UCLA
UCLA Previously Published Works

## Title

Integration of Electrochemistry with Ultra-Performance Liquid Chromatography/Mass Spectrometry

## Permalink

https://escholarship.org/uc/item/96d89194

## Journal

European Journal of Mass Spectrometry, 21(3)

## ISSN

1356-1049

## Authors

Cai, Yi
Zheng, Qiuling
Liu, Yong
et al.

## Publication Date

2015-06-01

## DOI

10.1255/ejms.1318

Peer reviewed

eScholarship.org Powered by the California Digital Library
University of California

# HHS Public Access

Author manuscript

*Eur J Mass Spectrom (Chichester, Eng)*. Author manuscript; available in PMC 2016 January 01.

Published in final edited form as:

*Eur J Mass Spectrom (Chichester, Eng)*. 2015 ; 21(3): 341–351. doi:10.1255/ejms.1318.

# Integration of Electrochemistry with Ultra Performance Liquid Chromatography/Mass Spectrometry (UPLC/MS)

**Yi Cai**[a], **Qiuling Zheng**[a], **Yong Liu**[b], **Roy Helmy**[b], **Joseph A. Loo**[c], and **Hao Chen**[a,*]

[a]Center for Intelligent Chemical Instrumentation, Department of Chemistry and Biochemistry, Edison Biotechnology Institute, Ohio University, Athens, OH, 45701 USA

[b]Department of Analytical Chemistry, Merck Research Laboratories, Merck & Co., Inc., Rahway, NJ 07065, USA

[c]Department of Chemistry and Biochemistry, Department of Biological Chemistry, David Geffen School of Medicine at UCLA, and UCLA/DOE Institute for Genomics and Proteomics, University of California-Los Angeles, Los Angeles, CA 90095, USA

## Abstract

This study presents the development of ultra-performance liquid chromatography/mass spectrometry (UPLC/MS) combined with electrochemistry (EC) for the first time and its application for the structural analysis of disulfide bond-containing proteins/peptides. In our approach, a protein/peptide mixture sample undergoes fast UPLC separation and subsequent electrochemical reduction in an electrochemical flow cell followed by online MS and MS/MS analyses. The electrochemical cell is coupled to MS using our recently developed desorption electrospray ionization (DESI) interface. Using this UPLC/EC/DESI-MS method, disulfide bond-containing peptides can be differentiated from those without disulfide bonds as the former are electroactive and reducible. Tandem MS analysis of the disulfide-reduced peptide ions provides increased sequence and disulfide linkage pattern information. In a reactive DESI-MS detection experiment in which a supercharging reagent was used to dope the DESI spray solvent, increased charging was obtained for the UPLC-separated proteins. Strikingly, upon online electrolytic reduction, supercharged proteins (e.g., α-lactalbumin) showed even higher charging, which would be useful in top-down protein structure analysis as increased charges are known to promote protein ion dissociation. Also, the separation speed and sensitivity are enhanced by approximately 1~2 orders of magnitude by using UPLC for the LC/EC/MS platform, in comparison to the previously used high performance liquid chromatography (HPLC). This UPLC/EC/DESI-MS method combines the power of fast UPLC separation, fast electrochemical conversion and online MS structural analysis for a potentially valuable tool for proteomics research and bioanalysis.

### Keywords

DESI-MS; UPLC; electrochemistry; disulfide bond reduction; protein; supercharging

[*]Corresponding author: Associate Prof. Hao Chen, Department of Chemistry and Biochemistry, Ohio University, Athens, OH 45701, 740-593-0719 (Tel), 740-597-3157 (Fax), chenh2@ohio.edu.

Prepared for the 20th Anniversary Special Issue of *European Journal of Mass Spectrometry*

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

## Introduction

Liquid chromatography/mass spectrometry (LC/MS) has become one of the most powerful techniques for the analysis of biomolecules and pharmaceuticals,[1–3] which combines the separation capability of LC with the mass analysis power of MS. The combination with electrochemistry (EC) further broadens LC/MS applications.[4–6] Previous studies showed that post-column electrochemical conversion in LC/MS can be used to increase the MS detection sensitivity of the target compounds. In such an experiment, an electrochemical flow cell is placed in between the LC and MS to convert the LC-separated compounds to more polar or even charged products, which are suitable for MS detection with increased ionization efficiency. For instance, Karst *et al.* employed ferrocenoyl piperazide to derivatize isocyanate analytes. After derivatization, charged ferrocinium ions could be generated via electrochemical oxidation of the derivatized analytes, which were easily detected by MS. Compared with the approach without electrochemical conversion, the detection sensitivity was increased by a factor of 50.[7] Similar sensitivity enhancement was observed for analytes such as phenothiazines and non-polar polycyclic aromatic hydrocarbons (PAH) that could be electrochemically converted into radical cations for MS detection.[8, 9] However, in such an LC/EC/MS experiment, atmospheric pressure chemical ionization (APCI) rather than electrospray ionization (ESI) was used in most cases. The drawback of APCI is that polar compounds cannot be ionized very well with APCI. In addition, the LC separation employing high performance liquid chromatography (HPLC) was time-consuming. In particular, the mobile phase flow rate adopted was limited to 200–600 μL/min to be compatible with the use of the electrochemical cell (a low electrochemical conversion yield would result from a higher flow rate),[9, 10] thus making fast elution difficult.

Ultra-performance liquid chromatography (UPLC) is a significant advance in the field of chromatography. With the packing particle diameter size reduced to less than 2.0 μm, the separation efficiency of UPLC is significantly improved and the separation time is typically shortened by 10 times in comparison to conventional HPLC.[11, 12] Importantly, UPLC does not require a high elution flow rate to achieve fast separation,[13] thus making it a better fit for LC/EC/MS experiments than HPLC. However, UPLC has not been implemented for LC/EC/MS to date.

In this study, we report the development of LC/EC/MS using UPLC for the first time. In addition, we adopted liquid sample desorption electrospray ionization (DESI)[14, 15] as the interface to couple the electrochemical cell with a mass spectrometer (Figure 1a). Unlike APCI, liquid sample DESI is a soft ionization method that can be used to ionize large proteins and protein complexes.[16–21] In addition, there are several additional advantages of using DESI in this study.[22] First, the use of DESI simplifies the coupling apparatus, as there is no need to separate the small potential applied to the electrochemical cell from the high voltage used for spray ionization. Second, the cell and DESI source can be connected with a very short piece of capillary as a conduit, thus minimizing the post-column dead volume in this LC/EC/MS apparatus (but a small post-column dead volume is essential for maintaining UPLC separation efficiency). Third, taking advantage of the flexibility of DESI to use different spray solvents, reactive DESI can be directly performed in the coupling of UPLC/EC/DESI-MS with post-column derivatization (e.g., to supercharge proteins in this

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

study). Using the new apparatus, we demonstrated the application of LC/EC/MS for protein/peptide structural analysis. For protein/peptide enzymatic digests, disulfide bond-containing peptides can be differentiated from those without disulfide bonds as the former are electroactive and electrochemically reducible. Tandem MS analysis of the reduced peptide ions provides increased sequence information and for pinpointing disulfide bond linkages. Reactive DESI-MS experiments using spray solvents doped with supercharging reagents, increased charging was observed for the UPLC-separated proteins.

