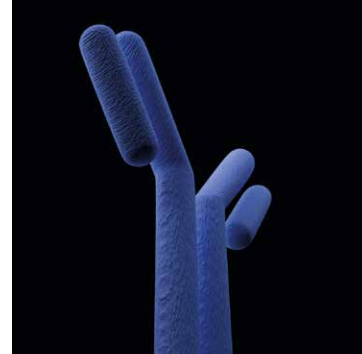
Antec® Scientific

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

A novel Approach to Antibody Engineering by Electrochemistry

- **Electrochemical reduction, conjugation and re-oxidation of antibodies**
- **Green technology – electrons instead of toxic chemicals**
- **Selective – generate light and heavy chains**
- **Fast – seconds vs. hours with chemical approaches (e.g. TCEP, DTT)**

Introduction

Antibodies are routinely used in molecular biology and medical research, but, with over 30 antibodies currently approved for clinical use, they already demonstrated their advantages as therapeutics for the treatment of conditions such as a range of cancers, Crohn's disease or transplant rejection. While antibodies are generally considered safer drugs than traditional small molecules, they also tend to show great efficacy. However, antibodies also suffer drawbacks such as the costs for developing new antibodies as well as interactions with the immune system. Currently, much effort is directed to optimize the selectivity and efficacy of antibodies toward a type of cells or molecules to optimally target a disease (1).

While antibodies were first produced using various cell lines after cloning the antibody genes, it is now possible to create chimera by fusing murine variable domain and human constant domain. A number of drugs on the market also consist of only a part of an antibody, a Fab fragment (that can be PEGylated), a Fc portion and a receptor, or a complete antibody bound to a cytotoxic compound (Antibody-Drug Conjugate or ADC). Recent technological developments have also allowed to generate antibodies binding two different antigens or bispecific antibodies (2).

Here, we demonstrate the capabilities of electrochemistry in reducing, conjugating and re-oxidizing complex proteins such as antibodies without affecting their binding affinity. Such an approach is likely to prove itself as a major technological advancement not only in the analysis of antibodies but also in the design of novel antibody-based therapeutics, such as bispecific antibodies or antibody-drug conjugates.

Summary

This novel electrochemical approach provides a fast and efficient method for the selective reduction, conjugation and re-oxidation of antibodies, thereby enabling to manipulate them with minimum chemicals. The reduction of antibodies into its Light Chains (L_C) and Heavy Chains (H_C) (Figure 1) is performed by a method previously published based on square-wave potential pulses applied to a proprietary Ti electrode (3,5). Subsequently, the free cysteine generated by the electrochemical reduction of the antibody can then be conjugated. Finally, the free cysteine on both L_C and H_C can be very rapidly and selectively re-oxidized to reform the antibody without any loss in binding affinity.

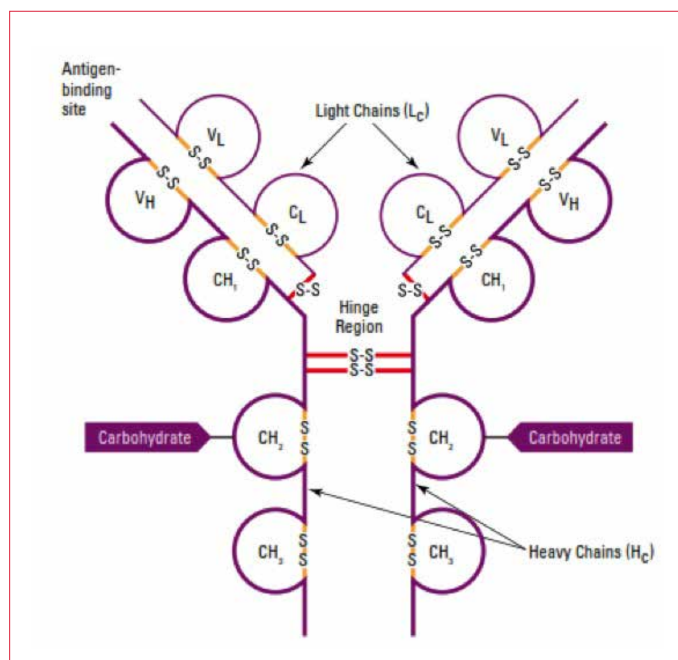


Figure 1: Schematic representation of an IgG1-type antibody, showing the 2 light chains (L_C), 2 heavy chains (H_C) and the position of the different disulfide bonds (DBs). There are 4 inter-chain DBs (red color) with two DBs located in the hinge region, connecting the two H_Cs, and two DBs connecting the L_C to the H_C. Additionally, there are 12 intra-chain DBs (yellow color), 2 in each of the L_Cs and 4 in each of the H_Cs.

