Electrochemical Reduction of Disulfide Bonds for the Analysis of Protein Therapeutics by Hydrogen-Deuterium Exchange Mass Spectrometry

Visit booth #61

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Introduction

Reduction followed by enzymatic digestion is a compulsory step in the characterization of protein therapeutics. Reduction is generally performed using chemicals such as TCEP at high concentrations, which interferes with the subsequent LC-MS analysis. Additionally, TCEP leads to very limited reduction efficiency when applied to proteins with a high disulphide bonds density.
A novel method for the efficient and fast reduction of disulphide bonds in peptides and proteins combined online with mass spectrometric (MS) detection is presented. It utilizes an alternating potential pulse, which is applied to an electrochemical (EC) flow-through cell, does not require any reducing chemicals or enzymes and is purely instrumental resulting in a fully automated platform for fast assessment and characterization of S-S bonds in biopharmaceuticals by Hydrogen-Deuterium Exchange Mass Spectrometry (HDX).

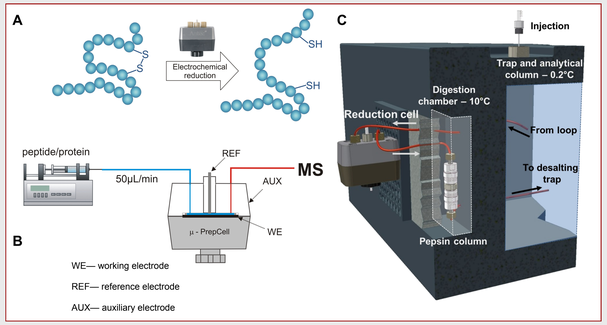


*Figure 1: **A.** Reduction of disulfide bonds using an electrochemical cell (µ-PrepCell™ with ROXY™ Potentiostat, Antec, Boston, USA): **B.** Instrumental set-up used in direct infusion EC-ESI-MS. **C.** Inclusion of the µ-PrepCell in the HDX-MS set-up (Waters).*

Methods

In infusion mode experiments (Fig 1B), typically 2 - 20 µM solutions of the target compound in 1% formic acid (FA) in water/acetonitrile (90/10 or 50/50, v/v) were pumped through the EC cell. Flow rates were either set at 50 or 100 µL/min. The outlet of the cell was directly connected to the ESI-MS.

In HDX experiments (Fig 1C), the sample was prepared in either 0.23% or 1% FA in water. The EC cell was positioned outside the HDX manager module, between the injection port and the pepsin column. The mobile phase flowing through the EC cell and the pepsin column was 0.23% FA in water, and the flow rate was set at 50 µL/min.

The cell was operating in pulse mode (Fig 2B) to reduce the compounds of interest. ROXY EC system (Antec, Boston, USA) and Dialogue software (Antec) were used to control the EC cell and to start the MS analysis. Infusion experiments were performed on a HCT Ion Trap, a solariX FT-ICR (Bruker Daltonics, Germany) or a Synapt G1 QTOF (Waters, USA) mass spectrometer, all equipped with an electrospray (ESI) source. The HDX experiments were performed using a Waters UPLC HDX MS system without any further modifications than the installation the EC cell.

Hepcidin, Insulin (Bovine pancreas) and Herceptin (a commercially-available monoclonal antibody, MAb) were used as model compounds for the electrochemical reduction of disulfide bonds in infusion and HDX experiments. They were selected to cover a broad range of molecular weights, approximately between 3000 & 150000 Da. Hepcidin is a 25 amino-acid peptide with a tight cystine-rich hairpin structure (4 disulphide bonds close to each other). Insulin is built of 51 amino acids forming two chains and contains 3 disulfide bonds (2 between A & B chains and 1 intra-chain on the A chain, Fig 2A). Herceptin is a MAb of the IgG1 type, and is therefore built up of 2 light chains (LC, ~ 25 kDa) and 2 heavy chains (HC, ~ 50 kDa).

