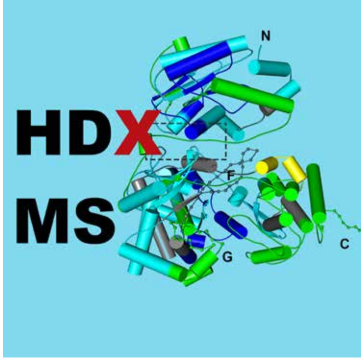
Antec Scientific

Application Note
Proteomics & Protein Chemistry



Electrochemical
Reactions upfront
MS – EC/MS

Proteomics &
Protein Chemistry
S-S bond reduction
HDX
Peptide bond cleavage
Na+, K+ removal
Drug-protein binding

Lipidomics & Fatty Acids
Cholesterol
Oxysterol
FAME Biodiesel

Drug Metabolism
Mimicking CYP 450
Phase I & II
Biotransformation

Synthesis (mg)
Metabolites & Degradants

Pharmaceutical Stability
Purposeful degradation
API testing
Antioxidants

Environmental
Degradation & persistence
Transformation products
Surface & drinking water

Food & Beverages
Oxidative stability
Antioxidants

Forensic Toxicology
Designer drugs
Illicit drugs

Healthcare & Cosmetics
Skin sensitizers

Genomics
DNA Damage
Adduct formation
Nucleic acid oxidation

Superior Reduction of Protein Disulfide Bonds in HDX-MS

- **Robust and efficient S-S bond reduction**
- **Fully automated and easy integration into HDX manager/workflow**
- **No influence on back exchange**

Introduction

Disulfide bonds present a challenge to HDX-MS as they increase the stability of proteins towards enzymatic digestion which can lead to low sequence coverage, especially in cysteine-rich regions. Traditionally used chemical reduction with reducing agents such as TCEP often cannot tackle highly disulfide bonded proteins due to slowed reduction rates at HDX-quench conditions (0°C and pH 2.5). Furthermore, these agents often need to be used at high concentrations, which can lead to extensive adduct formation and ion suppression.

Electrochemical reduction has successfully been applied to reduce heavily disulfide-bonded proteins under HDX compatible conditions, enabling sequence coverage of otherwise inaccessible regions and bypassing the disadvantageous ion suppression [1,2].

New developments of the electrochemical cell now offer high reduction efficiency, improved reproducibility and robustness, without unwanted protein oxidation.

This application note is by courtesy of Dr. Kasper Rand and Gerard Comamala Grau, University of Copenhagen, Department of Pharmacy, Copenhagen, Denmark

Superior Reduction of Protein Disulfide Bonds in HDX-MS

Instrumental Setup

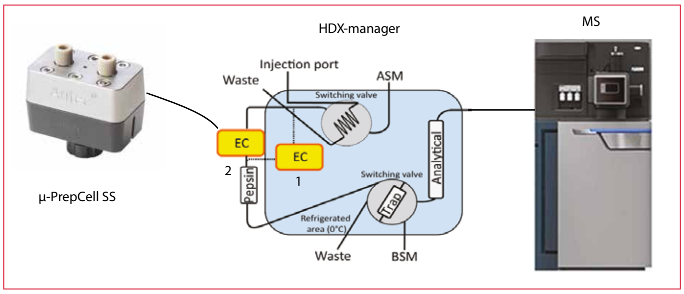


Figure 1: Schematics of a Waters HDX-MS (Synapt G2Si) system with an integrated µ-PrepCell SS, controlled by a ROXY™ potentiostat with Dialogue Elite software. The electrochemical cell can be placed inside the HDX manager at 0°C (1) or outside at room temperature (2).

New Dual Electrode Flow Cell

- Leak-free at 350 bar (tested for 7 consecutive days at the rated pressure)
- Ease of use and virtually maintenance-free
- Wide applicable flow rate range (20 - 200 µL/min) with high reduction efficiency
- Dualelectrode design for excellent long-term reproducibility and stability

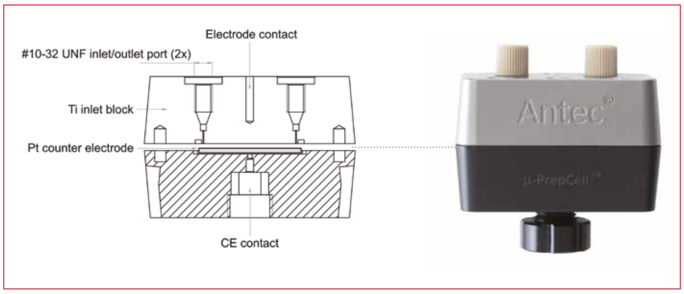


Figure 2: Figure 2: Dual electrode µ-PrepCell SS consisting of a Titanium inlet block and a Platinum counter electrode.

