

Online Mass Spectrometric Analysis of Proteins/Peptides Following Electrolytic Cleavage of Disulfide Bonds

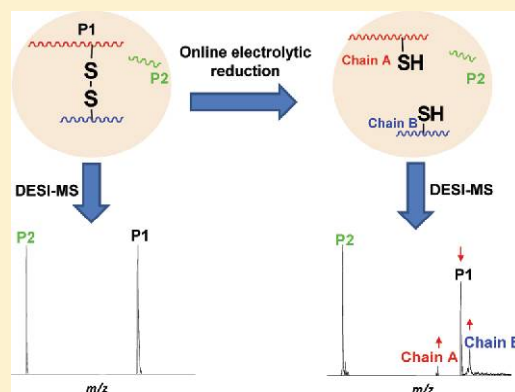
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Supporting Information

ABSTRACT: The disulfide bond bridge is an important post-translational modification for proteins. This study presents a structural analysis of biologically active peptides and proteins containing disulfide bonds using electrochemistry (EC) online combined with desorption electrospray ionization mass spectrometry (DESI-MS), in which the sample undergoes electrolytic disulfide cleavage in an electrochemical flow cell followed by MS detection. Using this EC/DESI-MS method, the disulfide-containing peptides can be quickly identified from enzymatic digestion mixtures, simply based on the abrupt decrease in their relative ion abundances after electrolysis. Peptide mass mapping and tandem MS analysis of the ions of the resulting free peptide chains can possibly establish the disulfide linkage pattern and sequence the precursor peptides. In this regard, the method provides much more chemical information than previous analogous electrochemical analyses. In addition, derivatization of thiols by selective selenamide reagents is useful for easy recognition of reduced peptide ions and the number of their free thiols. Furthermore, electrolytic reduction of proteins (e.g., α -lactalbumin) leads to increased charges on the detected protein ions, revealing the role of disulfide bonds on maintaining protein conformation. This electrochemical mass spectrometric method is fast (completed in few minutes) and does not need chemical reductants, potentially having valuable applications in proteomics research.

KEYWORDS: mass spectrometry, electrochemistry, desorption electrospray ionization, disulfide bond reduction, protein conformation



INTRODUCTION

Redox-active disulfide bonds are one of the most common protein post-translational modifications (PTM) and provide reversible covalent cross-linkages in native proteins for maintaining the three-dimensional structures of proteins and their biological activities.^{1,2} Such a linkage plays a critical role in the activity of enzymes and is also a key structural feature of biologically active peptide hormones such as somatostatin, oxytocin, and [Arg⁸]-vasopressin.³ The presence of the disulfide linkages increases the complexity for the protein structure elucidation by mass spectrometry (MS). The cleavage of disulfide bonds is often essential for the protein/peptide analysis as dissociation of a reduced protein/peptide ion can give rise to more structurally informative fragment ions than that of the intact counterpart.^{1,2} The traditional protocol to break a disulfide bond is chemical reduction using an excess amount of reagents like dithiothreitol (DTT) or tris(2-carboxyethyl)phosphine (TCEP). However, the reduction usually takes one-half to several hours and the removal of the excess amount of reductant is time-consuming and troublesome. Besides chemical reduction, other novel approaches include the cleavage of disulfide bonds via laser-based ionization,^{4,5} ultraviolet photodissociation,⁶ negative ion dissociation,^{1,7,8} electron-capture dissociation (ECD),⁹

electron-transfer dissociation (ETD),¹⁰ plasma-induced oxidation,¹¹ reactive electrospray-assisted laser desorption/ionization (ELDI),¹² or using new ion chemistry.^{13,14}

An alternative way for reducing disulfide bonds without involving chemical reductants is electrolytic reduction. It is well-known that disulfide bonds can undergo reductive cleavage on an amalgam electrode surface.¹⁵ Previous investigations^{3,16–18} showed that such a reduction followed with electrolytic oxidation of the resulting thiols back to disulfides in an electrochemical flow cell can be coupled with either HPLC or electrophoresis separation, which are useful for the simultaneous detection of thiol- and disulfide-containing peptides in mixtures.^{19,20} Although these techniques are elegant and quite sensitive, they do not provide structural information for the detected peptides. Also, the separation processes involved take a long time and the disulfide-containing peptides cannot be detected if they do not elute from the HPLC column.¹⁹

