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Dual reductive/oxidative electrochemistry/liquid chromatography/mass spectrometry: Towards peptide and protein modification, separation and identification

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ABSTRACT

A new hyphenated technique based on on-line dual (oxidative and reductive) electrochemistry coupled to liquid chromatography and high resolution electrospray mass spectrometry is presented. Two liquid streams are combined, with one containing a disulfide, which is reduced to the respective thiol in an electrochemical cell based on a titanium working electrode. The other stream contains phenol, which is electrochemically activated to benzoquinone on a boron-doped diamond working electrode. Upon combination of the two streams, a Michael addition takes places, leading to the covalent coupling of thiol to quinone. In continuous flow, the reaction mixture is transferred into an injection valve and the products are separated by reversed phase liquid chromatography and detected by electrospray-high resolution mass spectrometry. Proof of concept is demonstrated for low molecular mass disulfides and peptides, but further optimization will be required in future work to achieve efficient protein labelling.

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1. Introduction

The identification of proteins can either be carried out by identifying proteolytic peptides (bottom-up approach) or by analyzing intact proteins (top-town approach). One crucial step in both respective workflows is related to the reduction of disulfide bonds. These covalent dimers of the amino acid cysteine are the most common posttranslational modification of proteins (∼19% of all proteins contain multiple disulfide bonds [1]) and are important for their tertiary structure [2,3]. Within bottom-up experiments, the disulfide bonds have to be reduced quantitatively prior to enzymatic digestion in order to obtain the highest digestion yield possible [4]. In the top-down approach, reduction of disulfide bonds is necessary to achieve sequence information obtained from collision-induced dissociation (CID) experiments [5]. In contrast, methods as electron capture dissociation (ECD) [6] and electron transfer dissociation (ETD) [7] are capable of cleaving both disulfide bonds and the peptide backbone. Usually, the reduction step is performed using chemical reducing reagents such as dithiothreitol (DTT) [8] or *tris*(2-carboxyethyl)phosphine (TCEP) [9,10]. However, since an excess of these reagents is used, the protein sample has to be purified afterwards in order to prevent a modification and an activity loss of the respective applied protease. Due to this time-consuming, multi-step method, alternative, less laborious approaches are required. Electrochemistry as a purely instrumental setup, which allows fast and selective reduction of disulfide bonds by applying negative potentials to the working electrode can serve as an alternative.

Kraj et al. have shown a proof of principle by optimizing the parameters applied for the electrochemical reduction of insulin, which contains two intermolecular and one intramolecular disulfide bond [11]. They used a commercially available flow-through thin-layer cell equipped with titanium working and counter electrodes and a Pd/H_2 reference electrode. Square-wave pulses were applied to the working electrode in order to achieve a reduction efficiency of 100% for all three disulfide bonds. By varying the parameters of the square-wave pulses, Kraj et al. were able to control the reduction efficiency, which is advantageous for experiments where no quantitative reduction is required. Reduction of somatostatin (14 amino acids, one disulfide bond) allowed the online detection of almost the full series of y- and b-type ions by means of CID experiments. Without reduction, only one y-ion was identified. Finally, partial to complete reduction of α-lactalbumin (123 amino acids, 4 disulfide bonds) was performed, thus demonstrating that their developed method is also capable

Abbreviations: BDD, boron-doped diamond; FA, formic acid; GSH, glutathione; GSSG, glutathione dimer; H_2Q, hydroquinone; Q, benzoquinone; Q-GSH adduct, benzoquinone-glutathione adduct.

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to assign and localize disulfide bridges in proteins. Nicolardi et al. described an improved Fourier transform ion cyclotron (FTICR)-CID and −ETD sequence coverage of the disulfide containing peptides oxytocin and hepcidin after online electrochemical reduction [12]. In the group of Chen, electrochemical reduction of biomolecules in combination with desorption electrospray ionization (DESI)-MS as detection technique was investigated [13–19]. In a study by Zhang et al. based on the bottom-up approach, electrochemical reduction of enzymatically digested peptides containing disulfide bonds was performed using an amalgam working electrode [15]. They were able to identify disulfide containing enzymatic peptides based on the changes of their relative ion abundance during DESI-MS detection, when the cell was either switched on or off. In addition, several peptides and the protein α-lactalbumin were electrochemically reduced and subsequently labelled with selenium reagents, which are reactive towards free thiol functions. Thus, it was possible to distinguish between intra- (+2 tags) and intermolecular (+1 tag) disulfide bonds. Apart from these bottom-up experiments, Zhang et al. [19] investigated the electrochemical reduction of disulfide bonds in top-down proteomics experiments. Here, a significantly enhanced sequence coverage of the two proteins β-lactoglobulin A (24 vs. 75 backbone cleavages before and after electrochemical reduction) and lysozyme (5 vs. 66 backbone cleavages) could be obtained. Van Berkel et al. [20] developed a method, which allows to perform disulfide reduction, benzoquinone generation and follow-up reactions within only one experiment. They modified an ESI interface by applying porous flow-through electrodes instead of stainless-steel electrodes at the upstream grounding point and emitter. At the upstream grounding point a disulfide containing peptide was electrochemically reduced and the obtained free thiol groups were subsequently tagged with benzoquinone, which was generated by electrochemically oxidizing hydroquinone at the emitter.

