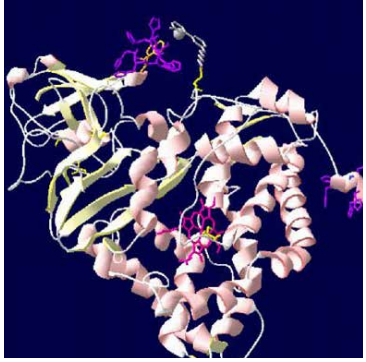
Antec® Scientific

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

Controlled Reduction of Disulfide Bonds in Proteins/Peptides using on-line EC/MS

- **Fast reduction of S-S bonds in an electrochemical flow-through cell**
- **Reagent free, no reducing chemicals, e.g., DTT, TCEP, etc.**
- **Ideal for TCEP resistant proteins**
- **Automated reduction by on-line EC/MS or LC/EC/MS**

Summary

The electrochemically-assisted reduction of disulfide bonds in peptides and proteins after HPLC and followed by on-line mass spectrometric detection is presented. The method is based on square-wave potential pulses applied on a new type of working electrode made from Titanium alloy. The method does not use any chemical agents and is purely instrumental resulting in a fully automated platform for fast assessment and characterization of S-S bonds in proteins/peptides [1-6].

ROXY Application Note # 210_006_05

Schematics Disulfide Bond Reduction

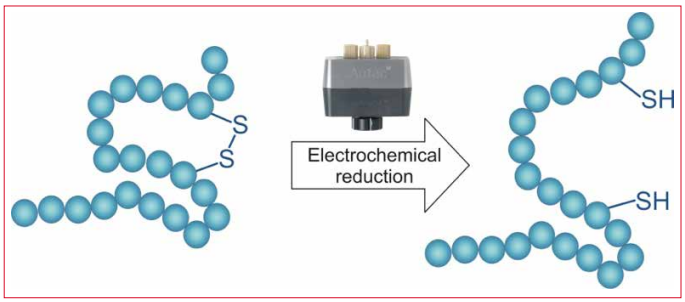


Figure 1: Schematics disulfide bond reduction: replacing interfering chemicals (e.g., DTT, TCEP) by an electrochemical reactor cell used on-line with LC/MS. Push-button reduction

Introduction

Reduction of disulfide bonds in peptides and proteins prior to MS analysis is done for several reasons. It is one of the steps in the determination of the disulfide bonds positions which is important for understanding the folding processes of a protein. Also disulfide bonds can hinder MS identification and their reduction is necessary for efficient sequence analysis in top-down proteomics, or prior to HDX exchange procedures.

We developed an electrochemical (EC) method for the efficient and fast reduction of disulfide bonds in peptides and proteins. The method utilizes an electrochemical flow-through cell. The cell can be directly connected to the ESI-MS for flow injection analysis or after the HPLC column in case of complex sample analysis by LC/EC/MS.

A complete or near to complete reduction of the disulfide bonds of the tested proteins and peptides has been demonstrated.

Method

All experiments were performed on a ROXY EC system (Antec, The Netherlands) consisting of a ROXY Potentiostat, equipped with a µ-PrepCell. The ROXY system was online hyphenated to a LTQ-FT mass spectrometer (Thermo Fisher Scientific, USA).

The thin-layer electrochemical reactor cell consisted of a titanium-based working electrode (WE) specifically optimized for efficient reduction [1-6], a titanium auxiliary (counter) electrode (AUX) and a Pd/H2 reference electrode (REF). A 100-µm spacer was used to separate the WE and the auxiliary electrode inlet block giving a cell volume of approximately 6 µL. The ROXY EC system was controlled by Dialogue software. An electrical grounding union was used to decouple the electrochemical cell from the ESI high voltage.