Method

All experiments were performed using a ROXY EC system (Antec, The Netherlands) consisting of a ROXY potentiostat, equipped with a µ-PrepCell and a syringe pump. The ROXY system was used in two different setups (Figure 2), where the µ-PrepCell outlet was either coupled to a Premier QTOF mass spectrometer (Waters, USA) or samples were collected for further reaction or analysis.

The µ-PrepCell is a thin-layer electrochemical cell consisting of an exchangeable working electrode (WE), a titanium auxiliary electrode (AUX) and a Pd/H2 reference electrode (REF).

A proprietary titanium-based WE was used for efficient and selective reduction (3-5), while a Boron-doped diamond (BDD) WE was used for the re-oxidation of free cysteine into disulfide bonds. The ROXY EC system was controlled using the Dialogue software (Antec). An electrical grounding union was used to decouple the electrochemical cell from the ESI high voltage.

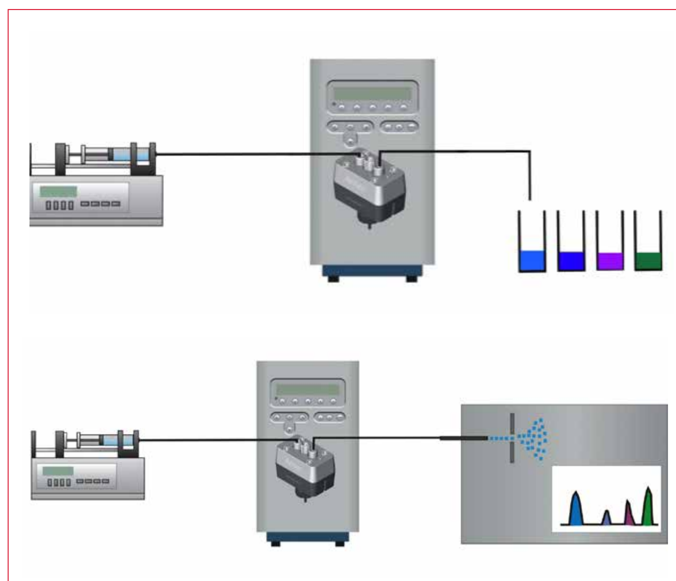


Figure 2: Schematic representations of the two experimental set-ups. Top: A syringe pump was used to deliver the sample solution to the µ-PrepCell positioned in the ROXY potentiostat before collecting fractions. Bottom: A syringe pump was used to deliver the sample solution to the µ-PrepCell positioned in the ROXY potentiostat and to the mass spectrometer.

Reduction

The electrochemical reduction of an antibody (Figure 1) results in the generation of L_Cs and H_Cs as exemplified by the spectra shown in Figure 3. Infusing a 0.2 mg/mL solution of antibody (prepared in 1% formic acid in water) in the mass spectrometer with the µ-PrepCell turned OFF (Figure 3A) resulted in a charge envelope spreading between m/z 2200 and

4000, with the intact antibody carrying approximately 40 to 60 charges. When the cell was turned ON (Figure 3B), a shift of the antibody charge envelope toward lower m/z (~1000-2500) was observed. The spectrum (Figure 3C) results from the deconvolution of the MS signal obtained with the electrochemical cell OFF and confirms that the antibody is intact. The spectrum in Figure 3D was obtained with the cell turned ON, and mostly shows species with molecular weights of approximately 25 and 50 kDa corresponding to the masses of L_C and H_C. The very low intensity of the MS signal for species with molecular weights greater than 50 kDa (75, 100, 125 and 150 kDa corresponding to the masses of L_C-H_C, H_C-H_C, H_C-H_C-L_C, and intact antibody) further confirms the selective reduction of the antibody inter-chain disulfide bonds in high yields. More intense reductive pulses would be necessary to reduce intra-chain disulfide bonds as well.