## Experimental

### Chemicals

Somatostatin 1–14 was purchased from American Peptide Company (Sunnyvale, CA). TPCK-treated trypsin from bovine pancreas, pepsin from porcine gastric mucosa, insulin from bovine pancreas, α-lactalbumin from bovine milk (type III, calcium depleted), formic acid (FA) and HPLC-grade acetonitrile (ACN) were all purchased from Sigma-Aldrich (St. Louis, MO). HPLC-grade methanol was obtained from Fisher Scientific (Fairlawn, NJ) and deionized water used for sample preparation was obtained using a Nanopure Diamond Barnstead purification system (Barnstead International, Dubuque, IA).

### Apparatus

A Waters ACQUITY UPLC® System with a Waters Xevo QTOF mass spectrometer (Milford, MA) was used. Our LC/EC/MS assembly employs a thin-layer μ-PrepCell™ electrochemical flow cell equipped with a magic diamond (MD) electrode as the working electrode (WE, Figure 1a). A platinum electrode and a titanium electrode served as the reference electrode (RE) and auxiliary electrode (AE), respectively. The cell was connected to the UPLC column using a piece of PEEK connection tubing (i.d.: 200 μm, length: 25 cm). An ACQUITY UPLC BEH $C_{18}$ column (1.7 μm, 2.1 mm × 50 mm) and an ACQUITY UPLC Protein BEH $C_4$ column (1.7 μm, 2.1 mm × 50 mm) were employed for peptide and protein separation, respectively. The mobile phase flow rate used was 200–300 μL/min. After UPLC separation, compounds in the eluent underwent electrochemical reduction in the electrochemical cell. The electrolyzed species flowed out of the thin-layer cell via a short PEEK tube (i.d. 510 μm; wall thickness: 530 μm, length: 3 cm) carrying a micro-orifice (i.d. 100 μm) that was located in the tube 2 cm downstream from the LC column. Approximately one-third of the eluent emerged out of the micro-orifice, which was then subject to DESI ionization. Such a DESI interface for LC/MS study was recently reported, in which the eluent splitting can be realized using the micro-orifice on the PEEK tube without introducing a large post-column dead volume.[14, 15] Unless specified, the spray solvent for DESI was $CH_3OH/H_2O/HOAc$ (50:50:1 by volume) and flowed at 10 μL/min. A 5 kV potential was applied to the spray solvent with $N_2$ nebulization (160 psi). The microdroplets from the DESI spray interacted with the eluent flowing out of the micro-orifice to effect eluent ionization. A Roxy™ potentiostat was used to apply a potential (pulsed mode, $E_1$= −2.0 V for 1990 ms, $E_2$ = −1.5 V for 1010 ms and $E_3$= 0 V for 20 ms) to the electrochemical flow cell to trigger redox conversion.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

### LC separation conditions

Somatostatin 1–14 was trypsin digested in 25 mM ammonium bicarbonate at a molar ratio of 1:25 (enzyme/protein) for 12 h at 37 °C. The digest products were separated using a gradient elution: solvent A was 0.1% FA in $H_2O$, and solvent B was 0.1% FA in ACN; 10% B was ramped to 40% in 3 min. The mobile phase flow rate was 300 μL/min.

Pepsin digestion of insulin was performed by incubating insulin and pepsin at a molar ratio of 25:1 in water containing 1% acetic acid at 37°C for 12 h. The digested insulin was diluted to 20 μM with water containing 0.1% FA and 6 μL of the sample was injected into the UPLC system using an autosampler. Solvent A was 0.1% FA in $H_2O$, and solvent B was 0.1% FA in ACN. Peptides were eluted by using a 5 min linear elution from 5% to 7% B, and from 7% to 15% B in 1 min, then from 15% to 30% B in 10 min. The flow rate of mobile phase was 300 μL/min.

For the protein mixture separation, 6 μL of a protein mixture of insulin, myoglobin and α-lactalbumin (20 μM each) was loaded onto the UPLC $C_4$ column with an elution flow rate of 200 μL/min. For the LC gradient program: solvent A was 0.1% FA in $H_2O$, and solvent B was 0.1% FA in ACN; 28% B was ramped to 32% in 3 min, and then increased to 45% in 3 min.

## Results and Discussion

The configuration of the UPLC/EC/DESI-MS apparatus is shown in Figure 1a. In this study, we focused on its application for the structural analysis of disulfide bond-containing proteins/peptides, taking advantage of the platform's capability for fast separation, online electroreduction and MS detection.

Disulfide bonds are one of the most common protein post-translational modifications, which provide reversible covalent cross-linkages in native proteins that maintain protein three-dimensional structures and their biological activities.[23, 24] However, the presence of disulfide linkages increases the complexity for protein structure determination by MS. Cleavage of disulfide bonds is often essential for protein/peptide analysis as dissociation of a disulfide-reduced protein/peptide ion can give rise to more structurally informative fragment ions than that of the intact counterpart.[23, 24] The traditional protocol to break a disulfide bond is chemical reduction using an excess amount of reducing chemical reagents like dithiothreitol (DTT) or tris(2-carboxyethyl)phosphine (TCEP). However, the reduction usually takes more than 30 min and the removal of the excess reductant can be time-consuming. In addition, the resulting protein/peptide thiols often need to be protected due to the possibility of being re-oxidized prior to MS analysis. Besides chemical reduction, other novel approaches include the cleavage of disulfide bonds via laser-based ionization[25–28], ultraviolet photodissociation[29–31], negative ion dissociation[32–35], electron-capture dissociation (ECD)[36], electron-transfer dissociation (ETD)[37–41], plasma-induced oxidation[42], or using new ion chemistry[43–49]. An alternative way for reducing disulfide bonds without involving chemical reductants is electrolytic reduction, which is fast and does not need chemical reductants[50, 51]. It would be useful if electrochemical conversion of disulfide bonds can be performed in the LC/MS or LC/MS/MS analysis of proteins and

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript



peptides, which is commonly used in the bottom-up approach for protein/peptide structure determination in proteomics research.

