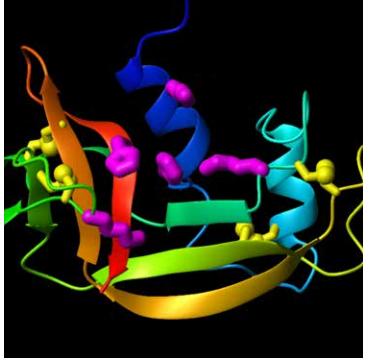
Antec Scientific

Application Note
Proteomics & Protein Chemistry



Electrochemical Reactions upfront MS – EC/MS

Proteomics & Protein Chemistry
S-S bond reduction
HDX
Peptide bond cleavage
Na+, K+ removal
Drug-protein binding

Lipidomics & Fatty Acids
Cholesterol
Oxysterol
FAME Biodiesel

Drug Metabolism
Mimicking CYP 450
Phase I & II
Biotransformation

Synthesis (mg)
Metabolites & Degradants

Pharmaceutical Stability
Purposeful degradation
API testing
Antioxidants

Environmental
Degradation & persistence
Transformation products
Surface & drinking water

Food & Beverages
Oxidative stability
Antioxidants

Forensic Toxicology
Designer drugs
Illicit drugs

Healthcare & Cosmetics
Skin sensitizers

Genomics
DNA Damage
Adduct formation
Nucleic acid oxidation

Monitoring the Electrochemical Reduction of Disulphide Bonds in Proteins for Enhanced MS and MS/MS Capabilities

Christopher A. Wootton[1] and Cookson Chiu,[1] Agnieszka Kraj,[2] Thomas Lambert,[3] Simon Lambert,[3] and Peter B. O'Connor[1]

1. University of Warwick, Coventry, UK
2. Antec, The Netherlands
3. ARC Sciences Limited, Hampshire, UK

Introduction

With the success of bottom-up proteomics in the study of entire host proteomes, mass spectrometry has become a key technique in studying whole biological systems. However, due to the inherent downsides of bottom-up proteomics; loss of post-translational modifications (PTM's), accurate protein identification, and observation of different proteoforms etc., attention has turned to the top-down approach for studying biological molecules/systems of interest.

The top-down approach requires accurate measurement of the protein's intact mass-to-charge ratio and successful fragmentation in subsequent tandem mass spectrometry (MS/MS) experiments. Top-down protein analysis and proteomics thus requires high-performance instrumentation to resolve and identify intact proteins, but also requires high-performance MS/MS to achieve extensive fragmentation of precursors of interest. Top-down protein analysis has already been shown to be able to reveal vital information on biological systems, including PTM locations (1), various proteoforms (2), mutations (3), important structural dynamics (4), and protein-protein interactions (5) to name a few. However, top-down protein analysis/proteomics can be significantly more difficult than bottom-up proteomic analysis and can be hindered by many natural factors (such as protein conformation/modification), and many user/instrument factors (such as incompatible sample preparation, challenging chromatography conditions etc.).

One particularly challenging hurdle for top-down analysis using mass spectrometry is the presence of disulphide bonds within proteins of interest. Cysteine-cysteine disulphide bonds are covalent linkages within protein structures and occur frequently in biological systems both in an intra-protein and inter-protein fashion. When studying proteins using MS/MS disulphide bonds

create cyclic regions within the protein sequence, hindering fragmentation and the production of sequence informative fragments, often creating so called "disulphide protected" areas within the protein sequence. For truly effective top-down analysis, techniques need to develop to access these areas and enable study of proteins both with and without disulphide-bonded regions.

Although combinations of chemical-based reducing and alkylating agents have been frequently utilised for both top-down and bottom-up analysis to break disulphide bonds and prevent them reforming, these reactions use very harsh reagents and conditions, which can easily damage certain protein/modification functional groups, and can easily modify analytes artificially providing misleading information during analysis and requiring further sample preparation/clean up steps downstream.