Desorption electrospray ionization (DESI)²¹ has recently been introduced for direct ionization of analytes with little or no sample preparation. This technique is successful in the fast

Received: October 20, 2010

Published: January 03, 2011

analysis of a variety of different analytes ranging from pharmaceuticals to tissue imaging.^{22–24} In addition to being used regularly for solid sample analysis of surfaces, DESI has been extended to the analysis of liquid samples in several laboratories.^{25–28} In liquid sample DESI experiments, ionization occurs via interactions of the sample with charged microdroplets generated in a pneumatically assisted DESI spray and subsequent desolvation of the resulting secondary microdroplets containing the sample analyte. It has been shown that liquid sample DESI allows convenient online coupling of MS with electrochemistry (EC),²⁹ microfluidics,³⁰ and single droplet microextraction.²⁷ It can also be used for monitoring protein conformational changes in solution.³¹ In the combined EC/DESI-MS experiments, previous data showed that the products generated from redox reactions such as dopamine oxidation and disulfide bond reduction can be directly desorbed and ionized from an electrochemical cell by DESI for online MS analysis.²⁹ The method is a new development in the field of electrochemical mass spectrometry (EC/MS, viz., online electrochemistry coupled with mass spectrometry) which has a history of 40 years.³² Different ionization methods such as thermospray (TS) and electrospray ionization (ESI)^{33–39} were employed in previous EC/MS studies, which has found numerous applications such as the identification of electrochemically generated species, mimicking biologically relevant electrochemical reactions, oxidative cleavage of peptide backbones, and online chemical tagging. In our EC/DESI-MS method,^{25,29} some unique and valuable features originating from using DESI as the coupling interface have been revealed, including a simplified coupling apparatus owing to no requirement to separate the small potential applied to the electrochemical cell from the high voltage used for spray ionization, tolerance to inorganic salt electrolytes, and the freedom to choose traditional solvents for electrolysis, as well as, to use either positive or negative ionization modes.

In this study, the EC/DESI-MS method has been explored for analysis of protein/peptide digest mixtures, various biologically active peptides (both intra- and inter- disulfide bond containing peptides) and intact proteins. It is one part of our effort toward the utilization of EC/DESI-MS in proteomics research, as motivated by the fact that many proteins (e.g., disulfide or metal ion containing proteins) are electroactive species and the investigation of protein electrochemistry is still very limited.⁴⁰ In this experiment, we first optimized the experimental conditions to improve the performance of an amalgam electrode for reduction by polishing its surface with a silk handkerchief and using low concentration of protein and peptide samples to reduce possible chemical adsorption on the electrode. Through the analysis of various types of samples by the EC/DESI-MS, several interesting findings are uncovered, including: (i) disulfide-containing peptides can be quickly identified from enzymatic digest mixtures, simply based on their large relative ion abundance changes before and after electrolysis; (ii) in conjunction with tandem MS analysis and peptide mass mapping, the disulfide linkage pattern and sequence information for the examined peptides could be determined; (iii) in comparison to traditional chemical reduction, the reported online EC/DESI-MS method is much faster (completed in few minutes) and has no requirement to remove chemical reductants for obtaining good ionization efficiency; (iv) in the case of peptides having symmetric chains or intrapeptide disulfide bonds, selenamide reagents can be used to label the reduced peptides, a rapid and highly selective thiol derivatization strategy recently developed in

our laboratory,⁴¹ which aids in the recognition of the reduced peptide ions, the number of free thiols in the reduced peptides, and the type of disulfide bond in the precursor peptides examined; (v) the role of disulfide bonds in maintaining protein folding can be revealed, based on the charge state distribution (CSD) changes after electrolytic reduction.

EXPERIMENTAL SECTION

Chemicals

Peptides [Arg⁸]-conopressin G and somatostatin 1–14 were purchased from Bachem (King of Prussia, PA). Ebselen was purchased from Calbiochem (Cincinnati, OH). TPCK-treated trypsin from bovine pancreas (MW ≈ 23.8 KDa), pepsin from porcine gastric mucosa (MW ≈ 35 KDa), *N*-(phenylseleno) phthalimide, ammonium bicarbonate, TCEP hydrochloride solution, mercury (triply distilled, 99.9999% purity), L-glutathione disulfide (GSSG), [Arg⁸]-vasopressin acetate salt, oxytocin, bovine pancreas insulin, α -lactalbumin from bovine milk (type III, calcium depleted, ≥ 85%), and HPLC-grade acetonitrile were all purchased from Sigma-Aldrich (St. Louis, MO). Acetic acid and HPLC-grade methanol were obtained from Fisher Scientific (Fair Lawn, NJ) and GFS Chemicals (Columbus, OH), respectively. The silk handkerchief was purchased from Royal Silk Direct, Inc. (Princeton, NJ). The deionized water used for sample preparation was obtained using a Nanopure Diamond Barnstead purification system (Barnstead International, Dubuque, IA).