In this study, a disulfide-containing peptide somatostain 1–14 (MW 1637.9 Da) was first chosen as a test sample for UPLC/EC/DESI-MS analysis. Somatostain 1–14 was digested by trypsin to produce a peptide mixture, AGCK TFTSC and NFFWK. In our experiment, 6 μL of the digest mixture (20 μM) was loaded onto the UPLC. As displayed in Figure 1b, the two peptides eluted at 1.20 and 2.08 min, respectively. Figure 1c–1d shows the DESI-MS spectra of the peptide AGCK TFTSC. When the cell was not turned on, the singly and doubly charged peptide ions for AGCK TFTSC were detected at *m/z* 933 and 467, respectively. When the negative reduction potential was applied to the working electrode of the cell, the electrochemical reduction of the peptide occurred. Two new ions of *m/z* 378 and 558 were observed, resulting from the reduction of the AGCK TFTSC. Tandem MS/MS was performed to elucidate the ion structures. Upon collision induced dissociation (CID), the observed fragment ions of the singly charged AGCK TFTSC of *m/z* 933 are limited, only producing B($b_2$), A/B($y_1$) (A/B($y_1$) refers to a fragment with $y_1$ ion of B chain linked with an intact A chain; the notation also is applicable to other fragment ions), A/B($y_2$), A/B($y_3$), and B/A($y_2$) (Figure S1a, Supporting Information). In contrast, CID of the electro-generated ions provides more fragment ions that cover all of the backbone cleavage sites. As shown in Figures S1b and S1c (Supporting Information), CID of the ion at *m/z* 378 gives rise to $b_3$, $b_3$-$H_2O$, $y_2$ and $y_3$ fragment ions and CID of the ion at *m/z* 558 yields $b_2$, $b_3$, $b_4$, $b_4$-$H_2O$, $y_3$ $y_3$-$H_2O$ and $y_4$-$H_2O$ fragment ions, which reveals that the two peptide sequences are AGCK and TFTSC. This result shows that the electrochemical reduction removes the disulfide bond linkage and produce linear peptides whose sequences are more easily determined by MS/MS. The sum of the MWs of the two products (378.2 + 558.2 – 2.0 = 934.4 Da) is higher than that of the precursor peptide (932.4 Da) by 2.0 Da, suggesting that the precursor peptide AGCK TFTSC has one disulfide bond. Furthermore, the result shown above is also helpful for pinpointing the disulfide bond linkage between the 3$^{rd}$ residue of one chain AGCK with the 5$^{th}$ residue of the other chain TFTSC. Interestingly, the protonated peptide NFFWK containing no disulfide bonds remained unchanged once the potential was applied (Figure S2, Supporting Information). This suggests that UPLC/EC/DESI could be used to differentiate disulfide bond-containing peptides from others, which is in agreement with our previous report.[52] Previously the somatostain 1–14 digest was analyzed by LC/EC/MS using HPLC. In that experiment, the retention times for AGCK TFTSC and NFFWK were 15.5 and 17.5 min, respectively, much longer than those observed in this study. Furthermore, 5 μL of 2.4 mM tryptic digested somatostain 1–14 was loaded into HPLC for obtaining reasonably good signal. By comparison, this UPLC/EC/MS method shortened the separation time by about 9 times and improved the sensitivity by about 100 times (12 nmol somatostain 1–14 digest used for HPLC separation in the previous study versus 0.12 nmol somatostain 1–14 digest used for UPLC separation in this study) [52].

After the successful trial with somatostain 1–14 digest, we further tested the method for the analysis of a protein pepsin digest and bovine pancreatic insulin was chosen as the test sample. Insulin is composed of A and B chains linked by two inter-peptide disulfide bonds, and the A-chain has an additional intra-peptide disulfide bond. As shown in the acquired

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

extracted ion chromatograms (EICs, Figure 2), the peptides were well separated within 15 min. The obtained MS spectra (Figures S3, Supporting Information) show the ions of the first six eluted peptides, including $[YTPKA+H]^+$ (*m/z* 579), $[FVNQ+H]^+$ (*m/z* 507), $[GIVE+H]^+$ (*m/z* 417), $[YQLEN+H]^+$ (*m/z* 666), $[YQLE+H]^+$ (*m/z* 552), and $[VEAL+H]^+$ (*m/z* 431). Comparing the mass spectra of these six peptides before and after the cell was turned on, there were no new peaks observed (Figure S3, Supporting Information), indicating that these are peptides without disulfide bonds. MS/MS analyses of these peptide ions (Figure S4, Supporting Information) provide the sequence information for all of these peptides except GIVE in which the first and second peptide bond cleavages were missing. For the additional four peptides that eluted later (denoted as P1, P2, P3, and P4, Figure 2), new peaks were observed when the cell was turned on (see example shown in Figures 3a and 3b), indicating that they carry disulfide bonds. As the UPLC separation was used prior to electroreduction, these new peptide ions would correspond to the reduced peptides of the UPLC-separated precursor peptides. Importantly, tandem MS analysis of the reduced peptides provides useful information for the sequencing and disulfide bond maping of the precursor peptide. For instance, as shown in Figure 3b, in the case of P3, the sum of the MWs of the two reduced products GIVEQCCASVCSL and HLCGSHL (2075.9 Da, calculated from the measured *m/z* of the corresponding product ions) is higher than that of P1 (2071.9 Da) by 4.0 Da, suggesting that the precursor peptide P3 has two disulfide bonds. The singly charged ion of chain B generated from electrolysis (*m/z* 766) gave rise to fragment ions $b_2, b_3, b_4, b_5, b_6, y_2, y_3, y_4, y_5,$ *and* $y_6$ upon CID (Figure 3e). This set of fragment ions originate from the cleavage of all the backbone amide bonds and determine its sequence to be HLCGSHL with one cysteine residue located at the 3$^{th}$ amino acid site. The fragment ions of the single charged ion of chain A from P3 reduction (*m/z* 1311) yielded $b_3$ $b_4, b_5, b_6, b_6\text{-}H_2O, b_7, b_8, b_9, b_{10}, b_{10}\text{-}H_2O, b_{11},$ and $b_{12}$ (Figure 3d), suggesting chain A sequence to be XXXEQCCASVCSL (X means the first three amino acids are unknown). But all three cysteine residues of chain A are known to be located at the 6$^{th}$, 7$^{th}$ and 11$^{th}$ amino acid sites. Thus, there are three possible disulfide bond linkages for P3 (chain B's sole cysteine residue links with one of the three cysteine residues of chain A). Interestingly, upon CID, the doubly charged P3 ion (*m/z* 1037, Figure 3c) dissociated into $A(b_2)$, $A(b_3)$, $A(b_4)$ $B/A(b_{11})^{2+}$, $B/A(b_{12})^{2+}$, $B/A(y_8)$, $B/A(y_{10})$, $B(b_2)$, $B(y_2)$, and $B(y_4)$, in which the backbone cleavage between $Cys^6$ and $Cys^{11}$ of the chain is missing. This result suggests that the $Cys^6$ in the chain A is paired up with $Cys^{11}$ of the chain A to form an intra-peptide bond and $Cys^7$ of chain A links with $Cys^3$ of chain B in P3. Furthermore, the appearance of fragment ions $A(b_2)$ and $A(b_3)$ in Figure 3c shows the 3rd residue of A is valine. Thus, chain A can be determined as XXVEQCCASVCSL. These results show that both the locations of disulfide bonds and most of the P3 sequence could be determined using the information acquired from this UPLC/EC/DESI-MS method. Likewise, two additional peptides, P1 and P4, were generated because of the low digestion specificity of pepsin used in the experiment. Their structures (shown in Figure 2) are similar to that of P3 and can be determined by MS/MS analysis (data not discussed here). The fragmentation patterns of P1 and P4 ions also agree with the disulfide bond assignment for P3 (Figure S5, Supporting Information).