Herein a study is presented using an instrumentation-based technique for reducing disulphide bonds within protein structures, enabling effective MS and MS/MS experiments, and showing the benefits readily achievable through the use previously optimised electrochemistry (6) and allowing effective top-down protein analysis without any further sample preparation. The ROXY electrochemical (EC) system not only allowed for the reduction of disulphide bonds for accurate determination of protein cysteine content, but also enabled a large increase in subsequent MS/MS fragmentation and sequence coverage. In addition, careful analysis of isotopic patterns shows that the EC reduction process is occurring in a sequential manner depending on the electrode potential used, enabling selective and sequential reduction of disulphide bonds within complex protein architectures, which is likely to further enable the study of protein structure and disulphide bond mapping using selective reduction-alkylation experiments (7) in the near future – a key target for top-down proteomics.

Method

All experiments were performed using a ROXY EC system (Antec, The Netherlands) consisting of a ROXY potentiostat, equipped with a µ-PrepCell2.0 fitted with a proprietary Ti-Blue electrode, and a syringe pump. The use of the newly optimised Ti-Blue electrode enabled the use of pure DC-mode electrochemistry, moving away from previously used pulsed-mode setups, and allowing use of the ROXY system readily with any mass spectrometer, rather than just fast-scanning setups which needed to keep up with the alternating pulses in pulsed mode. The Ti-Blue electrode was used for all reduction experiments and was found to be effective for many hours of continuous use, without pulsing and/or cleaning. The new µ-PrepCell2.0 is a thin-layer electrochemical cell consisting of exchangeable Ti-Blue working electrode (WE), a conductive polymeric auxiliary electrode (AUX), and a Palladium/hydrogen (Pd/H2) reference electrode (REF).

The ROXY-EC system was connected directly to a SolariX FT-ICR ultra-high resolution mass spectrometer (Bruker Daltonics, Bremen, Germany), equipped with a 12 Tesla superconducting magnet and an Apollo II ion source. The ROXY EC system was controlled using the Dialogue Elite software (Antec). The use of electrical grounding unions between the ROXY system and the ESI source were also not needed with the latest configuration, reducing the dead-volumes present within the system and allowing faster transitions between adjusting EC conditions and detecting the changes.

ESI flow rates were in the order of 1-2µL/minute, with minimal PEEK tubing between the sample syringe, µ-PrepCell, and ESI needle, transitions between voltages were observed at the MS within ~2 minutes.

Figure 1: ROXY EC system equipped with the µ-PrepCell2.0 and a proprietary Ti-Blue electrode was coupled with a Bruker SolariX 12T FT-ICR Mass Spectrometer for accurate protein MS and MS/Ms analysis

Results and discussion

Heavily disulphide-bonded proteins (such as lysozyme, RNAse A, and antibodies etc.) are readily ionised and observable using ESI-MS, but are often observed in lower charge states and more narrow charge state distributions compared to non-disulphide bonded analogous species due to their compact structures held together by these cysteine linkages. As a result of these effects observation of high charge states for lower limits of detection and for more extensive fragmentation in MS/MS experiments is often limited. In order to increase the charge of proteins in ESI-MS it is common practice to use acidified and/or denaturing solvents, however for heavily disulphide bonded proteins this can often have little effect. The standard ESI-MS spectra of denatured Lysozyme and RNAse A are shown in Figures 2a and 2c respectively.

Even in denatured conditions Lysozyme and RNAse A are only observed in relatively low charge states (Figure 2a and 2c respectively). However reducing the disulphide bonds in the protein structure enables more extensive denaturation of the otherwise compact protein structure, and allows the creation of much higher charge state protein ions in subsequent ESI-MS. The enhanced charge states for the Lysozyme and RNAse A species observed allow more sensitive detection of protein ions and allow access to higher charge states for subsequent MS/MS characterisation.