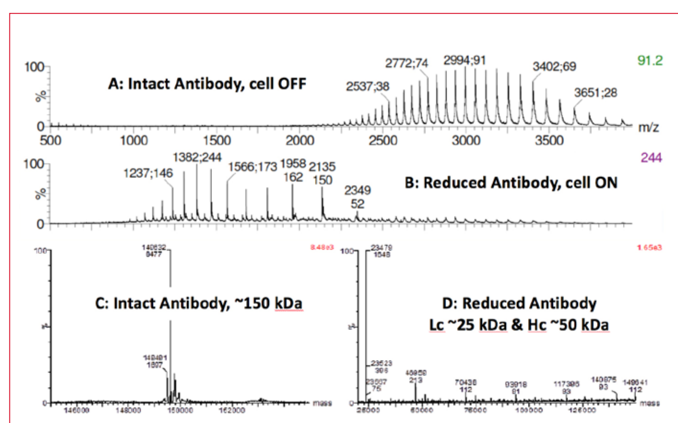


Figure 3: High Resolution Mass Spectra of a Monoclonal Antibody obtained with the electrochemical cell OFF (A) and ON (B). C and D are the deconvoluted spectra of the intact (A) and the reduced antibody (B) respectively

Conjugation

In order to prove that the L_Cs didn't rearrange instantaneously after reduction and the free cysteines remained available for conjugation, fractions were collected at the cell outlet, diluted in 0.1M phosphate buffer (pH 7.8) containing 5mM EDTA and subsequently conjugated using Maleimide-PEG2-Biotin.

The shift in mass of 526 Da (Figure 4) observed in the deconvoluted mass spectrum confirms that the L_C was conjugated and thus that the free cysteine resulting of the reduction remained free under acidic conditions and did not rearrange. The reduced antibody was kept in acidic medium for several hours without any reformation of the disulfide bonds being observed, therefore conjugation doesn't need to be performed directly after fraction collection and could be performed whenever convenient.

The conjugation yield was estimated based on the relative intensity of free and conjugated L_Cs in the deconvoluted mass spectrum to be ca. 90%. No control experiment was performed to determine the yield of conjugation of H_C with Maleimide-PEG2-Biotin but is expected to be similar to that of L_C.

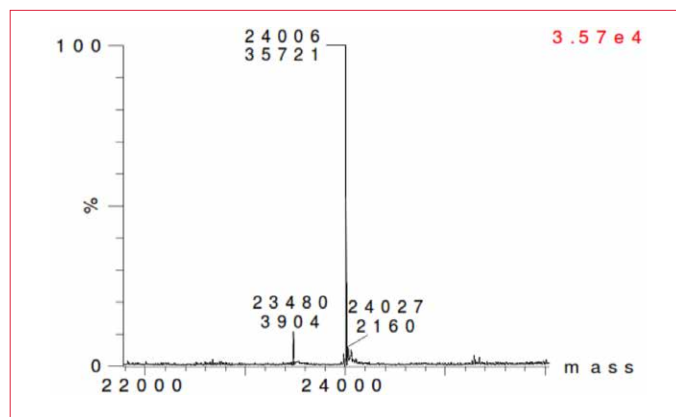


Figure 4: Deconvoluted spectrum of the light chain of an antibody after conjugation with Maleimide-PEG2-Biotin. Two peaks can be seen (L_C & conjugated L_C) with a mass difference of 526 Da indicating that conjugation with Maleimide-PEG2-Biotin occurred.

Conclusion

The use of an electrochemical reactor cell on- and off-line with MS for the easy, fast, efficient and specific electrochemical treatment of antibodies has been successfully demonstrated. It allowed the efficient and selective reduction of a monoclonal antibody into its light and heavy chains, the resulting free cysteine on the L_C remaining free for conjugation.