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

However, in the case of P2, one of its reduced products (i.e., A-chain) was missing in the acquired DESI-MS spectrum (Figure S6a–S6b, Supporting information) and only B-chain LVCGERGFF was detected. The reason for that is probably due to the lack of the basic amino acid residues, which causes low ionization efficiency. Similar phenomena of missing chain A in the spectra of reduced insulin has been reported before[53]. Nevertheless, regardless of the missing chain A, the sequence of P2 and its disulfide bond location could be still inferred based on examination of MS/MS data and MW analysis. CID of doubly charged P2 *m/z* 769 (Figure S6c, Supporting information) produced fragment ions $B/A(y_2)$, $B/A(y_2)\text{-}NH_3$, $B/A(y_3)$, $B/A(b_3)^{2+}$, $A(b_2)$, $B(y_1)$, $B(y_4)$, $A/B(y_7)$, $A/B(y_7)^{2+}$, and $B(b_2)$. The CID MS/MS spectrum of the doubly charged chain B of P2 (*m/z* 514) was further examined (Figure S6d, Supporting information), and its fragment ions of $b_2$, $b_3$, $b_6$, $b_7$, $y_1$, $y_4$, $y_5$, $y_6$, $y_7$ and $y_8$ suggest the chain B sequence to be LVCGERGFF. First, we assumed that P2 only has one disulfide bond. In this case, according to the MWs of P2 and chain B, the MW of chain A is calculated to be 512.1 Da. For sequencing chain A, the first two amino acids of A can be determined as asparagine and tyrosine, based on the MW of the A-chain and the fragment ions $B/A(y_2)$ and $B/A(y_3)$ generated from the CID of doubly charged P2 of *m/z* 769 (Figure S6c, Supporting Information). The appearance of $A(b_2)$ and $B/A(b_3)^{2+}$ in Figure S6c further suggests that the last two amino acid residues of A is cysteine (modified with chain B) and asparagine residues. Thus the chain A sequence is determined as NYCN. In the P2 peptide, chain A and B should be connected with one inter-peptide disulfide bond (i.e., the $Cys^3$ of chain A links with $Cys^3$ of chain B in P2), which is in agreement with our assumption above. This result reveals that the sequence of P2 can also be determined. Note that another peak of *m/z* 815 was observed after the cell was turned on (Figure S6b). It is likely to be caused by in-source CID of chain B ions. Indeed, doubly charged chain B (*m/z* 514) yields a major fragment ion $y_7$ of *m/z* 815 upon CID (Figure S6d). Thus, based on the structural analyses of the digested peptide, it can be seen that UPLC/EC/DESI-MS with MS/MS could obtain rich information for insulin sequencing (98% coverage) and disulfide bond location determination (i.e., all three disulfide bonds can be located).

Besides investigating the utility in the analysis of protein digests using our UPLC/EC/DESI-MS method, intact proteins were also tested. One aim would be to determine if UPLC/EC/DESI-MS can be used to identify the presence of disulfide bonds in proteins. Another purpose is to test if higher charging of protein ions with online electroreduction combined with online supercharging can be obtained. From previous work, electrochemical reduction can remove disulfide bond bridges with increased charges observed, probably due to protein unfolding upon disulfide bond removal[54–56]. Also, it is well known that supercharging reagents can be used to increase ion charges[57, 58]. It would be interesting to see if both supercharging and disulfide-reduction could further increase protein charging. One of the benefits of DESI is that post-column derivatization of separated analytes can be accomplished via a “reactive” DESI approach in which a chosen chemical reagent is doped into the DESI spray solvent and the reaction process occurs during the ionization process[53, 59]. This is in contrast to traditional derivatization protocols used for LC/ESI-MS coupling, in which a Tee mixer is used to introduce the derivatizing reagent and thus increases post-column dead volume. In this study, we extended this application to supercharge proteins following UPLC separation and electrochemical reduction. The charge

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

state distributions (CSDs) of protein ions will shift to higher charges with the appearance of a new population which could indicate a conformational change occurring to the protein due to disulfide-reduction[53, 55, 56]. A mixture of insulin (containing two inter-peptide and one intra-peptide disulfide bond), myoglobin (containing no disulfide bonds) and a-lactalbumin (containing four inter-peptide disulfide bonds) was chosen as a test example for this experiment. The three proteins were separated within 5.5 min (Figure 4a).

For insulin, comparing the mass spectra recorded before and after adding the supercharging reagent *m*-NBA (50 mM) into the DESI spray, the maximum charge state shifted from +5 to +6 (Figures S7a and S7b, Supporting information). It is known that insulin has three disulfide bonds as mentioned above. When the potential was applied the cell, two new peaks at *m/z* 851 and 1134 corresponding to the +4 and +3 charge states of chain B, respectively, could be observed (Figure S7c, Supporting information), indicating the presence of disulfide bonds. The missing of A chain ions in the spectrum may be again due to the lack of sufficient numbers of basic amino acid residues in chain A. For 16.9 kDa myoglobin, as shown in Figure 4b, when the DESI spray was doped with supercharging reagent *m*-NBA (50 mM), the maximum charge state of myoglobin shifted from +22 to +24; the average charge state increased from +15.1 to +18.9; and the highest abundance ion was shifted from +18 to +20. But since there are no disulfide bonds in myoglobin, the mass spectra before and after turning on the cell potential had no obvious differences (shown in Figure 4b). For the protein α-lactalbumin, which has 123 amino acid residues and contains four disulfide cross-links to maintain and stabilize its structure, the maximum charge state of α-lactalbumin shifted from +11 to +14 upon applying the supercharging reagent (Figure 4c). Once the EC potential was applied, interestingly, a newly produced +15 ion appeared. Compared to the mass spectra of α-lactalbumin before and after cell on with concurrent supercharging (Figure 4c), the maximum charge state of α-lactalbumin further shifted from +14 to +15 and the average charge state increased from +9.9 to +11.2. Strikingly, the ion with highest abundance was shifted from +8 to +12 when the cell was turned on. It is likely that the reduction of disulfide bonds to cause the protein to unfold. Unfolded proteins have a greater capability to carry larger number of charges on its surface than that of folded proteins[53, 60]. This result shows the combined effects of supercharging and disulfide bond reduction for increasing protein charge states. Increased charging should lead to increase ion dissociation efficiency, especially for proteins constrained by disulfide bonds[53, 59, 61].

## Conclusions

UPLC/EC/DESI-MS is a powerful tool for the structural elucidation of different types of disulfide-bond containing proteins/peptides. This method can identify disulfide bond-containing peptides in enzymatic digest mixtures. After UPLC separation, the reduced linear peptide chains can be assigned to their precursors and sequenced. Based on the tandem MS analysis, sequencing peptides and pinpointing the disulfide linkages are possible. Both electrolysis treatment and online supercharging reactive-DESI experiments could be used to increase charges for the proteins carrying intra-disulfide bonds (e.g., α-lactalbumin). As disulfide bonds play an important role in protein conformation and function, this UPLC/CE/DESI-MS method would have valuable utility in proteomics research.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript



## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

This work was supported by an NSF Career Award (CHE-1149367), NNSFC (21328502), Merck New Technology Research Licensing Committee (NTRLC) Fund and the Ohio Third Frontier Technology Validation and Start-Up Fund to HC. JAL acknowledges support from the National Institutes of Health (R01GM103479).