Tryptic Digestion

Digestion of peptides were carried out using TPCK-treated trypsin with a ratio of 1:100 (enzyme/peptide) in 25 mM ammonium bicarbonate aqueous solution for 3 h at 38 °C incubation to finish digestion.⁴²

Peptic Digestion

Digestion of insulin was performed by incubating insulin and pepsin at a molar ratio of 50:1 in water containing 1% acetic acid at 37 °C for 6.5 h.¹⁴ In the comparison experiment using chemical reduction, TCEP was added to the peptic digested insulin in the molar ratio of 1:20 (protein/TCEP) for 2 h at room temperature.

Online EC/DESI-MS Apparatus

A home-built apparatus for coupling a thin-layer electrochemical flow cell with either a Thermo Finnigan LCQ/DECA ion trap mass spectrometer (San Jose, CA) or a hybrid triple quadrupole-linear ion trap mass spectrometer (Q-trap 2000, Applied Biosystems/MDS SCIEX, Concord, Canada) by liquid sample DESI was used and described previously in detail.²⁹ The thin-layer electrochemical flow cell consisted of a working electrode (WE) embedded in PEEK and separated from a stainless steel auxiliary electrode (AE) by two Teflon gaskets (0.01 in. thick each) and a Ag/AgCl (3 M NaCl) reference electrode (RE) contacting the sample solution through a small hole in the AE. The WE used was a dual amalgam electrode (3 mm diameter) or an amalgam electrode (6 mm diameter) for reduction. The procedure used to polish the electrodes is as follow; the residual mercury was removed by 6 M nitric acid from the gold surface, and then the electrode was polished sequentially with water, 15, 3, and 1 μ m diamond and 0.5 μ m alumina powders on a grit pad or a nylon pad. Also the electrode block was rinsed using methanol and water and then air-dried. After that, the electrode was coated with a drop of mercury for 10 min, and the excess amount of mercury was removed by one edge of an index card. The mercury surface

Table 1. Sequence and MW Information of Precursor Proteins/Peptides, Digested Peptides, and Reduced Peptide Chains^a

Proteins/peptides	MW (Da)	Sequences	Sequences of peptides obtained from digestion	Denotation	MW (Da)	Sequences of reduced peptides and their MW (Da)		
[Arg ⁸]-Conopressin G	1062.3		CFIR	"Chain A"	P1	1023.5 (1023.9*)	CFIR	537.3 (537.0*)
			NCPR	"Chain B"			NCPR	488.2 (488.0*)
Somatostatin 1-14	1637.9		AGCK	"Chain A"	P2	932.4 (932.5*)	AGCK	377.2 (377.1*)
			TFTSC	"Chain B"			TFTSC	557.2 (557.3*)
			NFFWK			740.4 (740.3*)		
Insulin	5733.5		GIVEQCCASVCSL	"Chain A"	P3	2071.9 (2073.3*)	GIVEQCCASVCSL	1310.6 (1310.8*)
			HLCGSHL	"Chain B"			HLCGSHL	765.4 (766.1*)
			GIVEQCCASVCSL	"Chain A"	P4	2560.1 (2561.7*)	GIVEQCCASVCSL	1310.6 (1310.8*)
			FVNQHLCGSHL	"Chain B"			FVNQHLCGSHL	1253.6 (1254.3*)
			QCCASVCSL	"Chain A"	P5	2161.9 (2163.2*)	QCCASVCSL	912.4 (912.4*)
			FVNQHLCGSHL	"Chain B"			FVNQHLCGSHL	1253.6 (1254.3*)
			NYCN	"Chain A"	P6	1536.5 (1538.0*)	-	-
			LVCGERGFF	"Chain B"			LVCGERGFF	1026.5 (1027.1*)
			YQLENYCN	"Chain A"	P7	1922.8 (1920.9*)	-	-
			LVCGERGF	"Chain B"			LVCGLRGF	879.4 (879.6*)
			ALY			365.2 (365.4*)		
			GIVE			416.2 (416.4*)		
			VEAL			430.2 (430.4*)		
			FVNQ			506.2 (506.5*)		
YQLE			551.3 (551.5*)					
YTPKA			578.3 (578.6*)					
QLENY			665.3 (665.5*)					