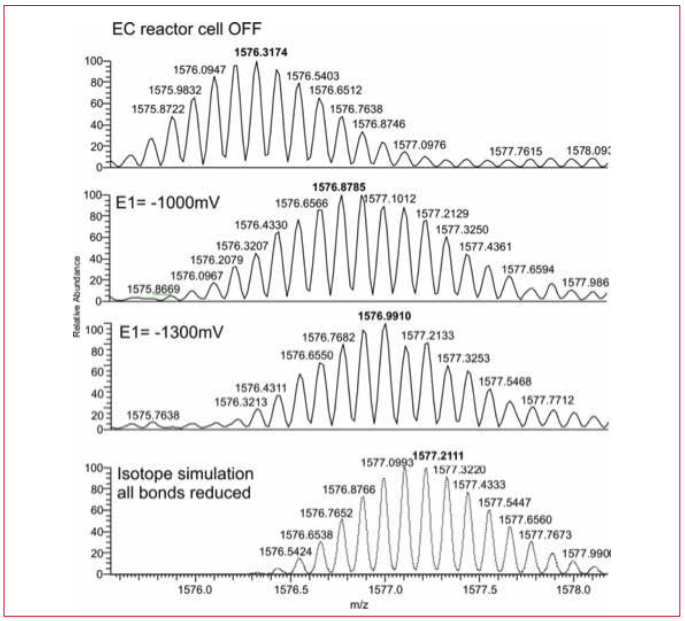


Figure 2: Partial and full reduction of disulfide bonds in α-lactalbumin. The overlapping isotopic pattern of the +9 ion measured with the EC reactor cell turned OFF (top), and ON at E1= -1000mV and E1= -1300mV.

Table 1

Conditions

HPLC	ROXY HPLC/EC system
Column	ALF C18, 150 x 1 mm, 3 um (Antec)
Mobile phase	1% Formic acid (250 mmol/L) in water with 5% acetonitrile. Gradient running from 10 – 50 % acetonitrile in 10 min.
Flow rate	50 µL/min
Vinjection	5 µL
Temperature	35 C
Flow cell	µ-PrepCell, Ti working electrode, HyREF electrode.
Potential waveform	E1, E2: -1.5, +1 V, t1, t2: 1990,1010 ms

In all experiments a square wave pulse was applied which has been described earlier [1].

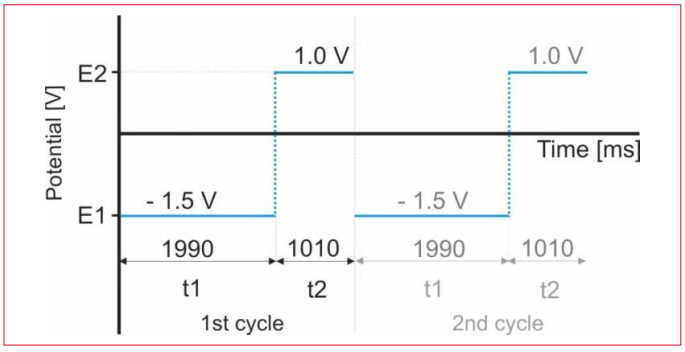


Figure 3: A schematic representation of the square-wave pulse. Under optimized conditions, the potentials were −1.5 V (E1) and +1.0 V (E2) and time intervals were 1,990 ms (t1) and 1,010 ms (t2), unless specified otherwise