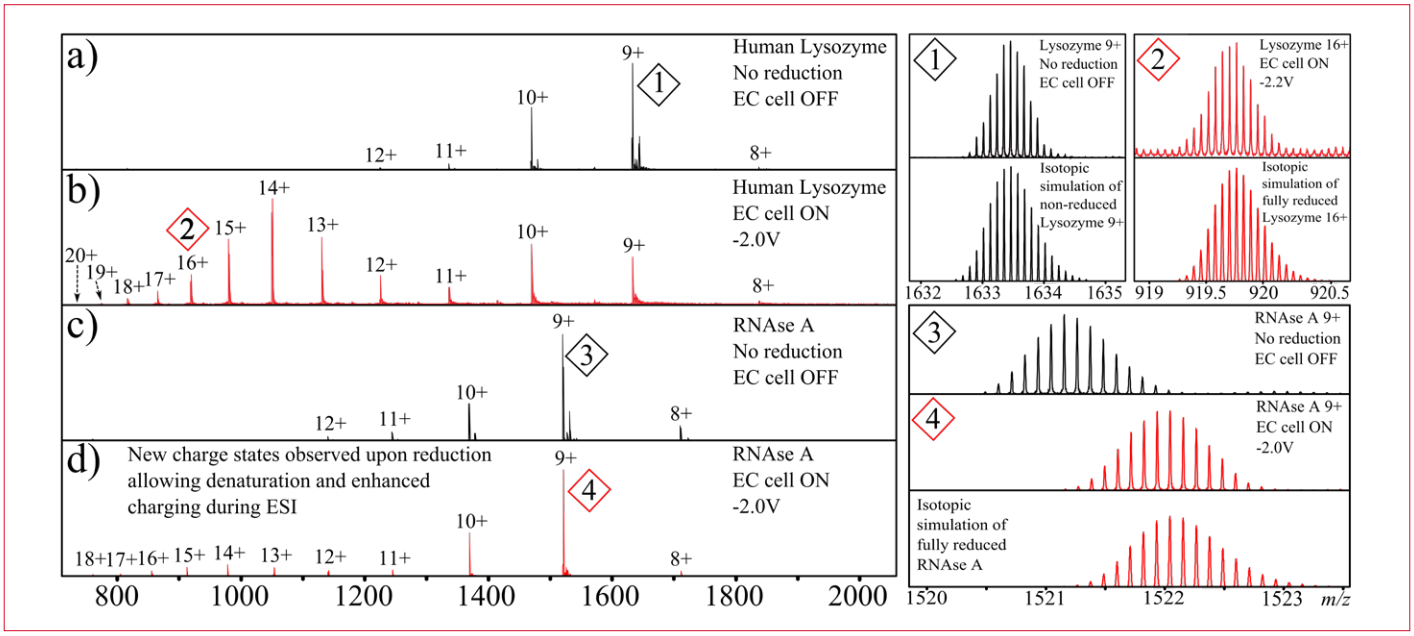


Figure 2a-d: ESI-MS spectra of Lysozyme under normal denaturing conditions (a) and then reduced using the ROXY EC system (b) and of RNAse A under normal denaturing conditions (c) and then reduced using the ROXY EC system (d)

Monitoring the reduction process

For species such as Insulin (6) and antibodies (8) the reduction of disulphide bonds results in the separation of separate peptide chains, causing distinctly different species to form and be observed in the resulting mass spectrum. For intramolecularly disulphide bonded species such as Lysozyme and RNAse A, reduction of disulphide bonds results in an addition of two nominal mass units (2H, 2.015650 Da). Due to the extended isotopic pattern of larger biomolecule species, separating the new reduced species (+2H) isotopic pattern from that of the unreacted (non-reduced) species is particularly challenging, even for ultra-high resolution instrumentation. Instead a statistical approach was adopted in which each individual isotope and its intensity was measured from each charge state observed for each reducing potential produced from the ROXY EC system, constituting ~2500 isotopes measured. At certain reducing potentials some disulphide bonds were reduced, while others remained, the isotopes could then be used to calculate how many disulphide bonds had been reduced by comparing the isotope intensities with that of the control (unreduced) isotopic pattern.