^a MW labeled with "*" in the parentheses refers to the measured MW from the m/z values; The small differences between the measured MW and the theoretic MW of peptides P3–P7 were caused by the low resolution of the ion trap instrument in the analysis of multiply charged ions of these peptides. "-" refers to "not detected".

was further polished with a piece of silk handkerchief until the smooth surface was obtained. After kept overnight (at least 6 h), the amalgam electrode was used for reduction.¹⁹ A ROXY potentiostat (Antec Leyden, Netherlands) was used to apply potentials to the electrochemical cell for reduction of analytes that flowed through the cell. Sample solutions were degassed by argon purging for 20 min to remove dissolved oxygen prior to the injection to the cell for electrolysis. The reduced species flowed out of the cell via a short piece of fused silica connection capillary (i.d. 0.1 mm, length 4.2 cm) and underwent interactions with the charged microdroplets from DESI spray for ionization. The capillary outlet was placed about 2–3 mm downstream from the DESI spray probe tip and kept in line with the sprayer tip and the mass spectrometer's inlet. The spray solvent for DESI was methanol/water (1:1 by volume) containing 1% acetic acid and injected at a rate of 5 $\mu\text{L}/\text{min}$ and a high voltage of +5 kV was applied to the spray probe. The flow rate for sample solutions passing through the electrochemical cell for electrolysis was 3 $\mu\text{L}/\text{min}$.

Safety Precautions. As mercury is toxic, the preparation for the amalgam electrode should be carried out in a hood. The mercury waste should be disposed in a special mercury waste container containing sublimed sulfur.

RESULTS AND DISCUSSION

1. Reduction of Peptides Containing Disulfide Bonds in Enzymatic Digests

a. Trypsin Digested [Arg⁸]-Conopressin G. Electrolytic reduction of disulfide bonds of trypsin digested [Arg⁸]-conopressin G (MW: 1062.3 Da) was first carried out using the EC/DESI-MS apparatus. The tryptic digestion breaks [Arg⁸]-conopressin G in its middle backbone to generate a peptide (denoted as P1) containing asymmetric chains linked by one disulfide bond (chain A: CFIR; chain B: NCPR, Table 1). Figure 1a illustrates the DESI-MS spectrum acquired when a solution of trypsin digested [Arg⁸]-conopressin G (0.1 mM) in methanol/water (1:1 by volume) containing 1% acetic acid flowed through