The formed L_C and H_C could be re-oxidized to selectively reform the antibody in high yield. Electrochemical treatment did not affect the binding affinity of antibodies, while eliminating the need for harsh chemicals and additional tedious cleaning steps.

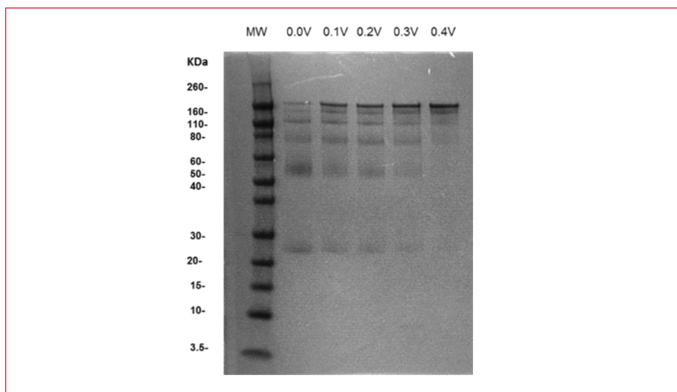


Figure 5: SDS gel showing the re-oxidation of a reduced antibody at different potentials using the uPrepCell equipped with a Boron-Doped Diamond (BDD) electrode. The sample was prepared in phosphate buffer at pH 7.8, and the flow rate used was 20 µL/min. Increasing positive potentials resulted in reforming the antibody in increased yields

Re-Oxidation

The same fractions collected after electrochemical reduction and diluted in 0.1M phosphate buffer (pH 7.8) were subjected to electrochemical oxidation at varying potential (0.0 to 0.4V at 20 µL/min) using a boron-doped diamond electrode. Fractions were again collected and further analyzed by SDS-PAGE (Figure 5). Band 1 shows the molecular weight markers, lanes 2 to 6 show samples subjected to increasing oxidizing potential of 0.0, 0.1, 0.2, 0.3 and 0.4V. The different band patterns show that even under very mild oxidizing potentials, L_Cs and H_Cs reformed their inter-chain disulfide bonds. Applying a potential of 0.4V allowed to produce in high yields an antibody with the same mass as the original intact antibody.

To prove that the electrochemical reduction and re-oxidation of the disulfide bonds didn't affect the antibody's binding activity, three antibodies were analyzed by ELISA, an antibody that was subjected to electrochemical reduction and oxidation, one that was also conjugated, and finally one that didn't undergo any electrochemical modification. The curves obtained in ELISA (Figure 6) for the three different antibodies show excellent agreement, thereby concluding that electrochemical treatment of antibodies does not affect their binding activity nor their biological activity. Additionally, keeping the reduced antibody for several hours in acidic medium did not affect its binding activity, giving the opportunity to scientists to time their work conveniently.

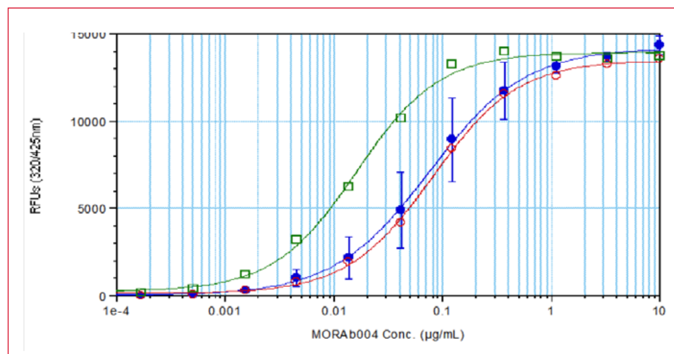


Figure 6: Evaluation of the ligand binding affinity by ELISA. The three traces were obtained with the same monoclonal antibody as starting material in its native form (green), electrochemically reduced and re-oxidized (red and blue)

Acknowledgements

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References

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4. Nicolardi S. et al., Anal Chem. 86 (2014) 5376
5. US & European patents and patent applications: Patent EP2706066A1, Patent US20140069822

Part numbers

210.0072A	ROXY EC system for S-S reduction
204.4300T	µ-PrepCell Ti incl. kit

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