Results

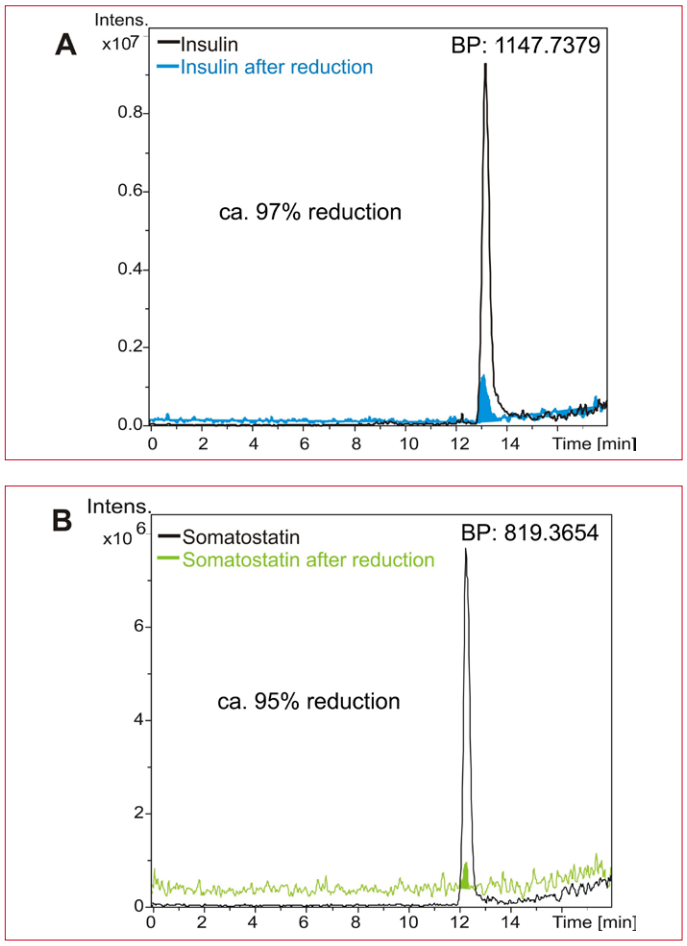


Figure 4: Reduction efficiency: Online HPLC/EC/MS analysis of a mixture of insulin (m/z 1147.7379) and somatostatin (m/z 819.3654). The overlays in A and B show the m/z traces of the non-reduced intact peptide with the µ-PrepCell "OFF" (main peak) and the almost fully reduced peptide with µ-PrepCell "ON" with reduction efficiencies of 95 and 97%.

Insulin, a small protein of 5733 Da containing 3 disulfide bridges, somatostatin with one disulfide bond (1638 Da), and α-lactalbumin with four bonds (14178 Da) were used as test substances to demonstrate the applicability of the method.

The reduction efficiency is affected by several parameters such as instrument set-up (i.e. infusion EC/MS vs. LC/EC/MS), flow rate, mobile phase composition (formic acid and acetonitrile content) peptide/protein concentration of the sample and the square-wave pulse settings (potential). By changing the applied potential the extent of disulfide bond cleavage is controlled. More negative potentials result in a shift of the charge state distribution indicating increased disulfide bond cleavage and unfolding of the protein (Fig. 2).

By increasing the flow rate and thereby shortening the residual time of the chromatographic peak in the electrochemical cell, a decrease of the reduction efficiency is inevitable.

Consequently, the flow rate can also be used to control the reduction efficiency beside the applied potential (square-wave pulse). Thus, by proper selection of the flow rate or potential it becomes possible to switch between complete and partial disulfide bond reduction.

Partial reduction is of particular importance to localize disulfide bonds and to study the impact of individual disulfide bonds on peptide and protein structures.

The effects of different experimental parameters are tested and the optimized protocol for the electrochemical reduction of disulfide bonds by online LC/EC/MS has been described in details elsewhere [1].

Figure 5: ROXY EC system for S-S reduction.

Conclusion

An electrochemical Flow-Through Cell for efficient reduction of disulfide bonds in proteins and peptides has been demonstrated. The new proprietary [6] titanium based working electrode provides high reduction efficiencies of 80 to almost 100% for the tested peptides. This electrochemical (EC) reduction can be done by direct infusion EC/MS or by LC/EC/MS. The applied potential can be used to control the degree of S-S bond reduction/cleavage and therefore offers new opportunities for faster and superior characterization of disulfide bonds in protein therapeutics. The chemical free EC reduction shows further great potential for the disulfide bond reduction of TCEP resistant proteins/peptides prior to HDX MS analysis.

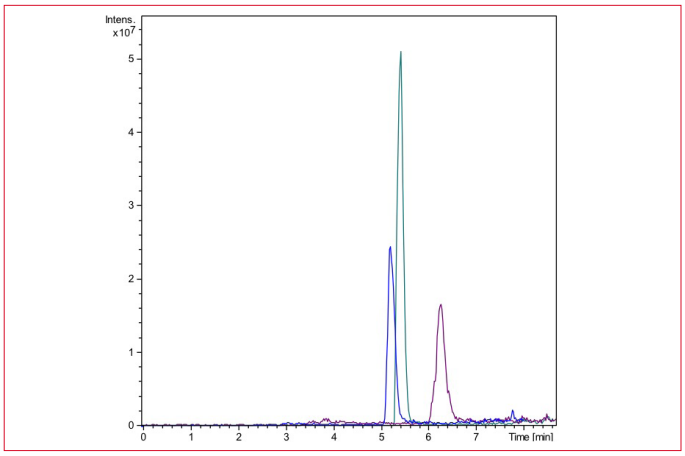


Figure 6: Peak broadening caused by the electrochemical cell. Separation of Insulin, without µ-PrepCell (green), and with µ-PrepCell: with a 100 µm spacer (blue) and with 150 µm spacer (purple). Flow rate was 50 (green) and 75 (blue, purple) µL/min.