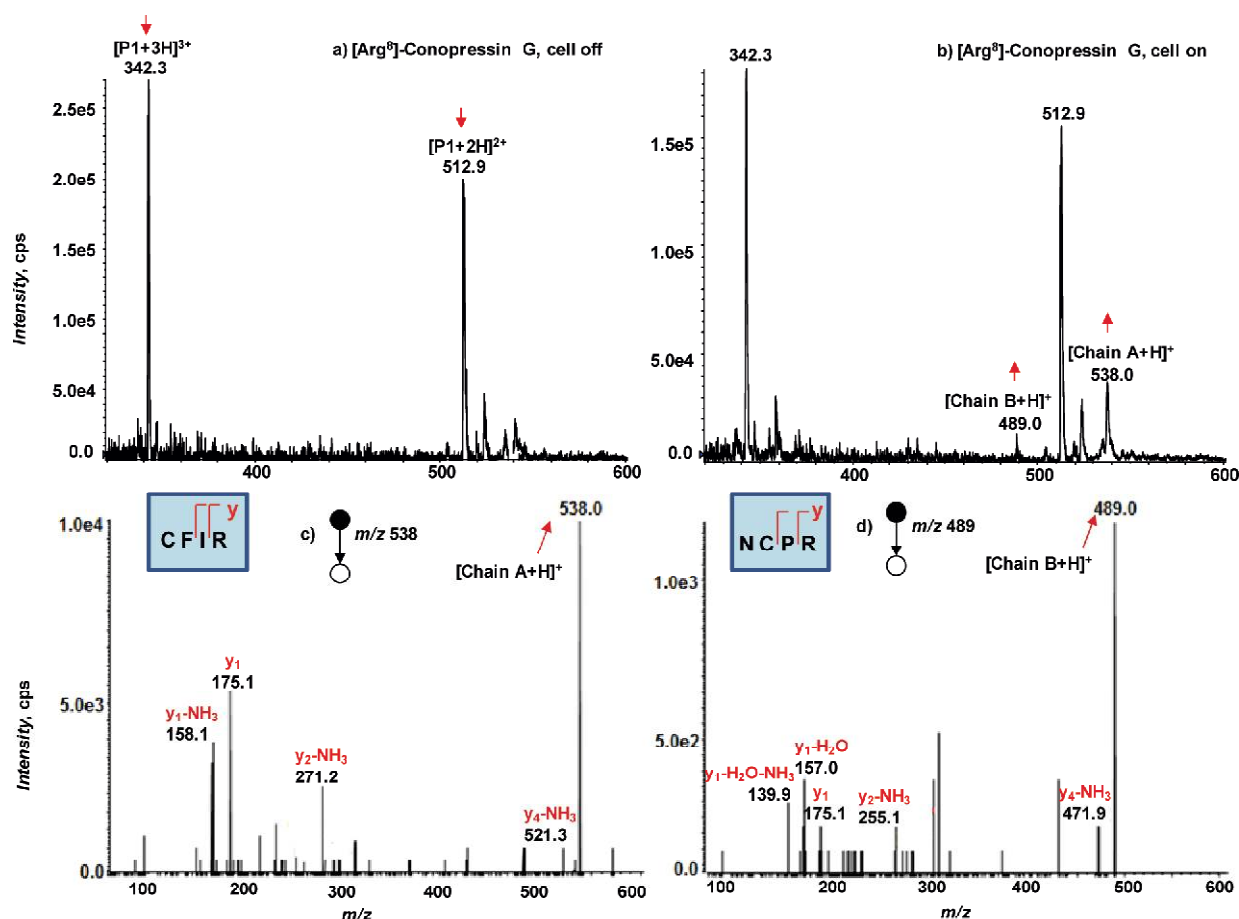


Figure 1. DESI-MS spectra acquired when a solution of 0.1 mM trypsin digested $[\text{Arg}^8]$ -conopressin G in methanol/water (1:1 by volume) containing 1% acetic acid flowed through the thin-layer electrochemical cell with an applied potential of (a) 0.0 V and (b) -2.0 V; CID MS^2 spectra of (c) the protonated chain A (m/z 538) and (d) the protonated chain B (m/z 489) resulted from the electrolytic reduction. The downward arrows in (a) indicate that the ion abundance will drop during electrolysis, the upward arrows in (b) indicate the appearance of new reduced peptide product ions. Such a labeling is applicable in Figures 2 and 3 as well.

the thin-layer electrochemical cell with no potential applied to the cell. Peaks corresponding to the doubly and triply charged P1 were detected at m/z 512.9 and 342.3, respectively. When a -2.0 V potential was applied to the amalgam working electrode, two new peaks at m/z 538.0 and 489.0 appeared, corresponding to the protonated free chain A, $[\text{CFIR}+\text{H}]^+$, and the protonated free chain B, $[\text{NCPR}+\text{H}]^+$ (Figure 1b), respectively. These assignments were consistent with their MS^2 spectra showing that m/z 538 dissociates into fragment ions of y_1 , $y_1\text{-NH}_3$, $y_2\text{-NH}_3$, and $y_4\text{-NH}_3$ ions (Figure 1c) and m/z 489 produces fragment ions of y_1 , $y_1\text{-H}_2\text{O}$, $y_1\text{-H}_2\text{O-NH}_3$, $y_2\text{-NH}_3$, and $y_4\text{-NH}_3$ ions upon CID (Figure 1d; other peaks seen in the spectrum are from the background contribution due to the low abundance of the precursor peptide ion). This result demonstrates that EC/DESI-MS is applicable for reducing the disulfide bonds of peptides in enzymatic digest and the resulting peptide chain products can be directly detected online by MS without separation, suggesting its utility in mixture analysis.