Insulin as System Suitability Test (SST)

The use of an optimized two-step pulse (Figure 3B) for disulfide bond reduction ensures efficient and reproducible performance of the new µ-PrepCell SS over time. Bovine insulin (BI) in 1% Formic acid (FA) was used as SST to verify the performance of the setup during a series of experiments. With BI under SST conditions consistently > 90 % reduction efficiency was obtained (red bars in figure 3A). No visible signs of wear and fouling were observed after several days of operation.

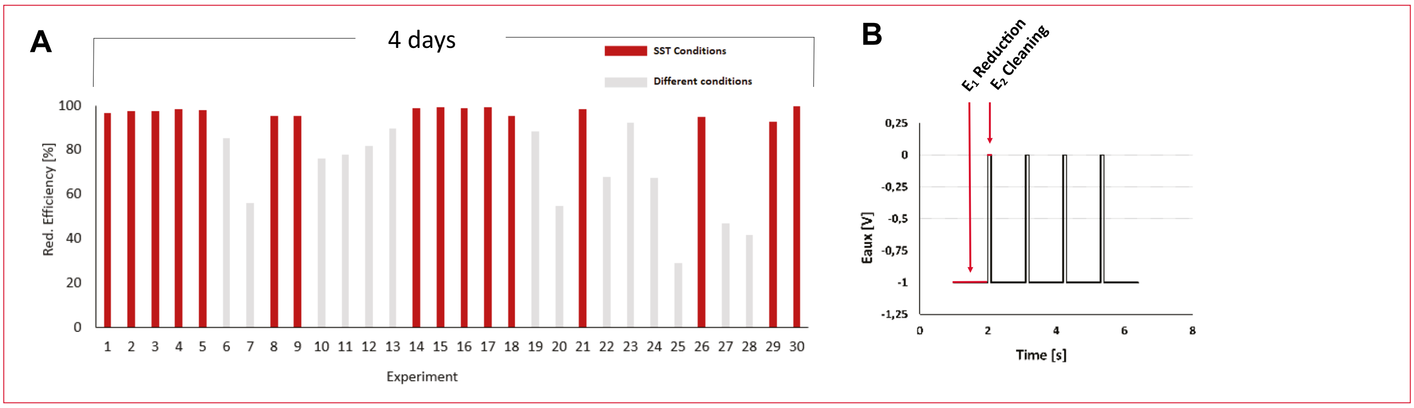


Figure 3: (A) Overview of the reduction efficiency of 10 pmol Bovine Insulin in a EC-HDX-MS set-up under SST (50µL/min and 1% FA at room temperature) and different conditions. (B) Standard pulse settings for disulfide bond reduction (E1, E2, t1,t2; 1.0V, 0.0V, 1.0s, 0.1s.). See next page for details about the experiments.

Influence of Excipients on Reduction Efficiency

Table 1

LC-EC Conditions

Exp#	Sample Matrix	Flow rate (µL/min)	E1 (Volts)	T (°C)	Reduction (%)
2	1% FA,5% ACN	50	1.0	20	97.6
11	1% FA	50	1.0	0	77.7
12	1% FA	25	1.0	0	81.8
13	1% FA	10	1.0	0	89.4
19	1% FA, 10 mM TRIS	25	1.0	20	88.2
20	1% FA, 10 mM TRIS	50	1.0	20	54.6
24	1% FA, 10 mM TRIS	50	1.2	20	67.5
25	1% FA, 25 mM NaCl	50	1.0	20	29.0
27	1% FA, 25 mM NaCl	25	1.0	20	47.0

Table 1: Influence of several parameters on reduction efficiency of 10 pmol Bovine Insulin in a EC-HDX-MS.

The reduction efficiency can be affected by the presence of excipients or the conditions at which the EC reduction is performed, such as temperature, potential and flow rate. Table 1 shows the conditions of the plotted experiments from Figure 3A. It is evident from Table 1 that flow rate and EC potential are important parameters for optimization of the reduction efficiency.

Oxidation-free Electrochemical Reaction

The µ-PrepCell SS with optimized 2-step pulse shows virtually no oxidative species (Figure 4B) during reduction experiments compared with previous versions of the µ-PrepCell.