Results - Insulin Reduction

Insulin was the first test compound to be reduced. Following optimisation of the pulse settings by direct infusion through the EC cell into the MS, insulin was reduced in high yields into the two chains, A & B. The intra-chain disulphide bond was also reduced in high yield (Fig. 3 A & B).

*Figure 2: **A.** Insulin sequence showing the two interchain chain disulphide bonds (orange) and the intrachain disufide bond (green). **B.** Example of pulse settings (E1, E2, t1 & t2 can all be*

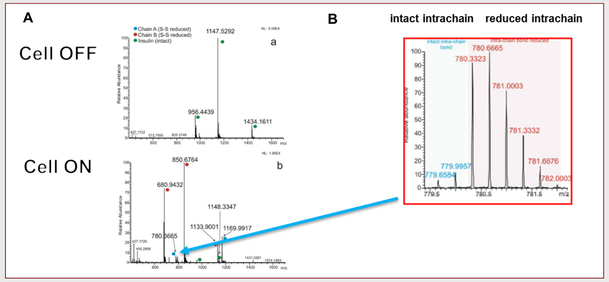


*Figure 3: Reduction of Insulin Disulfide Bonds **A.** Full-Scan Spectra of Insulin (Cell OFF) and reduced Insulin (Cell ON). The spectrum for Reduced Insulin is dominated by the signal corresponding to Insulin B chain **B.** Zoom-in view on the m/z corresponding to the A chain showing the complete reduction of the intra-chain A disulphide bond.*

Cystine "rich" Hairpin & MAb Reduction

More complex proteins - Hepcidin, with its cystine rich Hairpin, a MAb - were also electrochemically reduced as to demonstrate the capabilities of the EC cell. All disulfide bonds of hepcidin were fully reduced, resulting in the almost complete sequencing of its peptide backbone (Fig. 4). Although LC-MS/MS methods have been used to identify and quantify hepcidin in body fluids, never before such high sequence coverage of Hepcidin was achieved.

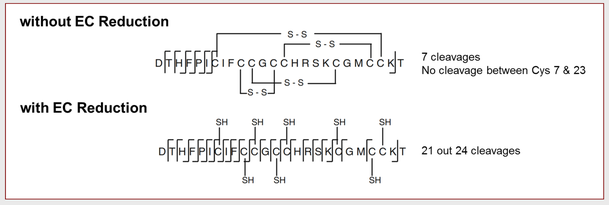


Figure 4: Sequence coverage of Hepcidin is increased by a factor 3 following electrochemical reduction of its disulphide bonds.

The MAb was efficiently and selectively reduced into LC & HC with only limited LC-HC, LC-2HC combinations (Fig. 5), allowing the profiling of its major glycoforms. The reduction of the disulphide bridges is demonstrated by the shift of the charge state distribution toward lower m/z indicating unfolding of the protein as result of the reduction of disulfide bridges. Only two intra-chain disulfide bonds couldn't be reduced, one in the variable region and one in the κ constant region.

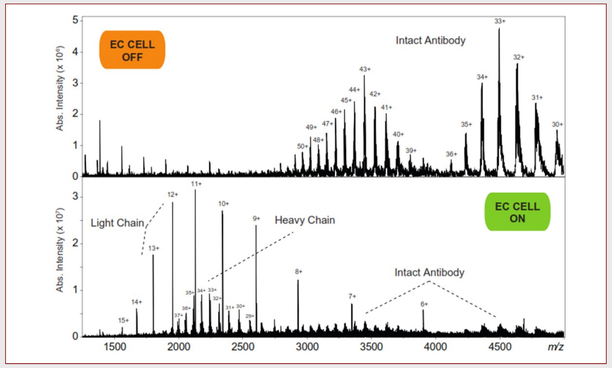


Figure 5: EC Reduction of the disulfide bonds of Herceptin monitored by on-line FTICR–MS.

