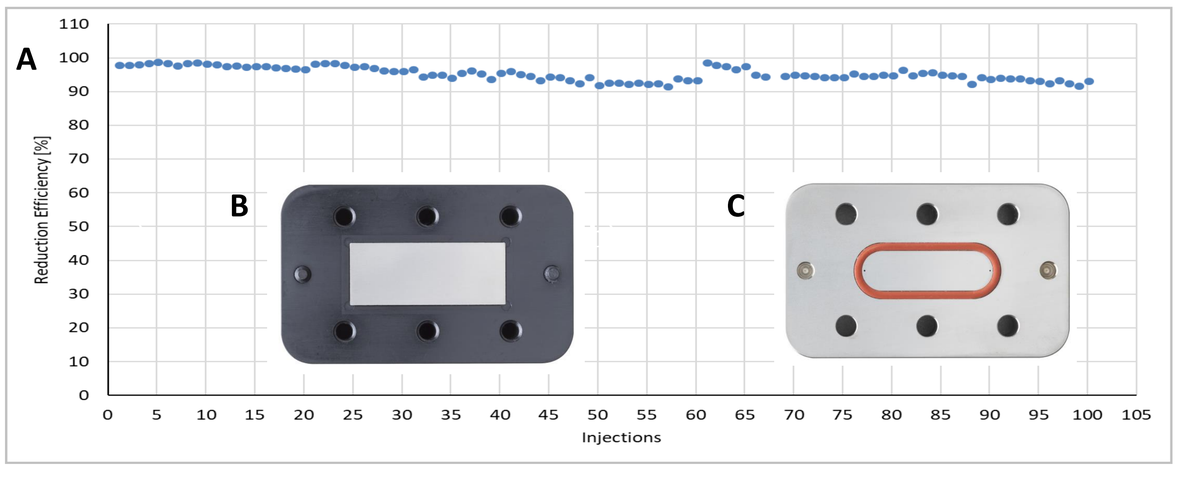

Figure 4: Long term reproducibility of reduction efficiency measured on 100 injections of insulin (A) and picture of Pt electrode (B) and Ti inlet block (C) after several days of operation.

Selective Reduction without Oxidation

The µ-PrepCell SS with optimized 2-step pulse shows virtually no oxidative species (Figure 5B) during reduction experiments compared with previous versions of the µ-PrepCell.

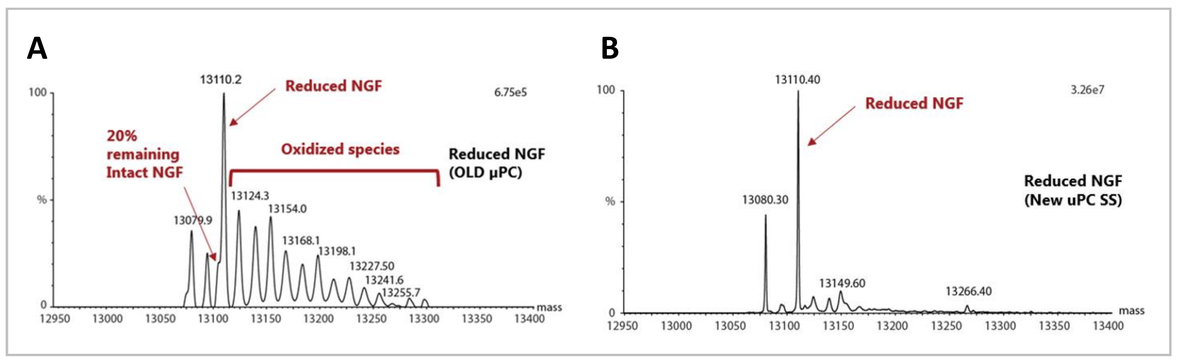

*Figure 5: Comparison of mass spectrum of reduced NGF using a previous model of the µ-PrepCell **(A)** and the new µ-PrepCell SS **(B)**.*

Comparison of TCEP vs EC Reduction in HDX-MS

Coupling electrochemistry with a HDX-MS setup enables sequence coverage in cysteine-rich regions that are not accessible by means of chemical reduction with TCEP under HDX quench conditions.

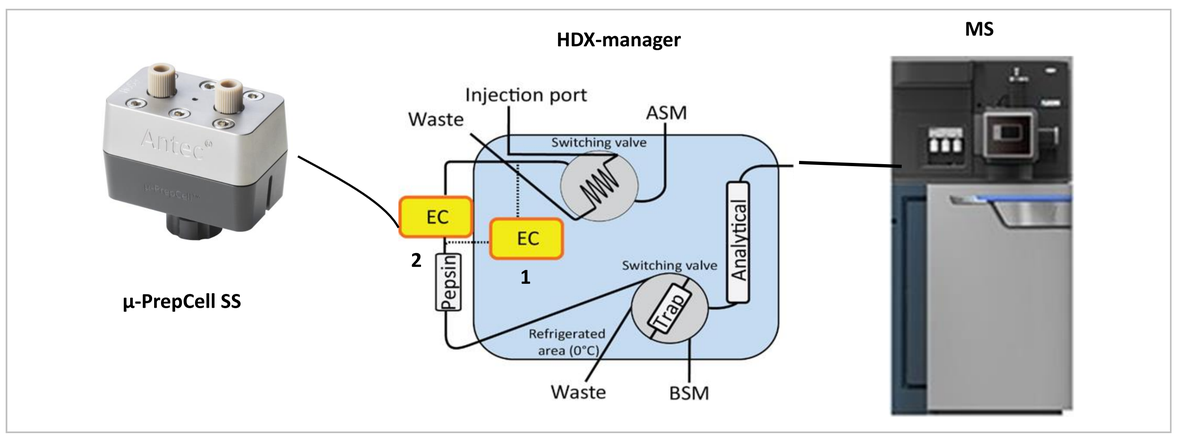

*Figure 6: Schematics of a Waters HDX-MS (*Synapt G2Si*) system with an integrated µ-PrepCell SS, controlled by a ROXY™ potentiostat with Dialogue Elite software. The electrochemical cell can be placed inside the HDX manager at 0°C **(1)** or outside at room temperature **(2)**.*