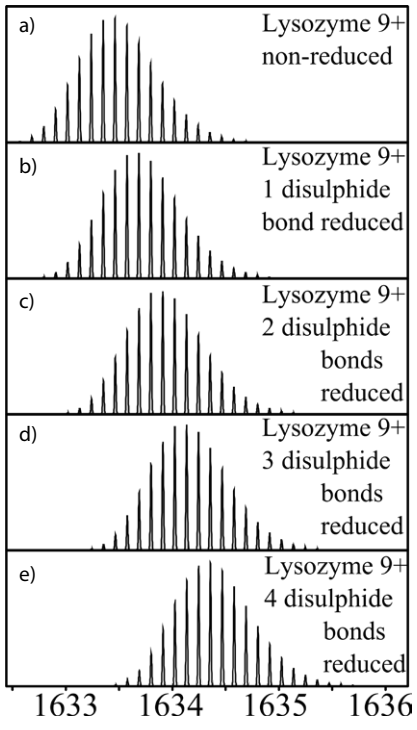


Figure 3: Isotopic pattern simulations of non-reduced Lysozyme (a) and reduced Lysozyme with 1 (b) 2 (c) 3 (d) or 4 (e) disulphide bonds reduced.

Isotopic peak measurements were weight-averaged and then scaled according to their difference between the non-reduced protein form (e.g. Figure 3a for Lysozyme) and the fully reduced protein form (Figure 3e for lysozyme), this scaling would provide a percentage of reduction measurement, which was easily converted to number of disulphide bonds via division with the number of bonds in the protein of interest; e.g. The 4 disulphide bonds in Lysozyme equates to 25% reduction per disulphide bond).

This analysis was carried out for each charge state of each protein, for each reducing potential over a wide range. The results (shown below in Figure 4) show that the reduction process is sequential, and that disulphide bonds can be selectively reduced by applying the correct potential. The results also show that as disulphide bonds are reduced they are able to unfold under the denaturing conditions used (50/50 v/v water/ACN +1% formic acid) and attain higher charge states during the ESI process.

