HDX-MS with Inline Electrochemical Reduction of Disulfide Bonds: State-of-the-Art

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Introduction

Reduction of disulfide bonds in proteins prior to MS analysis is an essential step in the determination of the disulfide bond positions, and the understanding of the proteins folding processes. Disulfide bonds also hinder MS identification of the proteins and their reduction is necessary to obtain high sequence coverage.
Electrochemical (EC) reduction of disulfide bonds integrated into the HDX-MS workflow has been shown in various publications resulting in significantly better sequence coverage.
In this poster the state-of-the-art of inline electrochemical reduction in HDX-MS workflows will be discussed on two recent examples/publications.

1. Instrumental Setup

All experiments were performed on a ROXY EC system (Antec Scientific, Boston, USA) consisting of a ROXY Exceed Potentiostat equipped with a µ-PrepCell SS. This new generation of flow cells allows working at higher pressure up to 500 bar (7300 psi) and the dual-electrode set-up (Pt working and Ti counter electrode) assures efficient and robust reduction over a longer period of time.
The new ROXY Exceed Potentiostat has further the advantage to be controlled out of different CDS, such as Empower and Chromeleon. For HDX-MS experiments the cell was placed into a HDX manager (LEAP/Trajan or Waters) consisting of a pepsin column, a trap column, and an analytical column, all cooled to 0°C and connected to a Synapt QTOF mass spectrometer (Waters, USA).

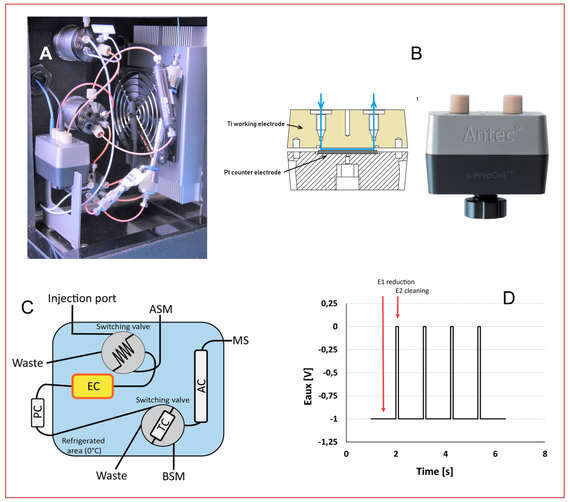

***Figure 1**: HDX manager with µ-PrepCell SS (A), schematics and picture µ-PrepCell SS (B), schematics flow path (C), and applied potential and pulse settings over EC cell (D).*

2. Influence of Buffers and HDX Additives on EC Reduction

The effect of several common protein buffers and HDX additives on the reduction efficiency of the electrochemical cell was investigated. In 1% FA the highest reduction efficiency is achieved with > 95%. In 10 mM $CaCl_2$ or 10 mM Bis-TRIS the reduction efficiency is about 90% and 70% respectively, and well acceptable.

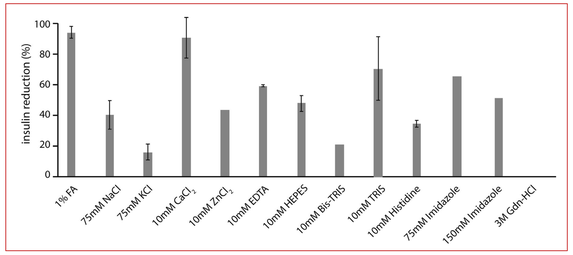

***Figure 2:** Influence of buffers and HDX additives on the efficiency of electrochemical reduction, measured on insulin. Most measurements are performed in duplicates, 10 mM TRIS is performed with n = 16. The mean with the standard deviation is shown by the error bars.*

3. Influence on Back-Exchange Using EC Reduction

Comparing the back-exchange during HDX-MS experiments using the µ-PrepCell SS was an important parameter to assess its suitability. Back-exchange levels were measured for model peptides using an HDX-UPLC system either with or without the electrochemical cell in turned OFF or ON mode. Peptides were labelled for 72 h at pH 7.4, quenched and injected into the cooled UPLC-HDX system.