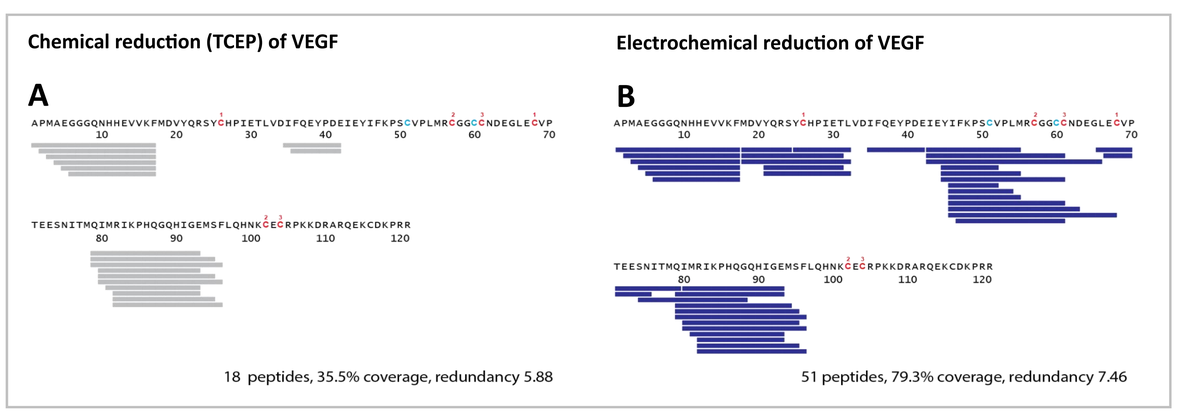

Figure 7: Sequence coverage maps of VEGF (Vascular endothelial growth factor) obtained by HDX-MS compatible reduction with (A) 0.25M TCEP (4min) and (B) Online Electrochemical reduction in 1% FA. Cysteine residues involved in intermolecular and intramolecular disulfide bonds are shown in light blue and red, respectively. Bound cysteines in VEGF structure are indicated by identical numbers. Reduced peptic peptides of the C-terminal end of VEGF could also be detected, however these yielded low S/N (<10) as they eluted in the void volume due to their very hydrophilic nature.

Reduction of Avastin® Fab Fragment

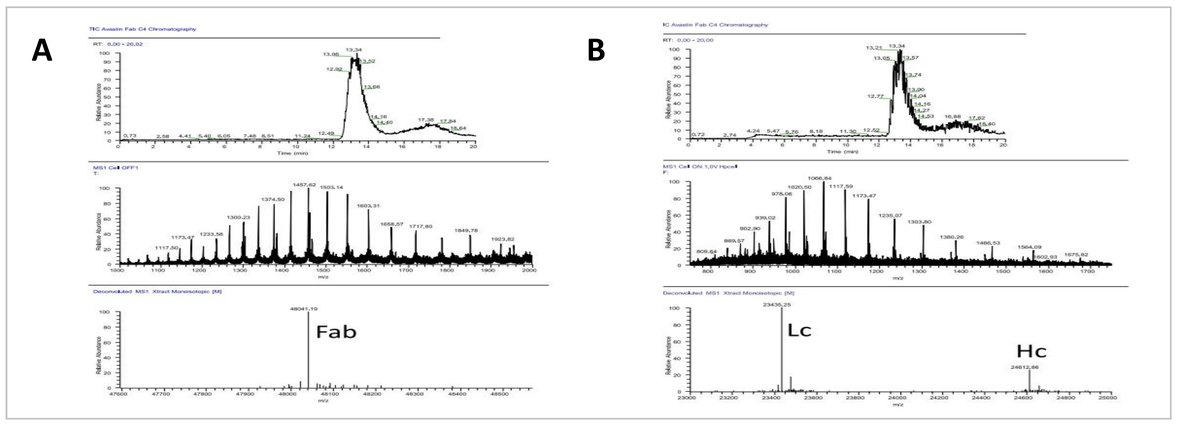

Figure 8: On-line LC-EC-MS of intact Fab fragment (A) and Fab fragment with electrochemically reduced S-S bonds (B). From top to bottom for (A) and (B): TIC, MS spectrum and deconvoluted MS spectrum with monoisotopic mass, measured on Orbitrap Fusion Lumos (Thermo)

Conclusion

The new µ-PrepCell SS offers a user-friendly and robust solution for efficient reduction of disulfide bonds in proteins and biopharmaceuticals, without the need of chemical reducing agents. The cell can be easily integrated into different workflows for automated S-S reduction of different samples. Even after several days of use no visible signs of wear & fouling were observed, demonstrating the robustness of the new cell.

Acknowledgements

Dr. Kasper Rand et al., University of Copenhagen, DK, for the data on HDX-MS on reduction of VEGF and Dr, Theo M. Luider et at., Erasmus MC, Rotterdam, NL, for the data on Avastin Fab reduction.