Another important aspect in protein analysis is related to the determination of the number of free and disulfide bound cysteines in proteins, which provides additional information for protein characterization [21]. Free cysteines contain a nucleophilic thiol group, which acts as a target for several drug compounds such as platinum(II) anti-cancer drugs [22,23]. Beyond this, thiol groups can be modified with reactive electrophilic intermediates or reactive oxygen and nitrogen species, which can be formed during the metabolism of a xenobiotic or during oxidative stress, respectively [24–27]. These modifications may lead to an activity loss or to toxic side effects. Therefore, analytical methods are required allowing the determination of the number and position of free and disulfide bound cysteine moieties. In the last years, electrochemistry in combination with mass spectrometry has shown to be a suitable tool for the specific modification of cysteine residues in peptides and proteins [20,28–38].

Within this study, a new method was developed, which combines for the first time a dual electrochemical approach (oxidative and reductive) on-line with reversed phase liquid chromatographic separations and electrospray mass spectrometric detection of the formed products. While a proof of principle of this method can be demonstrated, the approach should be suitable for automated labelling approaches after further optimization.

2. Experimental section

2.1. Chemicals

Glutathione (GSH), oxytocin, insulin from bovine pancreas and ammonium formate were obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Formic acid was ordered from Fluka Chemie (Buchs, Switzerland) and acetonitrile from VWR Chemicals (Darmstadt, Germany). Phenol was purchased from ABCR (Karlsruhe, Germany). All chemicals and solvents were used in the highest quality available. Water was purified before utilization with an Aquatron A4000D system (Barloworld Scientific, Nemours, France).

2.2. Electrochemical reduction of glutathione dimers and oxytocin by means of EC/MS

The reduction of oxidized glutathione (GSSG) was performed using an amperometric thin-layer cell (ReactorCell, Antec Leyden, Zoeterwoude, The Netherlands) equipped with a titanium working electrode, a graphite-doped Teflon counter electrode and a Pd/H_2 reference electrode. The potential was controlled by a home-made potentiostat. A schematic overview of the applied setup can be found in SI-1, Supporting information. In order to detect GSSG and its reduced form glutathione (GSH), the cell was directly coupled to a high-resolution mass spectrometer (Exactive Orbitrap, Thermo Fisher Scientific, Bremen, Germany) with an electrospray ionization (ESI) source. With a flow-rate of 10 μL/min, which was controlled by a syringe pump (model 74900, Cole Parmer, Vernon Hills, USA) a 50 μM solution of GSSG in 1% (v/v) formic acid (FA) in purified water containing 10% acetonitrile (ACN) (v/v) was pumped through the electrochemical cell. For reduction, a potential ramp from 0 to −3.0 V versus Pd/H_2 with a scan rate of 10 mV/s was applied. Mass spectra were recorded in the positive ion mode and were plotted as three-dimensional mass voltammograms (for detailed MS parameters see SI-1, Supporting information).

Electrochemical reduction of oxytocin was performed using a preparative electrochemical thin-layer cell (μPrepCell, Antec Leyden) equipped with working and counter electrodes made of titanium and a Pd/H_2 reference electrode. The potential was controlled by a home-made potentiostat. A solution with a concentration of 10 μM (in 1% FA (v/v) and 10% ACN (v/v)) was introduced into the EC cell, where a constant potential of −3.0 V vs. Pd/H_2 was applied. The effluent of the cell was continuously analyzed by means of ESI-MS using a time-of-flight (ToF) mass analyzer (micrOTOF, Bruker Daltonics, Bremen, Germany). Mass spectra were recorded in the positive ion mode (for detailed MS parameters see SI-1, Supporting information).

2.3. Electrochemical reduction of oxytocin and insulin by means of EC/LC/MS

Oxytocin (50 μM) and insulin (10 μM) were electrochemically reduced using the previously described preparative cell which was equipped with a titanium working electrode. The reduction potential was set to constant value of −1.2 V vs. Pd/H_2 using a home-made potentiostat. An LC separation using a C5 wide pore column (Discovery® BIO Wide Pore C5, 300 Å, 150 × 2.1 mm, 5 μm particle size, Supelco, Steinheim, Germany) and a binary gradient using 0.1% (v/v) FA and ACN was performed prior to MS analysis (for a schematic overview of the used setup see SI-1, Supporting information) in order to achieve a separation of the intact biomolecule and its reduced form. For online EC/LC/ESI-MS analysis, the cell effluent was collected in a 5 μL injection loop, which was mounted in a ten-port switching valve. By switching the valve to the injection position, the solution was transferred into the column. For this purpose, an Alexys LC system (Antec Leyden) and a microTOF-MS (Bruker Daltonics) equipped with an ESI source were used. The LC system included two LC 100 pumps, an OR 110 organizer rack with a degasser and a pulse dampener, an AS 100 autosampler, and a Roxy column oven. The flow-rate was set to 300 μL/min and the oven temperature was adjusted to 40 °C. The gradient profiles for reduced oxytocin and insulin are shown in Table 1.

In order to prevent salts from entering the MS, the effluent of the first 2 min of the LC separation was discarded. Mass spectrometric

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