Comparison of Reduction Efficiency - TCEP Vs EC Reduction

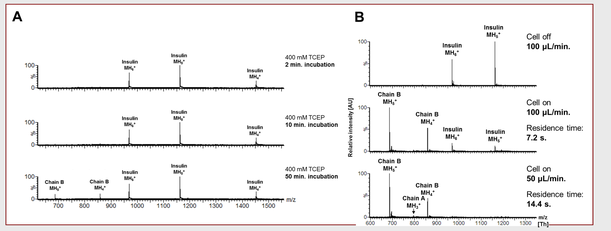
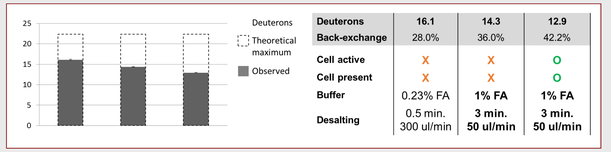


*Figure 6: Comparison of Reduction Efficiency at Room Temperature **A:** Incomplete Off-Line Reduction of Insulin using TCEP. **B:** Complete On-Line EC Reduction of Insulin within 14.4s.*



Deuterons	16.1	14.3	12.9
Back-exchange	28.0%	36.0%	42.2%
Cell active	X	X	O
Cell present	X	X	O
Buffer	0.23% FA	1% FA	1% FA
Desalting	0.5 min. 300 ul/min	3 min. 50 ul/min	3 min. 50 ul/min

Figure 7: Deuteration and back-exchange rate with EC cell on (O) and off (X) under standard HDX (mobile phase + T) conditions and optimized conditions for EC reduction.

Application to HDX Studies

The EC cell was positioned between the injector and the pepsin column outside the HDX manager (at room temperature). The rate of back-exchange (Fig. 7) when using this set-up was compared to analysis performed optimum conditions (0.23 % FA at 0°C). Even though back-exchange was higher in the present set-up than in the reference experiment, **deuteration patterns were qualitatively retained after EC reduction** (Fig. 8) and allowed to find that the B chain of Insulin is strongly protected (only ~ 50% of its backbone amides being deuterated at the longest exchange time (1000 s).
Decreasing both the %FA and temperature are not expected to jeopardize the reduction efficiency while improving the back-exchange.

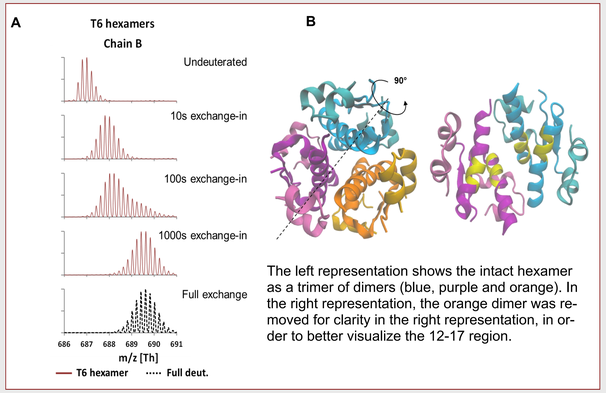


*Figure 8: **A:** Deuteration of Insulin T6 hexamers. **B**: Visualization of the region where the greatest changes in HDX were observed (12-17, labeled in yellow in the right representation).*

Conclusions

The superior reduction using ROXY EC (electrochemical reduction) was demonstrated by reducing both TCEP-resistant and large proteins in high yields. All disulfide bonds of a cystine-rich hairpin (hepcidin) were fully reduced, resulting in the highest sequence coverage ever reported. The MAb disulfide bonds were efficiently and selectively reduced into light and heavy chains, providing additional structural information.
The EC cell was also successfully implemented in a HDX workflow for the conformational study of Insulin. The EC cell gave higher reduction yields than the traditional TCEP approach in only a fraction of the time and deuteration patterns were qualitatively retained after EC reduction.
More data on the successful use of EC-HDX for the analysis of cystine knots and MAb will be presented at ***ASMS 2015: WOF pm 3:50: E. Trajberg , room #106.***

References

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