Controlled Reduction of Disulfide Bonds in Proteins and Peptides Using an Electrochemical Reactor Cell in online LC/EC/MS

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Introduction

The electrochemically-assisted reduction of disulfide bonds in peptides and proteins 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 biopharmaceuticals.

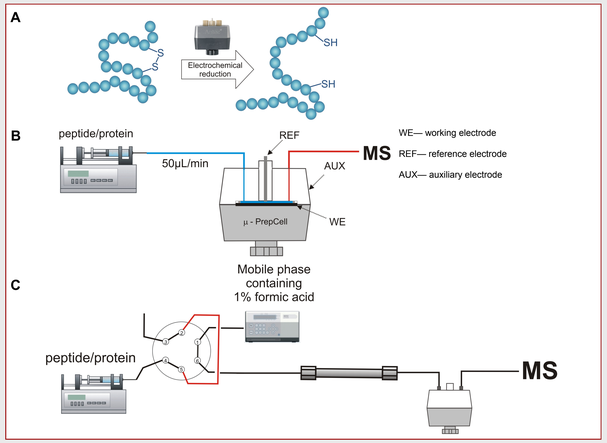

Figure 1: ***A.*** *Reduction of disulfide bonds using µ-PrepCell™:* ***B.*** *Instrumental set-up used in direct infusion.* ***C.*** *Post column reduction experiments.*

Methods/Instrumentation

In infusion mode experiments (Fig 1B), typically 2 - 20 µM solutions of the target compound in 1% formic acid/acetonitrile (90/10, v/v) were pumped into the electrochemical (EC) reactor cell at a flow rate of 50µL/min and the outlet of the cell was directly connected to the ESI-MS. In LC/EC/MS experiments (Fig 1C), the sample was introduced in 0.1% formic acid and 5% acetonitrile using 5µL injection loop. The gradient from 5% to 60% of acetonitrile was used. The mobile phase contained 1% formic acid. The flow rate was 50µL/min. The cell was operating in pulse mode to reduce the compounds of interest. ROXY EC system (Antec, The Netherlands) and Dialogue software were used to control reduction conditions and start MS analysis. HCT plus ion trap (Bruker Daltonics, Germany) or LTQ-FT (Thermo, USA) mass spectrometer equipped with electrospray (ESI) source was used to monitor the reduction products during the optimization steps and to confirm the presence of the reduced proteins/peptides in the control samples.

Somatostatin (synthetic), Insulin (Bovine pancreas) and α–Lactalbumin (Bovine milk) were chosen as the model compounds for electrochemical reduction of disulfide bonds. The peptides were selected to cover a broad range of molecular weights, 1637.88, 5731.61, 14176.81 Da, respectively. **Somatostatin** is a 14 amino acids peptide. Somatostatin has one intrachain disulfide bond that maintain the cyclic structure. Insulin is a hormone produced by pancreas. **Insulin** is build by 51 amino acids forming two chains, A and B, and contains 3 disulfide bonds. Two bonds connect chain A and B and one intrachain disulfide bond is located on chain A. **α–Lactalbumin** is 123 amino acids subunit of lactose synthase and may cause cow`s milk allergy in human. A globular structure of α–Lactalbumin is stabilized by four disulfide bonds (Cys25–Cys139, Cys47–Cys130, Cys80–Cys96, and Cys92–Cys110).

Results

Insulin and somatostatin was used for optimization of the electrochemical reduction of the disulfide bonds.

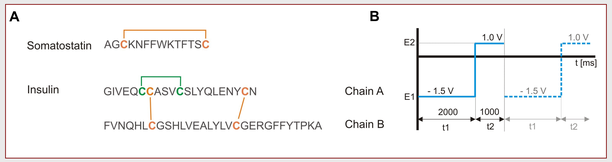

Figure 2: ***A.*** *Insulin & somatostatin sequence with indicated position of disulfide bonds.* ***B.*** *Pulse settings.*

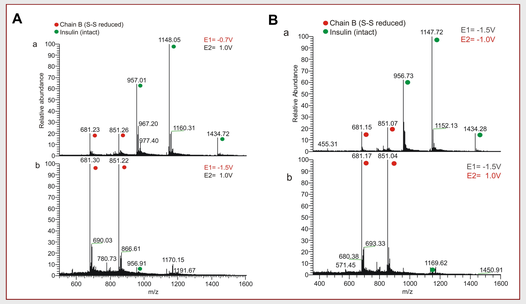

Figure 3: Pulse settings optimization. ***A.*** *Optimization of E1 potential. The application of potential below –1.5V resulted in near 100 % conversion of Insulin, E2 = 1V.* ***B.*** *Optimization of E2 potential. Only positive values of E2 potential resulted in near 100% reduction of Insulin. E1= -1.5V.*

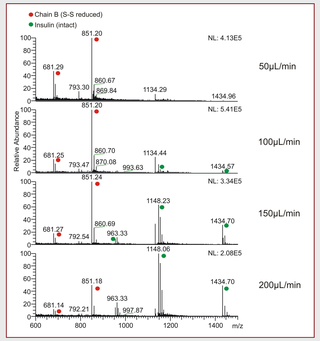

Figure 4: Electrochemical reduction of disulfide bonds in insulin presented at different flow rates. Reduction efficiency decreases gradually from 100% (50 µL/min) to 40% (200 µL/min).