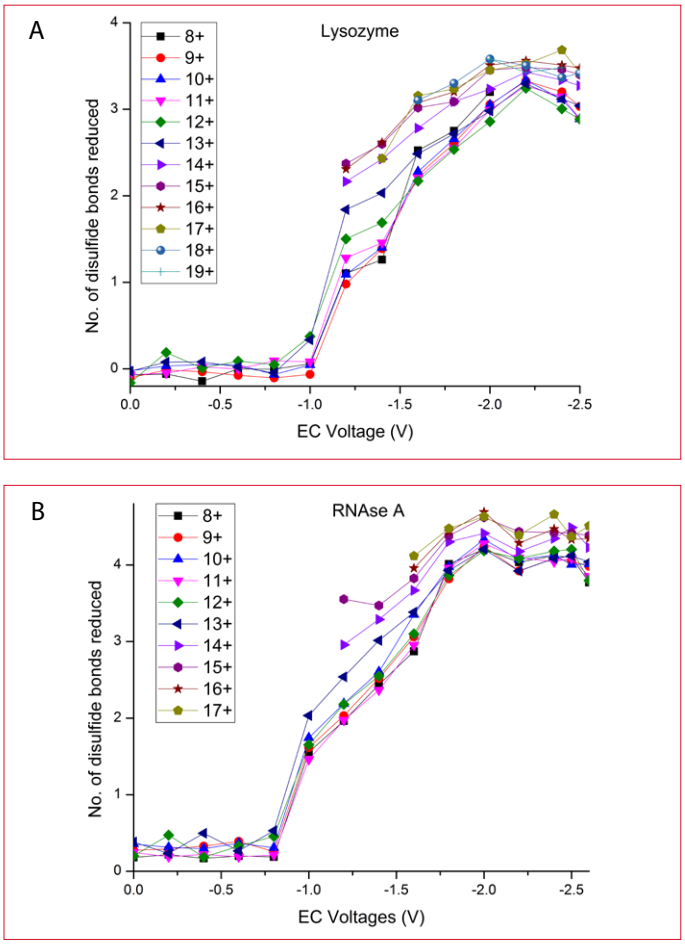


Figure 4: Number of disulphide bonds reduced as a function of EC potential using the ROXY EC system to reduce Lysozyme (A) and RNAse A (B) each charge state is shown separately to show transitions of proteins to higher charge states upon reduction.

Figure 4 clearly shows the more reduced protein species occupy the majority of the higher charge states, while the less reduced species still reside within the lower charge states more naturally seen for the control spectra. This approach also demonstrates the inaccuracy with using one charge state to monitor the reduction process, as reduced species adopt more highly charged states, monitoring