Introduction
The new µ-PrepCell SS consists of a dual electrode setup, i.e., working electrode and counter electrode. This new 2 electrode cell configuration results in distinguished advantages such as: superior stability and longevity, no undesired peptide/protein oxidation during the reduction and overall much easier in use. Another advantage of the new design is the much higher pressure stability of up to 350 bar, allowing for its use in HDX-MS and pre-column LC settings (EC-LC-MS).

- Fast and efficient reduction of S-S bonds
- Ideal for on-line reduction in top-down and bottom-up MS Proteomics
- Reagent free, no interfering DTT, TCEP
- Superior peptide sequencing and S-S bond assignment

ROXY Application Note # 210_015_01
Electrochemistry Discover the difference
Cell description

The inlet block of the cell (upper part) is made out of titanium and serves as working electrode, meanwhile the black lower part contains the rectangular counter electrode made of platinum, see Figure 1.

Pulse settings

For highest stability and reduction efficiency of the S-S bonds the μ-PrepCell SS should be operated in pulse mode. In Figure 2, a typical pulse setting is shown. For most peptides/proteins including mAbs a pulse (E1) of 1 to 1.5 V is sufficient for the reduction of inter- and intra-molecular disulfide bonds.

In Figure 3 the reduction of insulin is used to illustrate the stability and robustness of the new cell. The reduction efficiency remained unaffected and > 90% for over 100 injections without the need of any electrode maintenance. The pictures shown in Figure 1 were actually taken from such a long-term experiment. The clean, shiny surfaces of both the Pt counter electrode as well as the Ti working electrode demonstrate the cleanliness of the reduction condition, resulting in overall stable and robust S-S bond cleavage.

Reduction of Avastin

In Figure 4 the spectra of the intact and reduced Avastin (bevacizumab, Roche) Fab fragment after HPLC separation on a C4 column are shown. In Figure 4A the intact Fab fragment is shown with the post-column electrochemical cell off and in Figure 4B the reduced Fab fragment is depicted with the cell on (1 V, pulse mode).

From the deconvoluted MS spectrum in Figure 4B and the two fragments with mass 23435.25 Da for the light chain and mass 24612.86 Da for the heavy chain, the reduction of both the inter- and intramolecular disulfide bonds is unambiguously confirmed.
Electrochemical Reduction of Biopharmaceuticals

Cystine knot peptides (CKPs) are among a class of bioactive peptides with a highly conserved motif of six cysteine residues forming three knotted disulfide bonds. The constrained structure of CKPs makes these compounds especially stable against normal means of degradation, including thermal, chemical, and enzymatic, establishing them as promising candidates for drug discovery as pharmacological ligands capable of modulating protein function. Traditional approaches for peptide characterization via mass spectrometry oftentimes fall short and thus alternative approaches are in demand. In this study, a combination of inline electrochemical reduction, enzymatic digestion, UV photodissociation (UVPD), chemical derivitization, and ion mobility were explored as alternatives to characterizing these customizable biotherapeutic molecules.

Figure 4: On-line LC-EC-MS of intact Fab fragment (A) and Fab fragment with electrochemically reduced S-S bonds (B). From top to bottom for (A) and (B): TIC, MS spectrum and deconvoluted MS spectrum with monoisotopic mass, measured on Orbitrap Fusion Lumos (Thermo). Courtesy: Dr. Theo M. Luider, Yesim Ikiz and Dr. Martijn van Duijn, Erasmus Medical Centre, Rotterdam, The Netherlands

Figure 5: Structures of model cystine knot peptides, (a) EETI-II (PDB: 2IT7) and (b) Linaclotide. Linear single-letter sequences with disulfide bond connectivity are shown in (c) and (d) for each peptides, respectively.
Electrochemical Reduction of Biopharmaceuticals

Target CKP compound was introduced to the mass spectrometer via direct infusion flowing through the electrochemical cell (µ-PrepCell). The applied potential was controlled via the ROXY potentiostat. With the voltage on the ROXY system turned off, the 2+ charge state of CKP appears to have an expected molecular isotope distribution (Figure 6a). With a voltage applied to the CKP, the isotopic distribution shifts to higher mass, showing that one or more disulfide bonds were reduced (Figure 6b).

Collisional activation of the target CKP without (Figure 7a) and with (Figure 7b) applied voltage in the electrochemical cell showed differences in fragmentation. For the CKP without EC voltage, identifiable fragment ions were not observed when considering disulfide bonds both intact and reduced (Figure 7c,d). Limited fragmentation was observed with the EC voltage applied when considering a non-reduced CKP (Figure 7e), but extensive backbone fragmentation was observed when considering a fully reduced disulfide bond profile (Figure 7f).

Figure 6: MS1 spectra (zoomed into the 2+ charge state) of a target CKP with three disulfide bonds with the Antec Scientific ROXY EC system inline (a) turned off to prevent reduction and (b) turned on to promote disulfide bond reduction.

Figure 7: MS2 HCD spectra of a target CKP (z=2+) with the ROXY EC system turned (a) off and (b) on. Sequence coverage of a target CKP by HCD for non-electrochemically-reduced CKP are shown (c) with assumption that disulfide bonds are intact and (d) with assumption that disulfide bonds are reduced. Sequence coverage of the target by HCD for electrochemically reduced CKP are shown (e) with assumption that disulfide bonds are intact and (f) with assumption that disulfide bonds are reduced.

Figure 8: Obtained sequence coverage for a cystine knot peptide (CKP) using different fragmentation techniques (CID, HCD, ETD, UVPD), enzymatic digestion and electrochemical reduction (EC).

HCD and CID fragmentation techniques resulted in 0% cysteine knot peptide (CKP) sequence coverage at all collision voltages. ETD resulted in reduced charge on the peptide without any fragmentation of the precursor CKP. Ultraviolet photodissociation (UVPD) of CKP resulted in 68% peptide sequence coverage. Inline electrochemical reduction (EC) resulted in 100% CKP sequence coverage with HCD fragmentation after reduction.
Reference
1. All data related to the reduction of cystine knots peptides were in courtesy of Dr. Christopher M. Crittenden, Small Molecule Pharmaceutical Sciences (SMPS), Genentech Inc., South San Francisco, California 94080, USA, and presented as poster at ASMS 2019. Title: Advances in Structural Elucidation Techniques for the Characterization of Cystine-Knot Peptides

Conclusion
With the new μ-PrepCell SS efficient and robust reduction of S-S bonds in top-down and bottom-up proteomics becomes possible in routine. In case of mAbs full reduction of inter and intra molecular disulfide bonds was achieved by using an LC-EC-MS workflow.

For the reduction of cystine knots peptides (CPK) electrochemical reduction was the only technique resulting in 100% sequence coverage in combination with HCD fragmentation.

Ordering information

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<th>Code</th>
<th>Description</th>
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<tbody>
<tr>
<td>210.0072D</td>
<td>ROXY EC system for S-S bond reduction consisting of ROXY potentiostat, dual syringe pump, μ-PrepCell SS, Pt electrode, connection kit, Dialogue Elite software. All parts included for immediate use.</td>
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