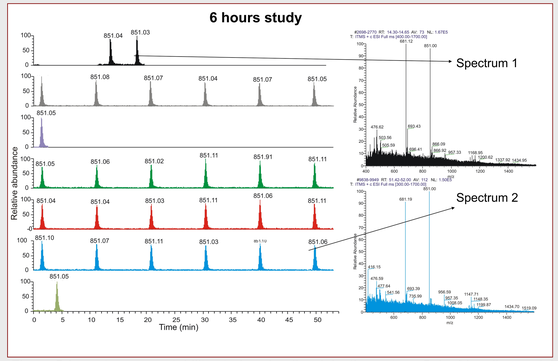

Figure 5: Long term repeatability study using flow injection set up. 5µL of Insulin was injected via injection loop. µ-PrepCell was continuously operating in optimized square wave pulse.

α–Lactalbumin

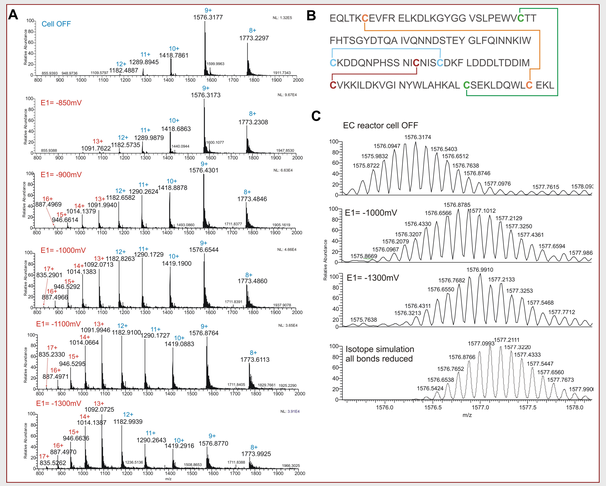

Figure 6: ***A:*** *Electrochemical reduction of disulfide bonds in α–Lactalbumin.* ***B:*** *EC reactor cell OFF (top) and ON with different E1 potentials. α–Lactalbumin sequence with indicated position of disulfide bonds.* ***C:*** *Zoom of the overlapping isotopic pattern of the +9 ion of α – lactalbumin measured with the EC reactor cell turned OFF (top), and ON at E1= -1000mV and E1= -1300mV.*

Lysozyme

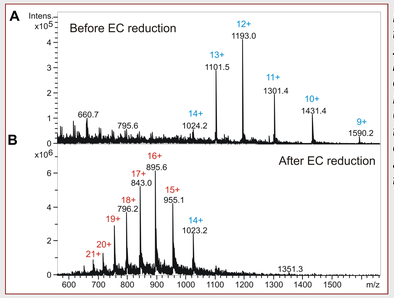

Figure 7: Electrochemical reduction of disulfide bonds in lysozyme. ***A:*** *Before reduction. A mass spectrum shows multiple charging up to the* $[M+14]^{14+}$ *ions.* ***B:*** *After reduction (Pulse: E1=-0.9V, t1=2000ms; E2=+1V, t2=1000ms). Shift to higher charges, with the appearance of a new population of the* $[M+15]^{15+}$ *to* $[M+21]^{21+}$ *species, is observed.*

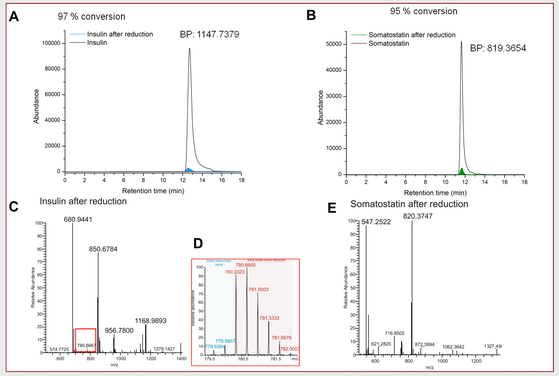

Figure 8: Somatostatin (m/z 819, ***A****) and Insulin (m/z 1147,* ***B****) before and after electrochemical reduction. Mass spectra of insulin and somatostatin after reduction, respectively* ***C*** *&* ***E****.* ***D.*** *Zoom of the [M+3H]3+ ion of chain A of insulin showing its isotopic pattern.*

Conclusions

In summary, we demonstrated new, electrochemically-based technique for efficient reduction of disulfide bonds in proteins and peptides. Compared to Magic Diamond (MD) working electrode with typical conversions rates of 20 to 30% the new proprietary [6] titanium based working electrode provides near 100% reduction of the S-S bonds in peptides and proteins, opening new opportunities for faster and superior characterization of disulfide bonds in biopharmaceuticals.

References

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[6] Patent appl. US 2014/0069822