a single, lower charge state from the control spectrum would lead to an underestimate of the overall reduction taking place.

Full reduction of all four disulphide bonds in each protein can be observed in each set of spectra. The RNAse A protein species was confirmed to be fully reduced at a potential of -2.0V, while the Lysozyme spectra showed fully reduced peaks (at certain charge states) at a potential of -2.2V. The average number of disulphide bonds reduced at a given potential, using the findings shown in Figure 4, are shown in Figure 5, emphasising the differences for the two proteins studied.

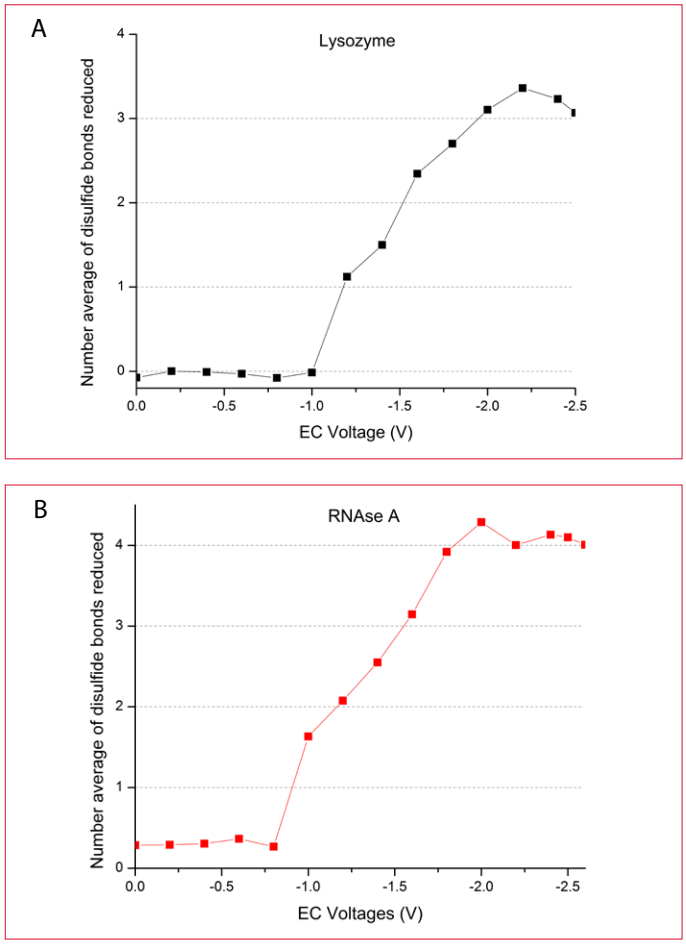
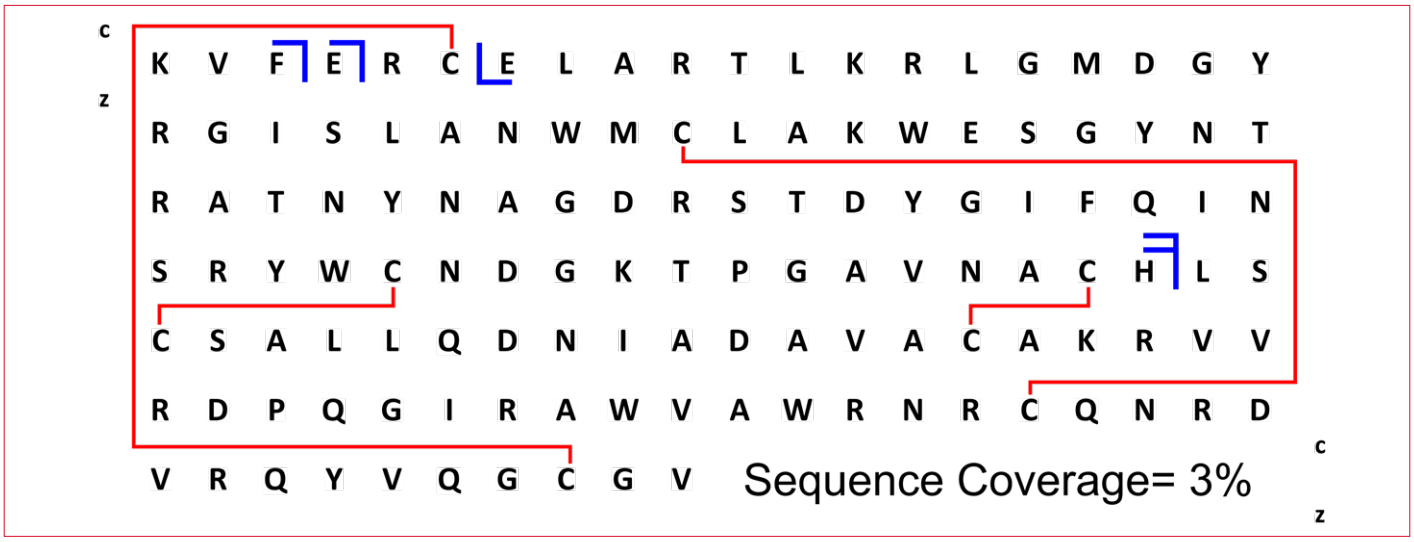


