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Electrochemical Reduction of Biopharmaceuticals for Superior Characterization by HDX-MS

- **Fast and Efficient Disulfide Bond Reduction**
- **Improved Characterization of the Hinge Region of Antibodies, Cystine Knots and Other Cysteine-Rich Proteins**
- **Not affecting back-exchange**
- **Electrons instead of harsh toxic chemicals**

Introduction

Conformational changes and protein dynamics play an important role in the activity of proteins. Hydrogen-deuterium exchange (HDX) coupled to mass spectrometry (MS) is used to study the changes in conformation and dynamics of proteins following the workflow depicted in Figure 1 (1). In solution, hydrogens bound to the protein backbone exchange protons with the surrounding solvent. Hydrogen atoms exchange with deuterium when the protein is dissolved in D2O. In very dynamic regions, exchange reactions take place in the millisecond-to-second timescale while other hydrogens exchange more slowly (2, 3). The deuteration pattern is frozen by quenching the HDX reaction by lowering the pH down to pH ~2.5. The quenching solution also contains a reducing agent - e.g. tris(2-carboxyethyl)phosphine (TCEP) or dithiothreitol (DTT) - present in large concentration in an attempt to compensate for the poor reducing activity at low pH of the chemicals traditionally used (4). Immediately after quenching and reduction of the disulfide bonds, the protein sample is digested using immobilized pepsin followed by chromatographic separation and mass spectrometric analysis.

A faster and more efficient reducing protocol is necessary to maximize the performance of HDX workflows. We demonstrate here the advantages electrochemical (EC) reduction of disulfide bonds for the analysis of Insulin, a cystine knot: Nerve Growth Factor-β (NGF) and a monoclonal antibody (MAb). Faster and more efficient EC reduction at low pH results in greatly improved sequence coverage, especially in the case of TCEP-resistant proteins such as cystine knots.

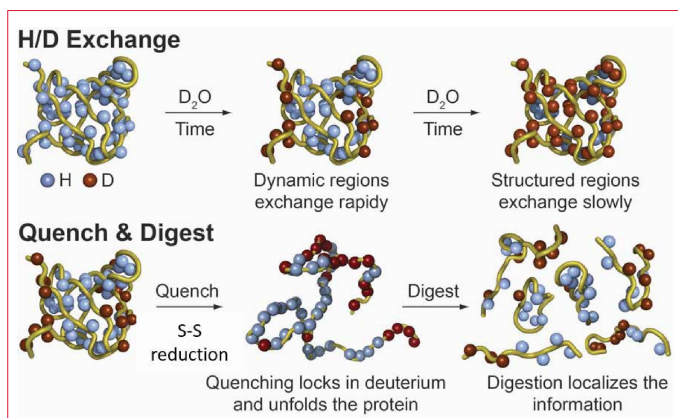**Electrochemistry Discover the difference**

Figure 1: HDX Workflow depicting the deuterium exchange and the influence of exchange time on the results. Label exchange is quenched by lowering the solution pH to ~2.5. Disulfide bond reduction is traditionally performed chemically concurrently with quenching prior to digestion with pepsin and analysis by LC-MS. In the case of EC reduction, it is performed after quenching and online with the pepsin and trap columns at low temperature. Adapted from https://mvsc.ku.edu/content/hydrogen-deuterium-exchange-mass-spectrometry.

Summary

The electrochemical reduction of disulfide bonds is fast, efficient and selective. It requires no chemicals other than those traditionally used in the LC-MS analysis of proteins and peptides, thereby making redundant the use of reducing and chaotropic agents. Reduction is performed by a method previously published (5) based on square-wave potential pulses applied to a proprietary Titanium electrode (6), allows for better reduction yields, and therefore greater sequence coverage of the protein of interest. The deuteration patterns were qualitatively retained and consistent with previously published data (7). EC reduction was successfully integrated in an automated HDX-MS workflow and proved to deliver significantly increased sequence coverage for a cystine knot containing protein and an antibody (8).

Method

All experiments were performed on a ROXY EC system (Antec, The Netherlands) consisting of a ROXY potentiostat equipped with a μ-PrepCell. The μ-PrepCell was integrated into a HDX system consisting of a pepsin column, a trap column and an analytical column μ-PrepCell, all cooled to 0 °C and connected to a Synapt G1 QTOF mass spectrometer (Waters, USA). Samples were flushed from the cooled sample loop, through the electrochemical reduction cell, the pepsin digestion column and onto the reversed-phase trap using 1% formic acid flowing at 50 μL/min (Figure 2).