## References

1. Hernandez F, Ibanez M, Bade R, Bijlsma L, Sancho JV. Investigation of pharmaceuticals and illicit drugs in waters by liquid chromatography-high-resolution mass spectrometry. TrAC, Trends Anal Chem. 2014; 63:140–157.10.1016/j.trac.2014.08.003
2. Kaufmann A. Combining UHPLC and high-resolution MS: A viable approach for the analysis of complex samples? TrAC, Trends Anal Chem. 2014; 63:113–128.10.1016/j.trac.2014.06.025
3. Cajka T, Fiehn O. Comprehensive analysis of lipids in biological systems by liquid chromatography-mass spectrometry. TrAC, Trends Anal Chem. 2014; 61:192–206.10.1016/j.trac.2014.04.017
4. Zhou F, Van Berkel GJ. Electrochemistry Combined Online with Electrospray Mass Spectrometry. Anal Chem. 1995; 67:3643–9.10.1021/ac00116a005
5. Deng H, Van Berkel GJ, Takano H, Gazda D, Porter MD. Electrochemically modulated liquid chromatography coupled on-line with electrospray mass spectrometry. Anal Chem. 2000; 72:2641–2647.10.1021/ac991461+ [PubMed: 10857648]
6. Faber H, Vogel M, Karst U. Electrochemistry/mass spectrometry as a tool in metabolism studies-A review. Anal Chim Acta. 2014; 834:9–21.10.1016/j.aca.2014.05.017 [PubMed: 24928240]
7. Seiwert B, Henneken H, Karst U. Ferrocenoyl piperazide as derivatizing agent for the analysis of isocyanates and related compounds using liquid chromatography/electrochemistry/mass spectrometry (LC/EC/MS). J Am Soc Mass Spectrom. 2004; 15:1727–1736.10.1016/j.jasms.2004.09.002 [PubMed: 15589750]
8. Hayen H, Karst U. Analysis of phenothiazine and its derivatives using LC/electrochemistry/MS and LC/electrochemistry/fluorescence. Anal Chem. 2003; 75:4833–4840.10.1021/ac0346050 [PubMed: 14674461]
9. van Leeuwen SM, Hayen H, Karst U. Liquid chromatography-electrochemistry-mass spectrometry of polycyclic aromatic hydrocarbons. Anal Bioanal Chem. 2004; 378:917–925.10.1007/s00216-003-2150-4 [PubMed: 12937883]
10. Karst U. Analytical methods: Electrochemistry/mass spectrometry (EC/MS) - a new tool to study drug metabolism and reaction mechanisms. Angew Chem, Int Ed. 2004; 43:2476–2478.10.1002/anie.200301763
11. Guillarme D, Ruta J, Rudaz S, Veuthey JL. New trends in fast and high-resolution liquid chromatography: a critical comparison of existing approaches. Anal Bioanal Chem. 2010; 397:1069–1082.10.1007/s00216-009-3305-8 [PubMed: 19998028]
12. MacNair JE, Lewis KC, Jorgenson JW. Ultrahigh-Pressure Reversed-Phase Liquid Chromatography in Packed Capillary Columns. Anal Chem. 1997; 69:983–989.10.1021/ac961094r [PubMed: 9075400]
13. Schappler, J.; Rudaz, S.; Veuthey, J-L.; Guillarme, D. Coupling UHPLC with MS: the needs, challenges, and applications. John Wiley & Sons, Inc; 2013. p. 95-131.
14. Cai Y, Adams D, Chen H. A New Splitting Method for Both Analytical and Preparative LC/MS. J Am Soc Mass Spectrom. 2014; 25:286–292.10.1007/s13361-013-0763-1 [PubMed: 24254577]
15. Cai Y, Liu Y, Helmy R, Chen H. Coupling of Ultrafast LC with Mass Spectrometry by DESI. J Am Soc Mass Spectrom. 2014; 25:1820–1823.10.1007/s13361-014-0954-4 [PubMed: 25023648]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript


16. Liu P, Zhang J, Ferguson CN, Chen H, Loo JA. Measuring Protein-Ligand Interactions Using Liquid Sample Desorption Electrospray Ionization Mass Spectrometry. Anal Chem (Washington, DC, U S). 2013; 85:11966–11972.10.1021/ac402906d
17. Ferguson CN, Benchaar SA, Miao Z, Loo JA, Chen H. Direct Ionization of Large Proteins and Protein Complexes by Desorption Electrospray Ionization-Mass Spectrometry. Anal Chem. 2011; 83:6468–6473.10.1021/ac201390w [PubMed: 21774530]
18. Miao Z, Chen H. Direct Analysis of Liquid Samples by Desorption Electrospray Ionization-Mass Spectrometry (DESI-MS). J Am Soc Mass Spectrom. 2009; 20:10–19.10.1016/j.jasms.2008.09.023 [PubMed: 18952458]
19. Miao Z, Wu S, Chen H. The Study of Protein Conformation in Solution Via Direct Sampling by Desorption Electrospray Ionization Mass Spectrometry. J Am Soc Mass Spectrom. 2010; 21:1730–1736.10.1016/j.jasms.2010.06.003 [PubMed: 20620076]
20. Moore BN, Hamdy O, Julian RR. Protein structure evolution in liquid DESI as revealed by selective noncovalent adduct protein probing. Int J Mass Spectrom. 2012; 330–332:220–225.10.1016/j.ijms.2012.08.013
21. Yao Y, Shams-Ud-Doha K, Daneshfar R, Kitova E, Klassen J. Quantifying Protein-Carbohydrate Interactions Using Liquid Sample Desorption Electrospray Ionization Mass Spectrometry. J Am Soc Mass Spectrom. 2015; 26:98–106.10.1007/s13361-014-1008-7 [PubMed: 25315460]
22. Li J, Dewald HD, Chen H. Online Coupling of Electrochemical Reactions with Liquid Sample Desorption Electrospray Ionization-Mass Spectrometry. Anal Chem. 2009; 81:9716–9722.10.1021/ac901975j [PubMed: 19873992]
23. Bilusich D, Bowie JH. Fragmentations of (M–H)- anions of underivatised peptides. Part 2: Characteristic cleavages of Ser and Cys and of disulfides and other post-translational modifications, together with some unusual internal processes. Mass Spectrom Rev. 2009; 28:20–34.10.1002/mas.20206 [PubMed: 18989895]
24. Gorman JJ, Wallis TP, Pitt JJ. Protein disulfide bond determination by mass spectrometry. Mass Spectrom Rev. 2002; 21:183–216.10.1002/mas.10025 [PubMed: 12476442]
25. Jones MD, Patterson SD, Lu HS. Determination of Disulfide Bonds in Highly Bridged Disulfide-Linked Peptides by Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry with Postsource Decay. Anal Chem. 1998; 70:136–143.10.1021/ac9707693 [PubMed: 9435472]
26. Qiu X, Cui M, Li H, Liu Z, Liu S. Prompt disulfide fragmentations of disulfide-containing proteins in a matrix-assisted laser desorption/ionization source. Rapid Commun Mass Spectrom. 2007; 21:3520–3525.10.1002/rcm.3230 [PubMed: 17922484]
27. Peng IX, Ogorzalek Loo RR, Shiea J, Loo JA. Reactive-Electrospray-Assisted Laser Desorption/Ionization for Characterization of Peptides and Proteins. Anal Chem. 2008; 80:6995–7003.10.1021/ac800870c [PubMed: 18683952]
28. Love CB, Tan L, Francisco JS, Xia Y. Competition of Charge- vs. Radical-Directed Fragmentation of Gas-Phase Protonated Cysteine Sulfinyl Radicals. J Am Chem Soc. 201310.1021/ja4008744
29. Shaw JB, Ledvina AR, Zhang X, Julian RR, Brodbelt JS. Tyrosine Deprotonation Yields Abundant and Selective Backbone Cleavage in Peptide Anions upon Negative Electron Transfer Dissociation and Ultraviolet Photodissociation. J Am Chem Soc. 2012; 134:15624–15627.10.1021/ja3032086 [PubMed: 22970927]
30. Parthasarathi R, He Y, Reilly JP, Raghavachari K. New Insights into the Vacuum UV Photodissociation of Peptides. J Am Chem Soc. 2010; 132:1606–1610.10.1021/ja907975v [PubMed: 20078132]
31. Stinson CA, Xia Y. Radical induced disulfide bond cleavage within peptides via ultraviolet irradiation of an electrospray plume. Analyst (Cambridge, U K). 2013; 138:2840–2846.10.1039/c3an00303e
32. Calabrese A, Good N, Wang T, He J, Bowie J, Pukala T. A Negative Ion Mass Spectrometry Approach to Identify Cross-Linked Peptides Utilizing Characteristic Disulfide Fragmentations. J Am Soc Mass Spectrom. 2012; 23:1364–1375.10.1007/s13361-012-0407-x [PubMed: 22644737]
33. Thakur SS, Balaram P. Fragmentation of Peptide Disulfides under Conditions of Negative Ion Mass Spectrometry: Studies of Oxidized Glutathione and Contryphan. J Am Soc Mass Spectrom. 2008; 19:358–366.10.1016/j.jasms.2007.12.005 [PubMed: 18258450]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript


34. Zhang M, Kaltashov IA. Mapping of Protein Disulfide Bonds Using Negative Ion Fragmentation with a Broadband Precursor Selection. Anal Chem. 2006; 78:4820–4829.10.1021/ac060132w [PubMed: 16841900]
35. Bilusich D, Bowie JH. Identification of intermolecular disulfide linkages in underivatised peptides using negative ion electrospray mass spectrometry. A joint experimental and theoretical study. Rapid Commun Mass Spectrom. 2007; 21:619–628.10.1002/rcm.2872 [PubMed: 17279483]
36. Zubarev RA, Kruger NA, Fridriksson EK, Lewis MA, Horn DM, Carpenter BK, McLafferty FW. Electron Capture Dissociation of Gaseous Multiply-Charged Proteins Is Favored at Disulfide Bonds and Other Sites of High Hydrogen Atom Affinity. J Am Chem Soc. 1999; 121:2857–2862.10.1021/ja981948k
37. Gunawardena HP, Gorenstein L, Erickson DE, Xia Y, McLuckey SA. Electron transfer dissociation of multiply protonated and fixed charge disulfide linked polypeptides. Int J Mass spectrom. 2007; 265:130–138.10.1016/j.ijms.2007.01.017
38. Cole S, Ma X, Zhang X, Xia Y. Electron Transfer Dissociation (ETD) of Peptides Containing Intrachain Disulfide Bonds. J Am Soc Mass Spectrom. 2012; 23:310–320.10.1007/s13361-011-0300-z [PubMed: 22161508]
39. Ni W, Lin M, Salinas P, Savickas P, Wu SL, Karger B. Complete Mapping of a Cystine Knot and Nested Disulfides of Recombinant Human Arylsulfatase A by Multi-Enzyme Digestion and LC-MS Analysis Using CID and ETD. J Am Soc Mass Spectrom. 2013; 24:125–133.10.1007/s13361-012-0510-z [PubMed: 23208745]
40. Wu SL, Jiang H, Hancock WS, Karger BL. Identification of the Unpaired Cysteine Status and Complete Mapping of the 17 Disulfides of Recombinant Tissue Plasminogen Activator Using LC-MS with Electron Transfer Dissociation/Collision Induced Dissociation. Anal Chem. 2010; 82:5296–5303.10.1021/ac100766r [PubMed: 20481521]
41. Wu SL, Jiang H, Lu Q, Dai S, Hancock WS, Karger BL. Mass Spectrometric Determination of Disulfide Linkages in Recombinant Therapeutic Proteins Using Online LC-MS with Electron-Transfer Dissociation. Anal Chem. 2008; 81:112–122.10.1021/ac801560k [PubMed: 19117448]
42. Xia Y, Cooks RG. Plasma Induced Oxidative Cleavage of Disulfide Bonds in Polypeptides during Nanoelectrospray Ionization. Anal Chem. 2010; 82:2856–2864.10.1021/ac9028328 [PubMed: 20196567]
43. Barlow CK, O'Hair RAJ. Gas-phase peptide fragmentation: how understanding the fundamentals provides a springboard to developing new chemistry and novel proteomic tools. J Mass Spectrom. 2008; 43:1301–1319.10.1002/jms.1469 [PubMed: 18819114]
44. Chrisman PA, McLuckey SA. Dissociations of Disulfide-Linked Gaseous Polypeptide/Protein Anions:3 Ion Chemistry with Implications for Protein Identification and Characterization. J Proteome Res. 2002; 1:549–557.10.1021/pr025561z [PubMed: 12645623]
45. Lioe H, Duan M, O'Hair RAJ. Can metal ions be used as gas-phase disulfide bond cleavage reagents? A survey of coinage metal complexes of model peptides containing an intermolecular disulfide bond. Rapid Commun Mass Spectrom. 2007; 21:2727–2733.10.1002/rcm.3122 [PubMed: 17654640]
46. Kim HI, Beauchamp JL. Identifying the Presence of a Disulfide Linkage in Peptides by the Selective Elimination of Hydrogen Disulfide from Collisionally Activated Alkali and Alkaline Earth Metal Complexes. J Am Chem Soc. 2008; 130:1245–1257.10.1021/ja075698w [PubMed: 18181621]
47. Gunawardena HP, O'Hair RAJ, McLuckey SA. Selective Disulfide Bond Cleavage in Gold(I) Cationized Polypeptide Ions Formed via Gas-Phase Ion/Ion Cation Switching. J Proteome Res. 2006; 5:2087–2092.10.1021/pr0602794 [PubMed: 16944919]
48. Mihalca R, van der Burgt YEM, Heck AJR, Heeren RMA. Disulfide bond cleavages observed in SORI-CID of three nonapeptides complexed with divalent transition-metal cations. J Mass Spectrom. 2007; 42:450–458.10.1002/jms.1175 [PubMed: 17295413]
49. Teesch LM, Orlando RC, Adams J. Location of the alkali metal ion in gas-phase peptide complexes. J Am Chem Soc. 1991; 113:3668–3675.10.1021/ja00010a006
50. Honeychurch MJ. The reduction of disulfide bonds in proteins at mercury electrodes. Bioelectrochem Bioenerg. 1997; 44:13–21.10.1016/S0302-4598(97)00062-7

