Protein disulfide bond mapping using online LC–Electrochemistry–MS applied to the characterization of notch3 protein fragments

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The identification of large numbers of proteins is, without doubt, powerful and useful, yet not sufficient in providing a full description of the proteome and its interactions. To this end, the analysis of protein isoforms, or proteoforms, experiences a growing interest in mass spectrometry (MS)-based proteomics. Post-translational modifications (PTMs) can be considered as an "additional layer" of information similar to the epigenetic code that may switch a gene on-and-off. Disulfide bonds, or "S–S bridges", are an important PTM because these bonds stabilize the three-dimensional structure of a protein and as a result are crucial elements for their biological function.

For example in CADASIL (Cerebral Autosomal-Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy), missense mutations in the NOTCH3 gene remove or introduce a cysteine residue and disrupt the triply-paired structure of the EGFR domains of the notch3 protein, see Figure 1. (*1*) This renders these proteins, which are expressed in vascular smooth muscle cells, prone to aggregation, a phenomenon believed to cause dementia and stroke in CADASIL patients. Currently, antisense oligonucleotide (AON) drug treatment is being developed based on exon skipping to generate a notch3 protein with an even number of cysteines and a predicted restored EGFR domain that should not form aggregates. In early phase research, cell models are used to produce skipped notch3 protein fragments to assess the aggregation properties and efficiency of exon skipping. Analytical

methodologies for the assessment of the presence and connectivity of disulfide bonds may contribute to the development of successful exon skipping strategies.

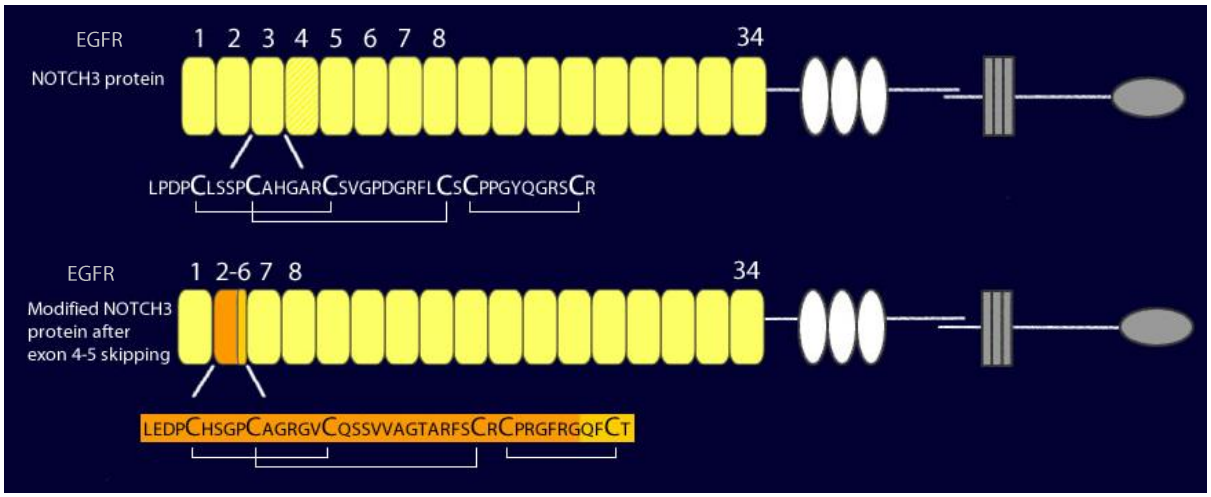

Figure 1, Schematic representation of wild-type and skipped notch3 protein.

Bottom-up proteomics is the most widely used technique for the analysis of proteins. However, the presence of disulfide bonds complicates the identification of peptides and proteins because it often hampers peptide backbone characterization by MS/MS. As a consequence, offline reduction of disulfides with a reagent, such as dithiothreitol, has become a pivotal step in obtaining full sequence coverage of a protein in a bottom-up experiment. However, this type of approach leads to a loss of information regarding the presence and connectivity of disulfide bonds. An alternative has recently been reported for top-down analysis of intact large peptides and small proteins that involves online reduction of disulfide bonds via electrochemistry coupled directly to high-resolution MS. In this way, the protein sequence coverage of lactoglobulin, lysozyme, oxytocin and hepcidin was increased and the disulfide bonds were mapped. (*2*, *3*)

In the current study, we further applied electrochemistry to overcome disulfide bridge complexity in the analysis of proteins. To this end, an electrochemical cell (microprep-cell, Antec) was used online *following* liquid chromatography (LC) and *prior to* electrospray ionization–Fourier transform ion cyclotron (ESI–FTICR) mass spectrometry, see figure 2. In order to prevent disulfide reshuffling proteins were digested at low pH, obviously without prior reduction and alkylation to keep the disulfide bonds intact. The (disulfide-linked) peptides from the digest were then separated using LC and passed through the electrochemical cell to the mass spectrometer. By performing two analyses, one run with online

electrochemistry-assisted reduction of disulfide bonds and one run without, peptides that initially contained S–S bridges could be identified based on accurate mass analyses. Additional LC–MS/MS experiments including online electrochemical reduction were performed for further validation of the identified disulfide-linked peptides.