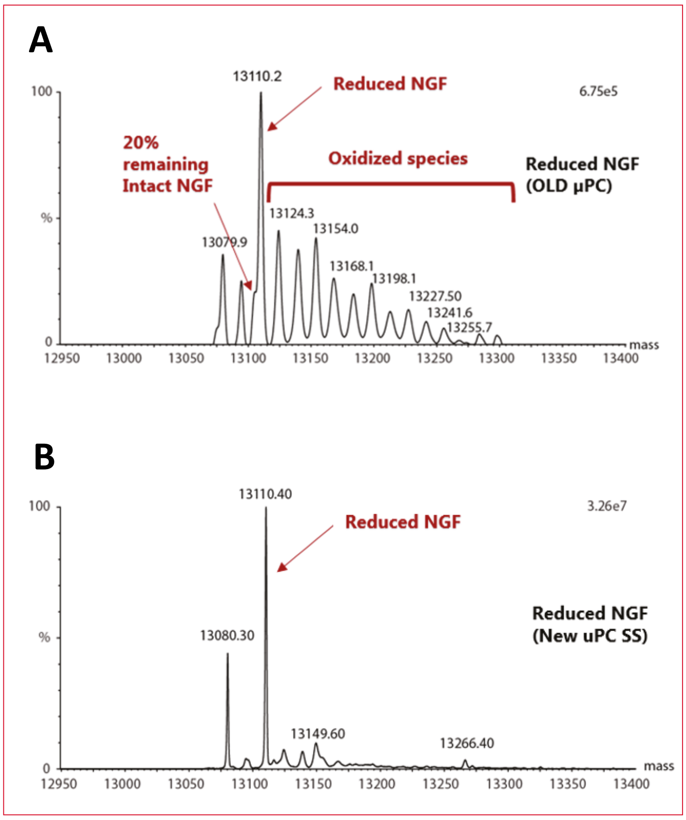


Figure 4: Comparison of mass spectrum of reduced Nerve Growth Factor (NGF) using a previous model of the µ-PrepCell (A) and the new µ-PrepCell SS (B).

Back-Exchange Influence

Peptides were labelled for 72h at pH 7.4, quenched, and injected into the cooled UPLC and thus exposed to 6 min desalting and 9 min gradient (2-40%). No significant differences could be observed between any of the conditions, indicating that the cell does not have a significant effect on back-exchange.

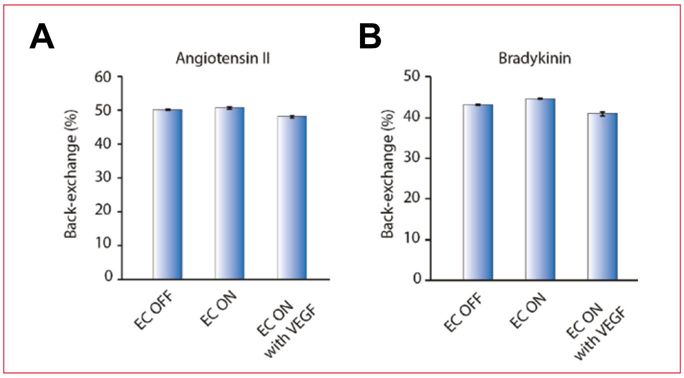


Figure 5: Back-exchange levels displayed by maximally labelled model peptides angiotensin II (A) and bradykinin (B) with the electrochemical cell OFF and ON