The proprietary μ-PrepCell is a thin-layer electrochemical reactor cell consisting of an exchangeable working electrode (WE), a titanium auxiliary (AUX) electrode and a Pd/H2 reference (REF) electrode (6). A 150-μm spacer was used to separate the WE and the AUX inlet block. Square-wave potential pulses were applied to the WE. The ROXY EC system was controlled using the Dialogue software (Antec).
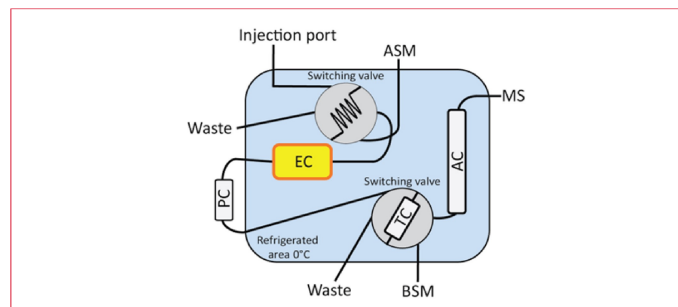

Figure 2: Schematics illustrating how the different components are connected in the HDX setup. Adapted from Mysling S. et al., Anal. Chem. 86 (2014) 340.

Reduction Efficiency

Reduction of disulfide bridges in proteins is traditionally performed using DTT or TCEP at pH ~7-8. However, HDX requires that reduction is performed at pH~2.5 to minimize deuterium back-exchange, pH at which both TCEP and DTT show limited activity (4). Most HDX users compensate TCEP's lack of efficiency at low pH by using high concentrations of TCEP with the drawback that TCEP accumulates on the LC column and hides peptides, potentially lowering the sequence coverage. EC reduction was investigated as an alternative to the conventional chemical approach using 400 mM TCEP. TCEP reduction was performed in a batch setup and aliquots were analyzed by LC-MS while EC reduction was performed online with LC-MS. The reduction yields were based on the decrease in intensity of the 6+ ion of insulin. After reduction with TCEP for 50 min, only 20% of insulin was reduced while reduction yield of virtually 100% were achieved within 15s with EC (Figure 3). It is noteworthy that the reduction yield was not affected by the low temperature of the HDX manager (7). Additionally, the use of guanidine or urea (or other protein denaturants) becomes obsolete and should be avoided due to the risk of protein oxidation (8).

Deuterium Back-Exchange

To minimize deuterium back-exchange, exchange kinetics are slowed down by performing all post-quench steps at low temperature (10 °C or lower) and rapid LC-MS analysis. Most back-exchange occurs because protic solvents are used for the analysis of deuterium levels (2, 3, 7). Proteolytic digestion is also known to increase deuterium-label back-exchange,
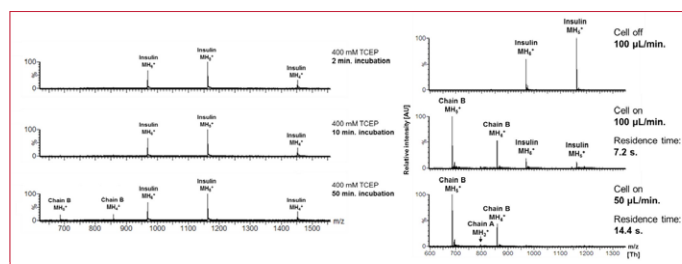
Figure 3: Comparison of the reduction efficiency of TCEP (left) and EC (right) under HDX-compatible conditions. TCEP reduction of Insulin was performed in a batch setup while EC reduction of Insulin was performed online with LC-MS. (Adapted from Supplemental Information Mysling S. et al., Anal. Chem. 86 (2014) 340).

sometime considerably, depending on the support material used to immobilize pepsin (9). Back-exchange was estimated in a previous study to vary between ~30 and 40% for insulin depending on the setup used. The use of the EC cell only slightly increased deuterium back exchange (36% vs. 28% when reduction was performed chemically) and the deuteration patterns were qualitatively retained and consistent with previously published data (7). The effect of formic acid content in the mobile phase on back-exchange was investigated in a HDX workflow where reduction was performed electrochemically (~45% back-exchange). After optimizing the flow path and positioning the cell in the cooling unit, back-exchange was down to 32% (data not shown) and compared favorably with previous studies (7).

Sequence Coverage

Often is back-exchange considered the most important parameter in HDX experiments, and practitioners satisfied with any sequence coverage they can obtain as it is understood that reduction is limited under HDX conditions. As discussed earlier, EC reduction performs optimally (near complete reduction) under acidic conditions, making the use of reducing and chaotropic agents obsolete. Furthermore, EC reduction can also easily be integrated and automated in a HDX setup.

The performance of EC and chemical reduction was compared by analyzing a commercially available MAb by HDX. Chemical reduction was performed in the sample vial with 0.25M TCEP and 3M guanidine HCl. The sequence coverage of the MAb heavy chain is presented in Figure 4. EC reduction resulted in a substantially higher sequence coverage (~90% vs. 79%) than chemical reduction.

