Electrochemical Reduction of Disulfide Bonds in Proteins for Enhanced Characterization by LC-MS and HDX-MS

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1. Objective

To develop a new Electrochemical Cell for disulfide bond reduction in proteins/peptides to assure:

- Excellent long term reproducibility and stability
- Avoid contamination/fouling of electrode surfaces
- High pressure stability for use in HDX-MS

2. Introduction

Electrochemical reduction of the S-S bonds has been successfully applied for the unfolding of larger proteins such as antibodies, replacing the harsh chemical reduction that often jeopardized the H/D exchange.
Until now most electrochemical flow cells used for the reduction were based on a 3-electrode configuration consisting of:
1. Working electrode, mostly Titanium (Ti) based
2. Reference electrode, Pd/H_2
3. Auxiliary electrode, Ti or Carbon-PEEK

To fulfill the objectives mentioned above a new flow cell was constructed.

3. New: Dual Electrode Flow Cell

Figure 1: Dual electrode µ-PrepCell for S-S bond reduction consisting of:
1. Titanium inlet block
2. Platinum counter electrode

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4. Instrumental Setup and Conditions

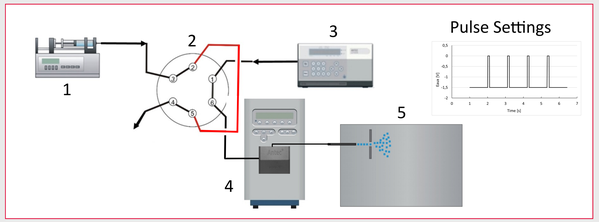

Figure 2: Schematics of Flow Injection Analysis EC-MS : 1) Sample infusion pump, 2) injection valve, 3) HPLC loading pump , 4) ROXY Potentiostat equipped with dual electrode µ-PrepCell, 5) MS.
Pulse settings: E_1 = 1.5V, t_1 = 1s, E_2 = 0V, t2 = 0.1s, ts = 40 ms

5. Results

5.1. Reduction Efficiency

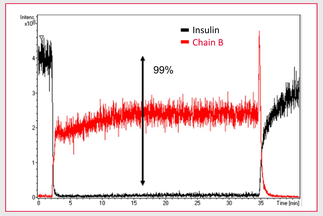

Figure 3: Reduction of insulin into chain A and B. Only chain B displayed. Insulin 10 µg/mL, diluted in 1%FA 50% Acetonitrile/ H_2O, 50 µL/min, E = 1.5 V

In Figure 3 the reduction of insulin into chain A and B is shown. Almost complete (99%) reduction of the two inter chain S-S bonds was observed as soon as the cell was turned on.

5.2. Flow Rate vs. Reduction Efficiency

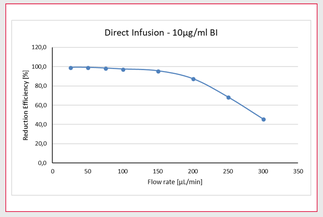

Figure 4: Influence of the flow rate through the cell on the reduction efficiency.
Optimal flow rate range: 20 - 150 µL/min

5.3. Acidity vs. Reduction Efficiency

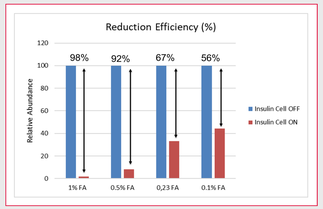

Figure 5: Influence of % Formic Acid (FA) in mobile phase on reduction efficiency. At 1% almost full reduction (98%), at 0.1% only 56%

5.4. Reproducibility

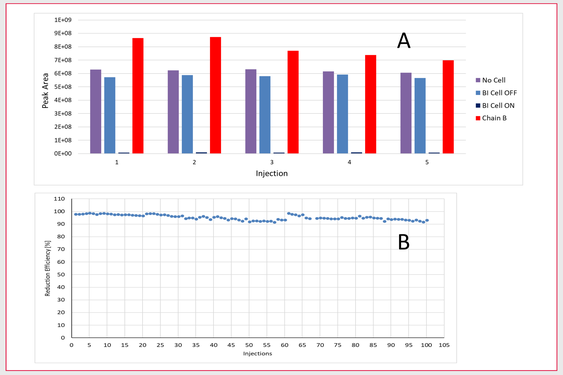

*Figure 6: (**A**) Short term reproducibility of 5 consecutive injections of insulin with no cell, cell off, cell on and chain B.*
*(**B**) Long term reproducibility of reduction efficiency measured on 100 injections of insulin.*

5.5. Cell to Cell Reproducibility

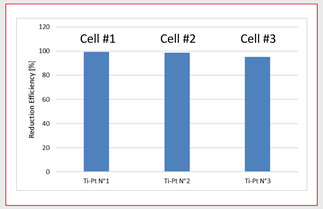

Figure 7: Reproducibility of 3 different cells, randomly assembled

5.6. Fouling of Cell and Electrode

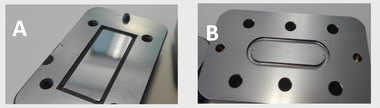

Figure 8: Picture of (A) Pt electrode and (B) Ti inlet block after several days of operation.

5.7. Reduction of Avastin® Fab Fragment

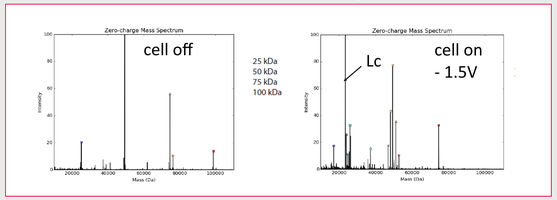

Figure 9: Electrochemical reduction of Avastin Fab digest consisting of intact 100 KDa fragment and different digest forms of 75, 50 and 25 kDa. Separated and reduced by on-line Capillary LC-EC-MS. Flow rate Cap LC: 1 uL/min + 19 ul/min makeup flow prior to on-line reduction. After reduction, clearly visible light chain (Lc) with mass of 23437.44 Da.

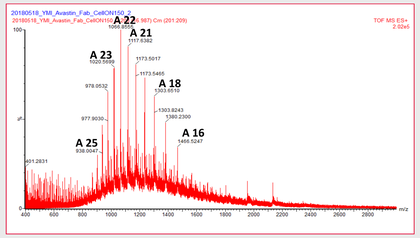

Figure 10: MS Spectrum of Lc with annotation of some charge states, A16-A25

6. Results

The new dual electrode µ-PrepCell for disulfide bond reduction in proteins provides the following features:

- Excellent reduction efficiency
- Robust and stable reduction w/o cell fouling
- Good cell to cell reproducibility

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