Comparison of TCEP vs. EC Reduction

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

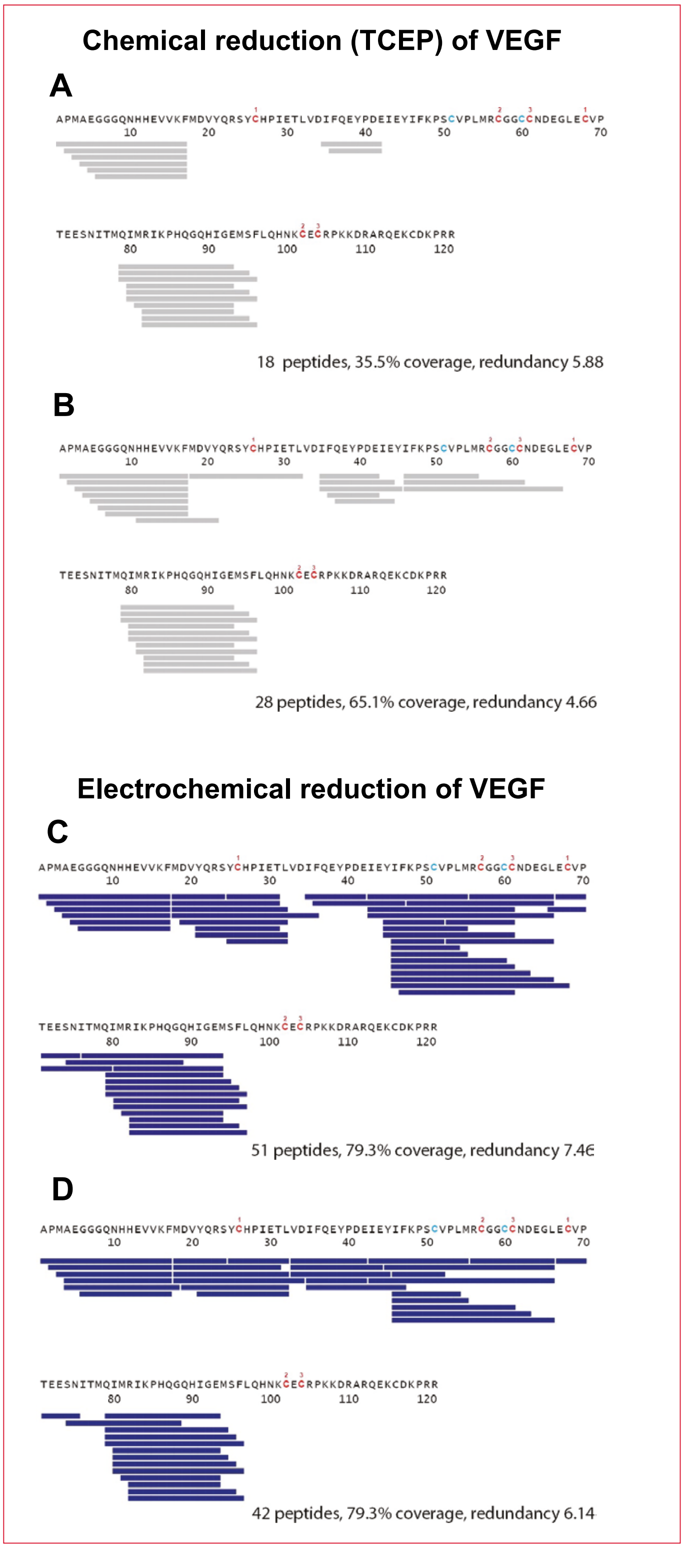


Figure 6: Figure 6: Sequence coverage maps of Vascular Endothelial Growth Factor (VEGF) obtained by HDX-MS compatible reduction with (A) 0.25M TCEP (4min), (B) Overnight reduction with 0.15M TCEP, (C) Electrochemical reduction in 1% FA. (D) shows an effective sequence coverage of 72h labelled VEGF using electrochemical reduction under HDX-MS compatible conditions. 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.

References

1. Mysling S., Salbo R., Ploug M., Jørgensen T. J. D., Anal. Chem., 2014, 86(1), p 340-345
2. Trabjerg E., Jakobsen R. U., Mysling S., Christensen S., Jørgensen T. J. D., Rand K. D., Anal. Chem., 2015, 87(17), p 8880–8888

Conclusion

The new µ-PrepCell SS offers a user-friendly and robust solution for efficient reduction of disulfide bonds in proteins, without the need of chemical reducing agents. The cell can be easily integrated into a HDX-MS work flow for automated S-S reduction of protein samples prior to digestion. Successful reduction of Vascular Endothelial Growth Factor (VEGF) with sequence coverage up to 79.3 % has been obtained under HDX-MS compatible conditions, which cannot be achieved with TCEP. No oxidative species were formed during reduction experiments with NGF, demonstrating that unwanted oxidation is effectively suppressed using the cell with an optimized 2-step pulse. Labelling experiments with peptides have indicated that reduction with the µ-PrepCell SS has no effect on back-exchange. Excellent reproducibility has been shown using insulin as SST. Even after several days of use no visible signs of wear & fouling were observed, demonstrating the robustness of the new cell.

Ordering information

210.0073D	ROXY EC system for S-S reduction in HDXMS, consisting of ROXY Potentiostat, µ-PrepCell SS, Pt electrode and Dialogue Elite software for system control

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