A most important difference between the two methodologies is the lack of information between residues 200 & 240 when us-

ing chemical reduction (8). This part of the heavy chain could not be sequenced using chemical reduction, and correspond to the hinge region of the antibody, which is responsible for the flexibility of the MAb and is therefore critical to any antibody efficacy. NGF, a cystine knot containing protein that proved to be very difficult to reduce, was also analysed by HDX. Cystine knots consist of 3 disulfide bonds forming loops through each passes one of the cystine knot's disulfide bonds resulting in a very tight structure. 26 reduction protocols were investigated over a period of 2.5 years involving high TCEP concentrations, chaotropic agents, one- and two-steps reduction and reaction times up to 12h. The best sequence coverage (Figure 5) that could be achieved using chemical reduction was 46% while 99% sequence coverage was achieved using EC reduction (8) in only a few minutes. The tightly-knit structure of NGF might preclude chemicals to reach the disulfide bonds while electrons do not suffer from steric hindrance. This would explain why EC reduction is much more effective at reducing cystine knots than the chemical approach.

Conclusion

The use of an EC reactor cell has been demonstrated for the fast, automated and efficient reduction of therapeutic proteins. The µ-PrepCell was easily integrated in a HDX-MS workflow and demonstrated superior performance compared to the traditional chemical approach. The back-exchange of deuterium label did not affect the deuteration patterns, which were consistent with previously published data. Sequence coverage was greater with EC reduction than with chemical reduction for both the heavy chain of a MAb and NGF, resulting in better characterization of these proteins.
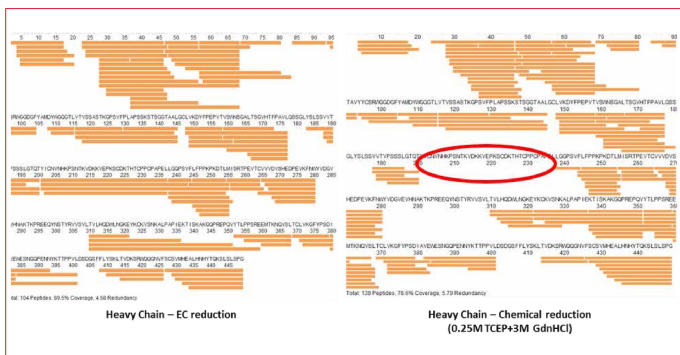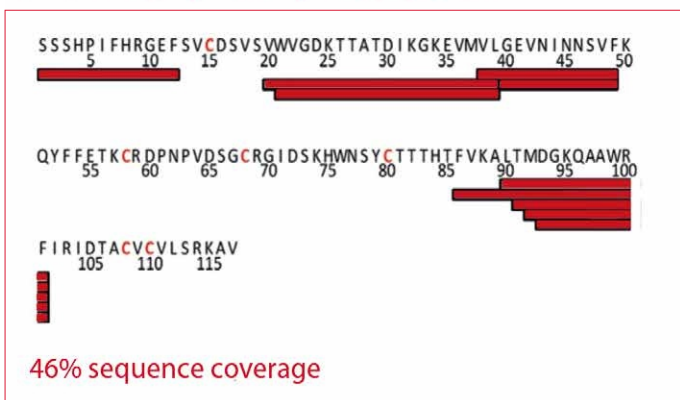
Figure 4: Sequence coverage for the Heavy Chain of a MAb with EC reduction (left) and chemical reduction (right). EC reduction resulted in a substantially higher sequence coverage (~90& vs. 79%) than chemical reduction. The most important difference between the methodologies is the lack of information between residues 200 & 240 (corresponding to the hinge region of the MAb) when using chemical reduction. (Adapted from data courtesy of K.D. Rand at the University of Copenhagen).
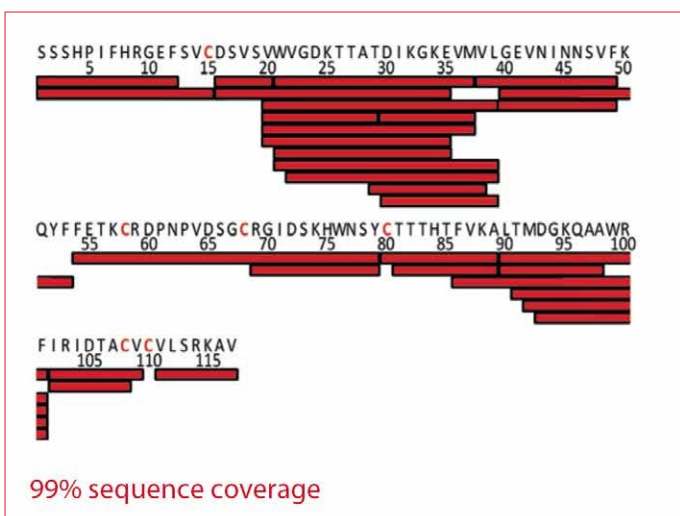

Figure 6: Sequence coverage for NGF-β with chemical (top) and EC reduction (bottom). EC reduction resulted in a much higher sequence coverage (~99& vs. 46%) than chemical reduction allowing a much more comprehensive characterization of NGF. (Adapted from Trajberg E. et al., Anal. Chem. 87 (2015) 8880).

Part numbers

211.0073	ROXY EC system for HDX
204.4304*	μ-PrepCell SS

*) Note: The presented work was performed with an older version of the μ-PrepCell.

Acknowledgements

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