b. Trypsin Digested Somatostatin 1–14. In the experiment described above, the conversion yield was low and the working amalgam electrode had a short lifetime. By polishing the electrode surface with silk handkerchief and also lowering the concentration of the samples injected into the electrochemical cell, an enhanced reduction yield and elongated lifetime of the

amalgam were achieved. Presumably, the polishing made the amalgam surface more uniform and using diluted sample solution reduced the possible chemical adsorption onto the amalgam surface. Under these optimized conditions, electrolytic reduction of disulfide bonds of trypsin digested somatostatin 1–14 (MW: 1637.9 Da) was examined. The sequence of somatostatin 1–14, a peptide containing an intrapeptide disulfide bond, is shown in Table 1. After tryptic digestion, peptide (denoted as P2) containing asymmetric chains linked by one disulfide bond (chain A, AGCK; chain B, TFTSC) was obtained, together with another peptide NFFWK. Figure 2a illustrates the DESI-MS spectrum acquired when a solution of trypsin digested somatostatin 1–14 (35 μM) in methanol/water (1:1 by volume) containing 1% acetic acid flowed through the thin-layer electrochemical cell with no potential applied to the cell. The singly, doubly and triply charged P2 were detected at m/z 933.3, 467.3, and 312.3, respectively (these peaks are listed in Figure 2a inset for clarity). In addition, one sodium adduct peak $[\text{P2} + \text{Na}]^+$ (m/z 955.3) was seen, probably due to ubiquitous sodium ions (e.g., from the water used for preparing the sample solution). Furthermore, ions of the peptide NFFWK, $[\text{NFFWK} + \text{H}]^+$ (m/z 741.3), $[\text{NFFWK} + \text{Na}]^+$ (m/z 763.3), and $[\text{NFFWK} + 2\text{H}]^{2+}$ (m/z 371.4), were also observed. When a -1.8 V potential was applied to the cell for reduction, two new ions m/z 378.1 and 558.3