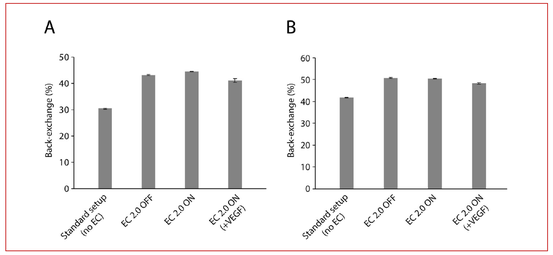

***Figure 3:** Influence of the EC reduction on the back-exchange. Samples were prepared in the exact same way as in the HDX-MS study of VEGF with the exception of the addition of 5 pmol of angiotensin II (A) and bradykinin (B) in each sample. The increase of ca. 10% in back–exchange is acceptable.*

4. Example #1

HDX-MS with electrochemical (EC) reduction enables comprehensive epitope mapping of a therapeutic antibody to the cysteine-knot containing vascular endothelial growth factor [1].

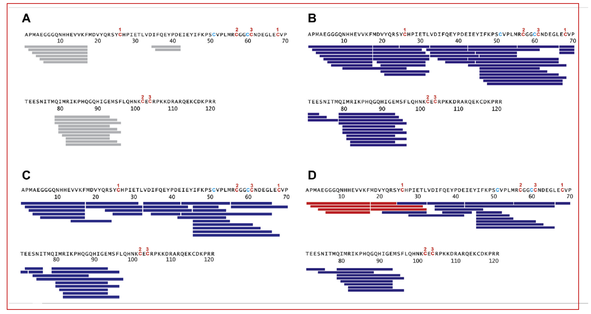

***Figure 4:** Sequence coverage of vascular endothelial growth factor (VEGF) obtained through different reduction strategies:*
(A) HDX-MS compatible reduction with 0.25 M TCEP (4 min),
(B) EC reduction in 1% FA and
(C) EC reduction in 10 mM TRIS and in the presence of the Fab fragment in a 2:1 ratio
(D) Effective sequence coverage of VEGF using EC reduction under full HDX-MS compatible conditions and labelled samples.
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. Peptides in grey are obtained by chemical reduction and EC, are shown in grey and blue respectively. Peptides shown in red are obtained by EC reduction and show decreased HDX in the presence of the Fab fragment.

Results

Electrochemical reduction (EC) provides an attractive solution to tackle disulfide-bonded proteins that are resistant to conventional chemical reduction during HDX-MS.
The new µ-PrepCellSS enables improved HDX-MS analysis with the following benefits:

- The EC reduction proceeds with high robustness and without oxidation side-reactions and the need for electrode polishing.
- The compatibility of electrochemical reduction with buffers commonly used in HDX-MS is established.
- HDX-MS with improved electrochemical reduction enables epitope mapping of a mAb to a challenging S-S bonded protein target e.g., VEGF.

Overall, our findings demonstrate that EC reduction and HDX-MS can be combined to enable analysis of the conformation and interactions of challenging disulfide-rich proteins.

[1] Gerard Comamala, et al. Analytica Chimica Acta 1115 (2020) 41-51
https://doi.org/10.1016/j.aca.2020.04.014

5. Example #2

HDX-MS based epitope mapping of antibody targeting the cysteine-rich region of IGF1R enabled by electrochemical (EC) reduction [2].

The insulin-like growth factor-1 receptor (IGF1R) has emerged as a target for receptor-mediated transcytosis, a process by which antibodies are shuttled across the blood–brain barrier (BBB). IGF1R is large and heavily disulfide bonded, and comprehensive HDX analysis was achieved only through the use of **online electrochemical (EC) reduction** coupled with a multiprotease approach, which identified an epitope for VHH-IR4 within the cysteine-rich region (CRR) of IGF1R spanning residues W244-G265. This is the first report of an sdAb binding the CRR.

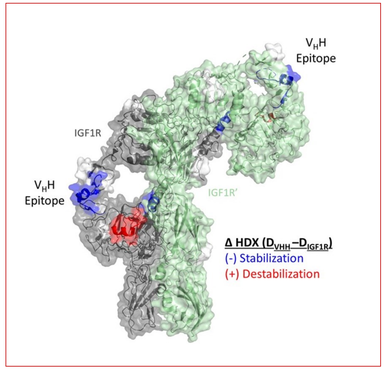

***Figure 5:** IGF1R with indication epitope V_HH and stabilization and destabilization domains.*

Results

In this paper the biophysical characterization of VHH-IR4, a BBB-crossing single-domain antibody (sdAb) is described.

- To our knowledge, this is the first known single-domain antibody (sdAb) binding to the cysteine rich-region.
- This data clearly demonstrates the utility of online EC reduction in large protein systems in both manual and automated HDX-MS operating modes.
- This platform will enable the selection of anti-IGF1R nanobodies with improved functionality, and will ultimately open up new research opportunities in the area of protein trafficking and transcytosis.

[2] Joey Sheff, et al. The Journal of Biochemistry 173.2 (2023) 95-105.
https://doi.org/10.1093/jb/mvac088