Figure 5: Average number of disulphide bonds reduced at each potential using the ROXY EC system for Lysozyme (black A) and RNAse A (red B)

Improvements to MS/MS efficiency and sequence coverage

Higher charge states have been shown (9) to provide more extensive fragmentation during MS/MS experiments, and thus improve sequence/cleavage coverage and overall protein characterisation. These benefits are also highlighted for the species observed above. The Lysozyme species detected were subjected to ECD MS/MS fragmentation in both their non-reduced and reduced forms (Figure 6a and 6b, respectively). There is a clear enhancement in fragmentation and detection of sequence informative fragments. An increased sequence/cleavage coverage of 22% (7-fold increase) for Lysozyme was observed, allowing much more extensive characterisation of the protein species, including "di-sulphide protected" regions.

Before ROXY EC reduction



After ROXY EC reduction

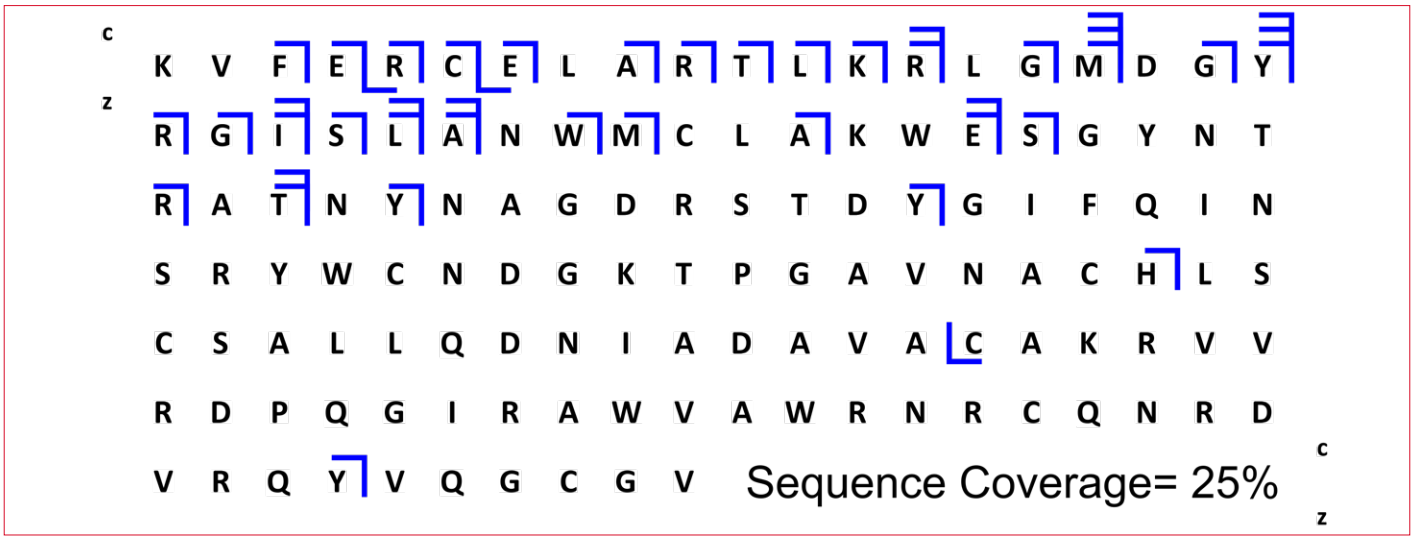


Figure 6: ECD MS/MS coverage of Lysozyme under using denaturing conditions (a) and then reduced using the ROXY EC system (b). Disulphide bonds are shown in red, MS/MS cleavages in blue.

References

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Conclusion

Disulphide bonds within protein architectures, though critical for biological function, present distinct challenges for effective MS and MS/MS characterisation of target proteins. Electrochemical reduction of disulphide bonds using the ROXY EC system provides an effective, chemical free method for reduction which requires no further sample preparation and can provide complete disulphide bond reduction in many protein targets.

Herein this reduction process was monitored using individual isotopes from each charge state observed for model proteins human Lysozyme and RNAse A, heavily disulphide bonded, medium sized proteins. The ROXY EC reduction was shown to occur in a sequential process, and could be used to selectively reduce certain disulphide bonds in stages. The reduced protein species were shown to be more effectively ionised during ESI-MS and attain much higher charge states than non-reduced species. These reduced species were shown to fragment much more readily in subsequent MS/MS experiments and showed over a 7-fold increase in sequence/cleavage coverage compared to the non-reduced samples. The ROXY EC system is an effective tool for top-down analysis and can greatly assist in protein identification and characterisation.

ROXY EC system consisting of dual syringe pump, ROXY Potentiostat and Dialogue software

New µ-PrepCell2.0 made of conductive polymeric inlet block (top), enables to work in simple DC-mode for efficient reduction (no more pulsing).

New TiBlue working electrode in blister package, set of 2 electrodes/blister

Ordering information

210.0072B	ROXY EC system for S-S reduction, consisting of: ROXY Potentiostat, dual syringe pump, µ-PrepCell2.0 with pre-mounted TiBlue electrode and Dialogue software
210.5010A	TiBlue working electrode in blister package, set of 2

For research purpose only. The information shown in this communication is solely to demonstrate the applicability of the ROXY system. The actual performance may be affected by factors beyond Antec's control. Specifications mentioned in this application note are subject to change without further notice.

Antec Scientific (USA)
info@AntecScientific.com
www.AntecScientific.com
T 888 572 0012

Antec Scientific (worldwide)
info@AntecScientific.com
www.AntecScientific.com
T +31 71 5813333

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