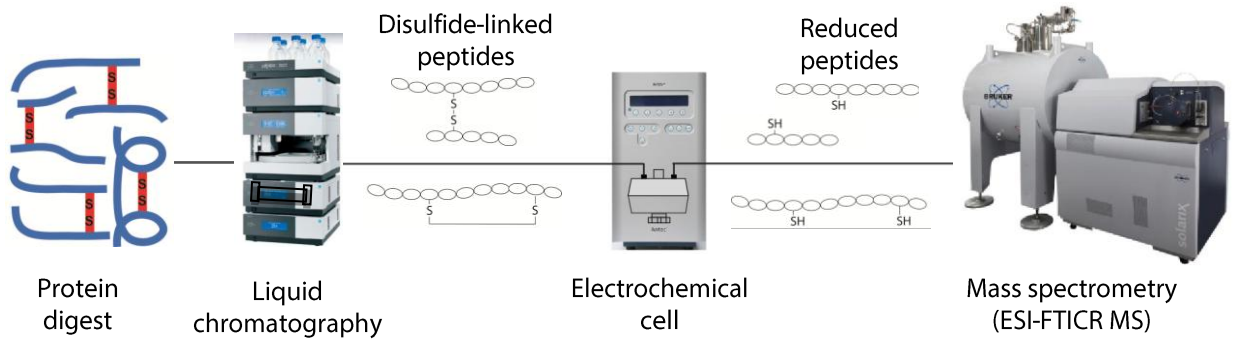

Figure 2, LC–Electrochemistry–MS system for online reduction of disulfide bonds.

The method was optimized and tested with two standard proteins, beta-lactoglobulin (18.3 kDa, 2 disulfide bonds, 1 free cysteine) and ribonuclease B (13.7 kDa, 4 disulfide bonds). Digestion of these proteins resulted in several types of disulfide-linked peptides, including an intrachain disulfide peptide and interchain disulfide peptides with two or three peptides connected via one or two disulfide bonds, respectively. All types of disulfide-containing peptides were detected and characterized using our online LC–Electrochemistry–MS system. Fragmentation of the disulfide-containing peptides resulted in characteristic double cleavage fragment ions, including S–C cleavage leading to fragment ions -32 or +32 Da (– or + sulfide), see figure 3.

Current experiments include the analysis of larger proteins containing more disulfide bonds and mixtures of disulfide bond-containing proteins. Moreover, in order to deal with the large amount of complex MS data that is generated with such experiments, we are developing a software tool to aid in the identification of disulfide-linked peptides. And finally, notch3 protein fragments, such as EGFR1-5 wild-type and EGFR1-9 skip, are under investigation.

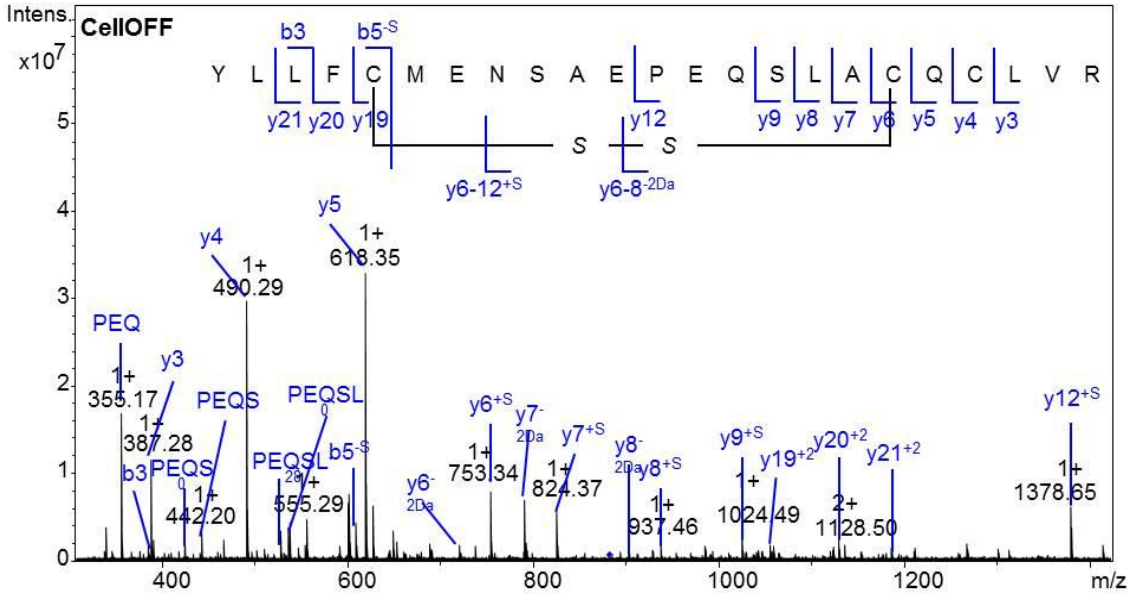

Figure 3, annotated MS/MS spectrum of an intrachain disulfide peptide (m/z 882.77 $^{3+}$) from beta-lactoglobulin.

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References:

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