Author Manuscript Author Manuscript Author Manuscript Author Manuscript


51. Stankovich MT, Bard AJ. The electrochemistry of proteins and related substances: Part II. Insulin. J electroanal chem interfacial electrochem. 1977; 85:173–183.10.1016/0368-1874(77)80458-9
52. Zhang Y, Yuan Z, Dewald HD, Chen H. Coupling of liquid chromatography with mass spectrometry by desorption electrospray ionization (DESI). Chem Commun. 2011; 47:4171–4173.10.1039/c0cc05736c
53. Zhang Y, Dewald HD, Chen H. Online Mass Spectrometric Analysis of Proteins/Peptides Following Electrolytic Cleavage of Disulfide Bonds. J Proteome Res. 2011; 10:1293–1304.10.1021/pr101053q [PubMed: 21197958]
54. Zhang Y, Cui W, Zhang H, Dewald HD, Chen H. Electrochemistry-Assisted Top-Down Characterization of Disulfide-Containing Proteins. Anal Chem (Washington, DC, U S). 2012; 84:3838–3842.10.1021/ac300106y
55. Kraj A, Brouwer HJ, Reinhoud N, Chervet JP. A novel electrochemical method for efficient reduction of disulfide bonds in peptides and proteins prior to MS detection. Anal Bioanal Chem. 2013; 405:9311–9320.10.1007/s00216-013-7374-3 [PubMed: 24077854]
56. Nicolardi S, Giera M, Kooijman P, Kraj A, Chervet JP, Deelder AM, van der Burgt YEM. On-Line Electrochemical Reduction of Disulfide Bonds: Improved FTICR-CID and -ETD Coverage of Oxytocin and Hepcidin. J Am Soc Mass Spectrom. 2013; 24:1980–1987.10.1007/s13361-013-0725-7 [PubMed: 24018861]
57. Iavarone AT, Williams ER. Mechanism of charging and supercharging molecules in electrospray ionization. J Am Chem Soc. 2003; 125:2319–27.10.1021/ja021202t [PubMed: 12590562]
58. Lomeli SH, Yin S, Ogorzalek LRR, Loo JA. Increasing charge while preserving noncovalent protein complexes for ESI-MS. J Am Soc Mass Spectrom. 2009; 20:593–6.10.1016/j.jasms.2008.11.013 [PubMed: 19101165]
59. Liu Y, Miao Z, Lakshmanan R, Loo RRO, Loo JA, Chen H. Signal and charge enhancement for protein analysis by liquid chromatography-mass spectrometry with desorption electrospray ionization. Int J Mass Spectrom. 2012; 325–327:161–166.10.1016/j.ijms.2012.06.006
60. Loo JA, Edmonds CG, Udseth HR, Smith RD. Effect of reducing disulfide-containing proteins on electrospray ionization mass spectra. Anal Chem. 1990; 62:693–8.10.1021/ac00206a009 [PubMed: 2327585]
61. Zhang J, Loo RRO, Loo JA. Increasing fragmentation of disulfide-bonded proteins for top-down mass spectrometry by supercharging. Int J Mass Spectrom. 2014 Ahead of Print. 10.1016/j.ijms.2014.07.047

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

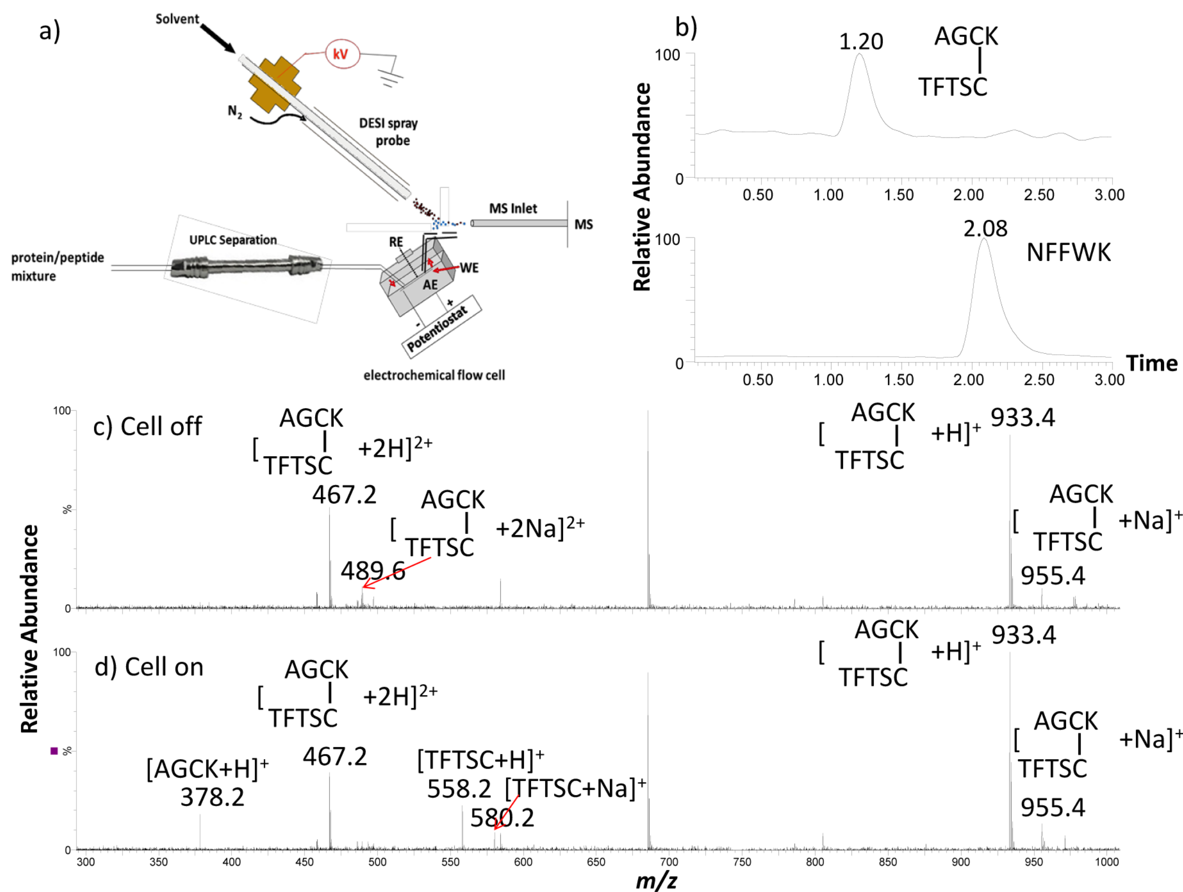


**Figure 1.**

a) The apparatus of UPLC/EC/DESI-MS. b) Extracted ion chromatograms (EICs) showing the UPLC separation of b) AGCK TFTSC (upper panel) and NFFWK (lower panel). DESI-MS spectra of AGCK TFTSC c) when cell was off and d) when cell was on.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript



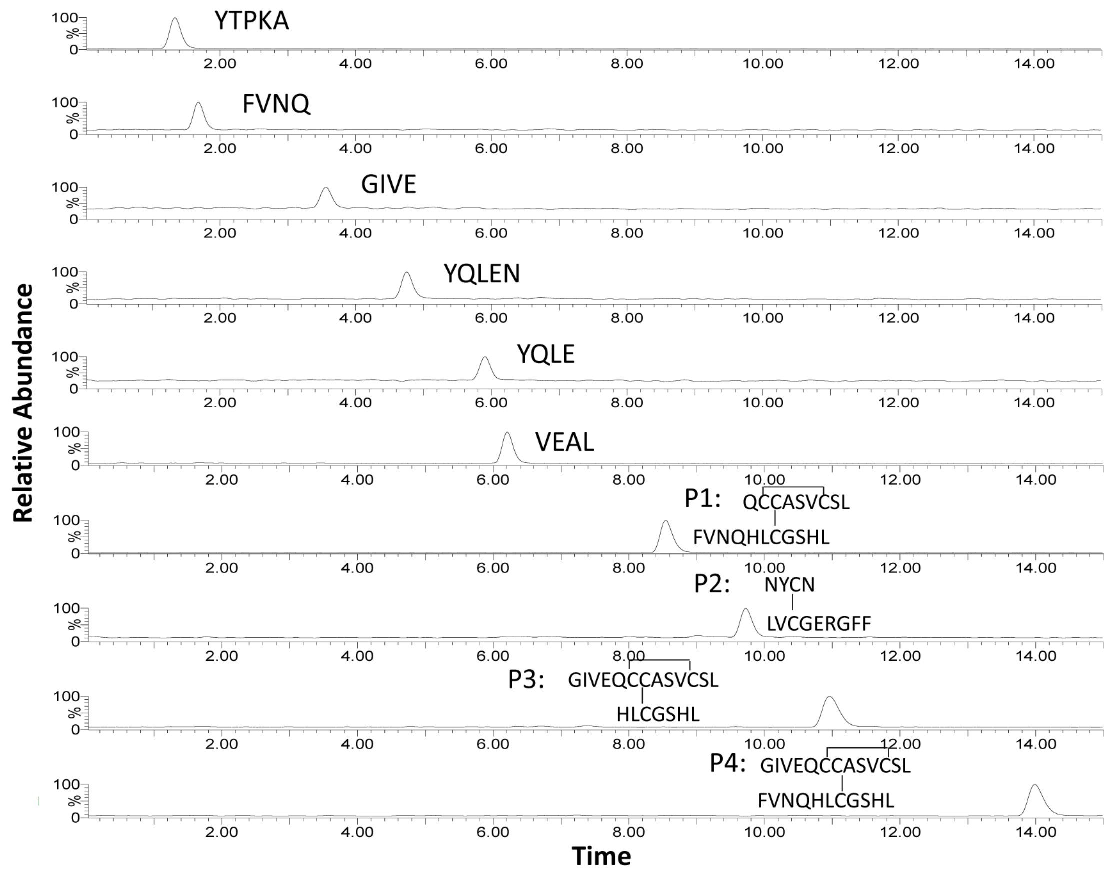


**Figure 2.**

EICs of the pepsin-digested insulin

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript



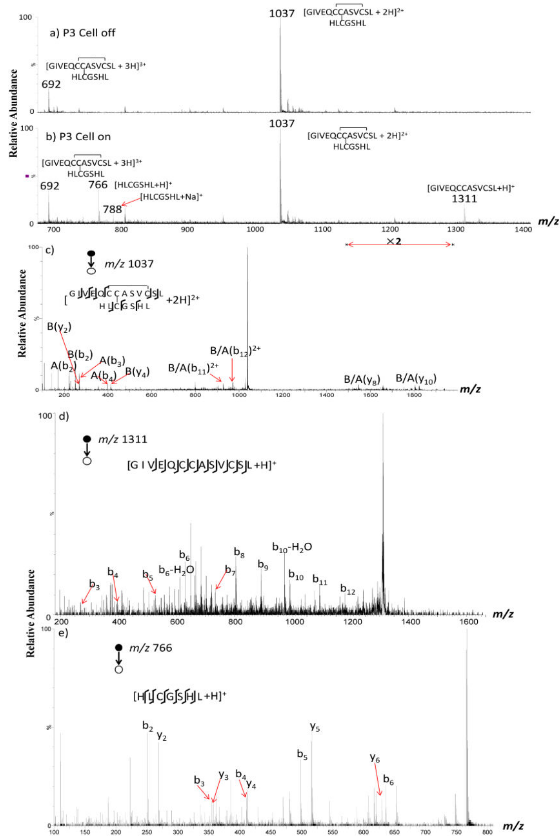


**Figure 3.**

DESI-MS spectra of P3 with a) cell off and b) cell on, c) CID MS$^2$ spectrum of doubly charged P3 at *m/z* 1037, d) CID MS$^2$ spectrum of singly charged P3 chain A at *m/z* 1311, and d) CID MS$^2$ spectrum of singly charged P3 chain B at *m/z* 766.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

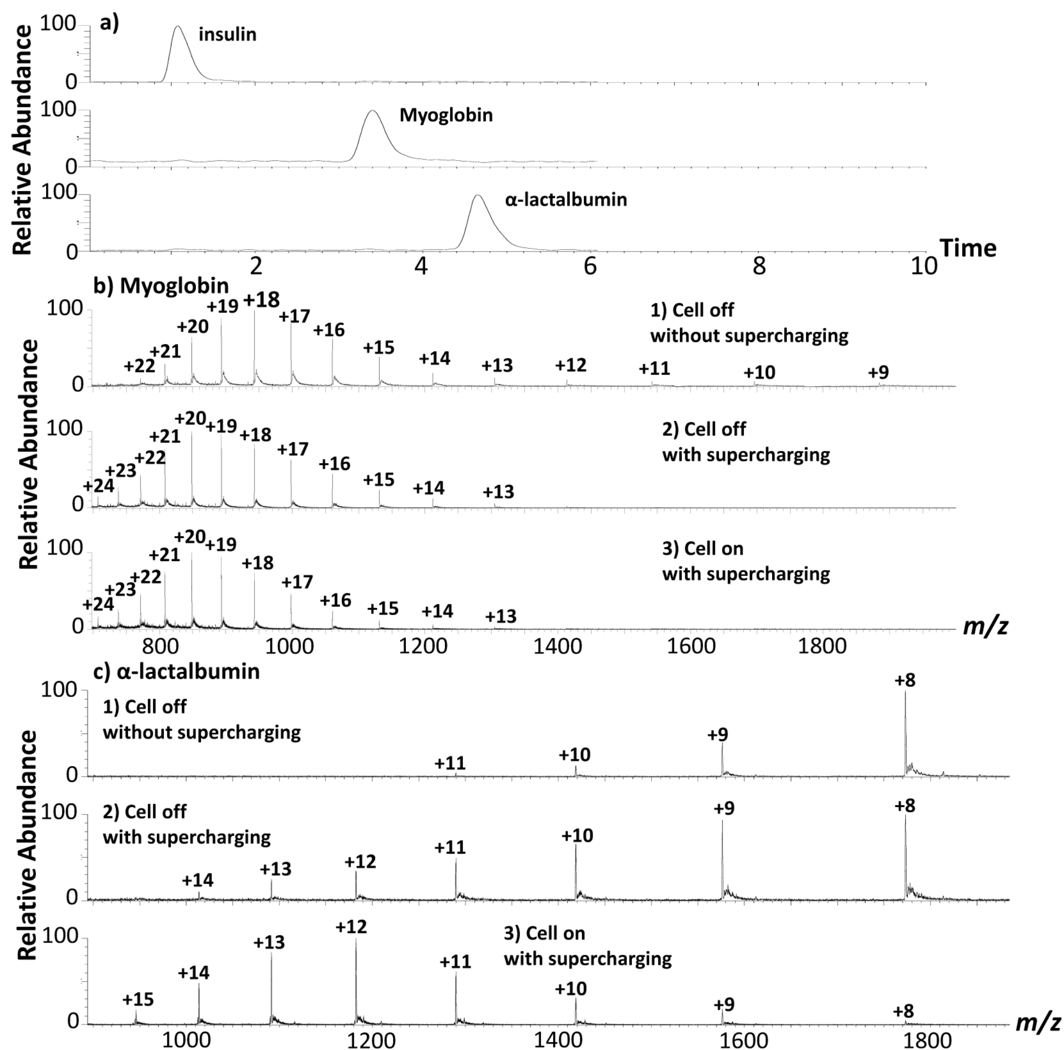


**Figure 4.**

a) EICs of the protein mixture containing insulin, myoglobin and α-lactalbumin, b) DESI-MS spectra of the UPLC-separated myoglobin 1) when cell was off without supercharging, 2) when cell was off with supercharging and 3) when cell was on with supercharging. c) DESI-MS spectra of the UPLC-separated α-lactalbumin 1) when cell was off without supercharging, 2) when cell was off with supercharging and 3) when cell was on with supercharging.