The electrochemical cell was positioned post-column resulting in a fully automated platform for fast characterization of disulfide bonds in protein/peptide samples. HPLC mass spectra of two test substances are shown in Fig. 4. After separation the peptides are reduced in the µ-PrepCell. The peak broadening has been investigated by comparing analysis with and without the µ-Prepcell (Fig. 6). The plate numbers changed from 6000 to about 4000 when applying the µ-PrepCell. Changing the spacer from 150 to 100 µm improved the platenumber to 5000 and this configuration was used for further experiments. In Figure 6A the amino acid sequence of Insulin with its 3 disulfide bonds is shown. Figure 6B and C show the mass spectra of intact and reduced insulin and in Figure 6D the ions of the intact and reduced intrachain disulphide bond of chain A are shown.

References

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4. Zhang Y. et al., J. Proteome Res. 10 (2011) 1293
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6. Patent appl. US 2014/0069822

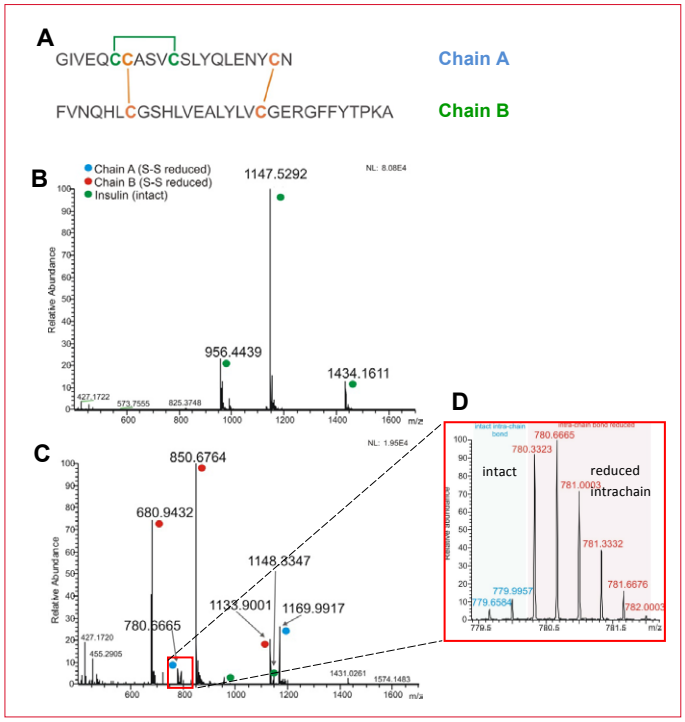

Figure 7:

A) Amino acid sequence of Insulin consisting of 51 amino acids with two interchain disulfide bonds between Chain A and B and one intrachain disulfide bond located in chain A.

B) Mass spectra of intact insulin with 3 major fragments (green dots) with m/z 1434.1611, m/z 1147.5292 and m/z 956.4439 for $[M+4H]^{4+}$, $[M+5H]^{5+}$, $[M+6H]^{6+}$ ions, respectively.

C) Mass spectra of reduced insulin with two low abundant fragments for chain A (blue dots): m/z 1169.9917 and m/z 780.6665 which are in agreement with the theoretical masses of reduced $[M+2H]^{2+}$ and $[M+3H]^{3+}$ ions of chain A, and three higher abundant fragments for chain B (red dot), m/z 1133.9001, m/z 850.6764 and m/z 680.9432, detected as $[M+3H]^{3+}$, $[M+4H]^{4+}$, $[M+5H]^{5+}$ ions of the reduced B chain.

D) Zoom of the $[M+3H]^{3+}$ ion with m/z 780.6667 of chain A of reduced insulin with its isotopic distribution showing ions from the intact and reduced intrachain disulphide bond.

Ordering information

210.0072A	ROXY EC system for S-S reduction
210.4300T	µ-PrepCell, Ti WE, HyREF

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