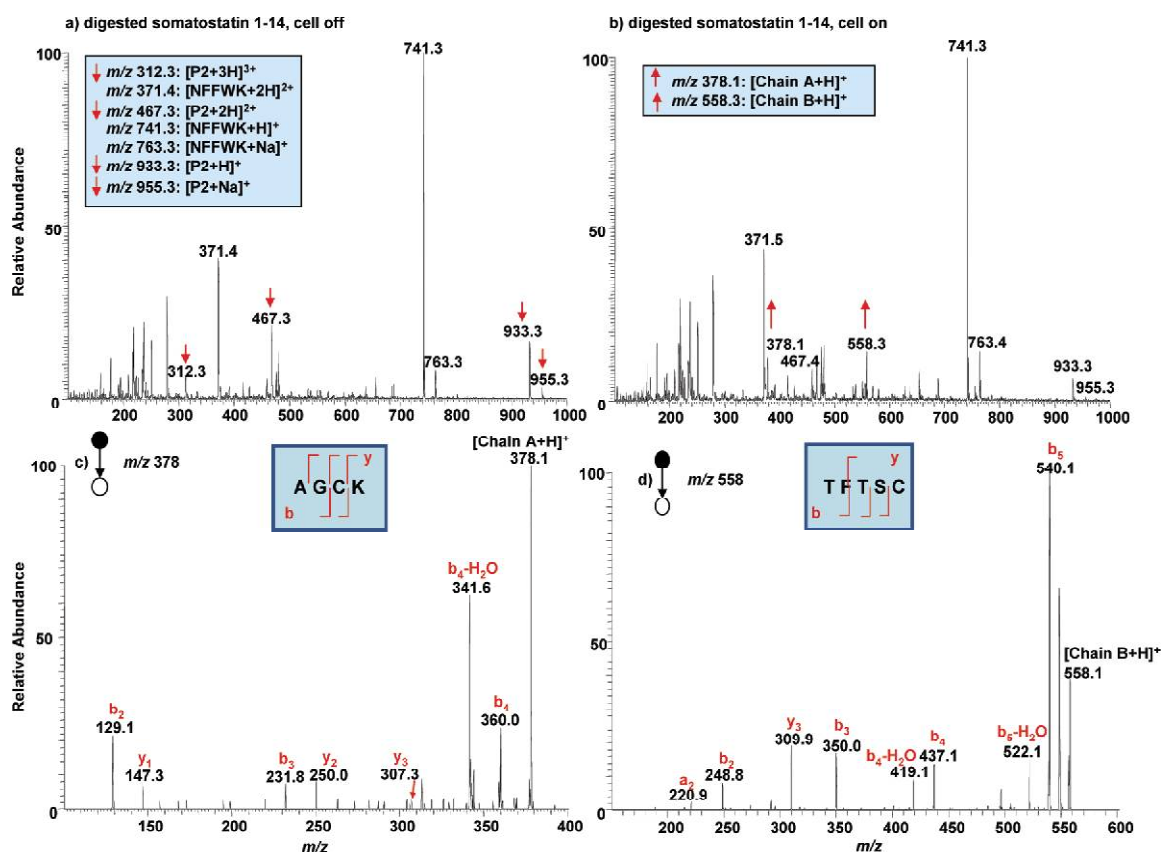


Figure 2. DESI-MS spectra acquired when a solution of 35 μ M trypsin digested somatostatin 1–14 in methanol/water (1:1 by volume) containing 1% acetic acid flowed through the thin-layer electrochemical cell with an applied potential of (a) 0.0 V and (b) -1.8 V; CID MS² spectra of (c) the protonated chain A (m/z 378) and (d) the protonated chain B (m/z 558).

were generated, corresponding to the protonated ions of free chain A and chain B of P2, respectively (Figure 2b). Strikingly, abrupt changes in relative abundances of disulfide containing peptide ions before and after electrolysis were observed (the data are summarized in Table 2). For instance, the relative ion abundances of ions associated with the peptide P2, $[P2 + H]^+$, $[P2 + Na]^+$, $[P2 + 2H]^{2+}$ and $[P2 + 3H]^{3+}$, decreased to a considerable extent (by 59–100% reduction) when the cell was turned on (Table 2). In particular, the triply charged P2 $[P2 + 3H]^{3+}$ (m/z 312.3) completely disappeared after electrolysis (Figure 2b). By contrast, the relative abundances for ions associated with the peptide NFFWK without disulfide bonds, $[NFFWK + H]^+$, $[NFFWK + Na]^+$, and $[NFFWK + 2H]^{2+}$, remained nearly unchanged (instead, a small increase of 2–5% were observed for the latter two ions probably caused by signal fluctuation during ionization, Table 2). This result is exactly in line with the fact that NFFWK is not electroactive while the disulfide-containing peptide P2 is. The pronounced changes in relative ion abundance due to electrolysis are useful to distinguish disulfide containing peptides from those without disulfide bonds in the enzymatic digest mixtures. As the electrolysis is simply controlled by turning on and off the cell potential, such a differentiation is simple and fast.

In addition, as shown in Figure 2c, the electro-generated chain A ion (m/z 378) gives rise to the fragment ions of b_2 , b_3 , b_4 , b_4-H_2O , y_1 , y_2 , and y_3 ions upon CID, from which its sequence can be determined as AGCK (the cysteine residue is in the third position in chain A). Likewise, the protonated chain B (m/z 558) dissociated into a_2 , b_2 , b_3 , b_4 , b_4-H_2O , b_5 , b_5-H_2O and y_3 ions

(Figure 2d), which reveals its sequence to be either TFTSC or FTTSC (in other words, the cysteine is located in the fifth position in chain B). In comparison to the fact that the CID of the protonated intact P2 (m/z 933) only produces A1–A4/B(y_1), A1–A4/B(y_2), A1–A4/B(y_3), B1–B5/A(b_3) and B1–B5/A(y_2) ions (spectrum not shown; A1–A4/B(y_1) refers to a fragment with y_1 ion of B chain linked with an intact A chain; the notation is applicable to other fragment ions), the electrolytic reduction of disulfide bond allows one to obtain more sequence information of the examined peptide. More importantly, in this experiment, the connectivity of the disulfide bond in P2 can be inferred, based on the measured molecule weight (MW) information of the precursor peptide and its reduced peptide chains (both theoretic and measured MWs of the peptides and their reduced peptide chains are listed in the Table 1 with the measured MWs labeled with “*”). As discussed above, we know that electroactive P2 in the mixture contains disulfide bonds and the two new peptides AGCK and TFTSC are generated after the sample electrolysis. It is found that the sum of the measured MWs of AGCK and TFTSC (934.4 Da) is 1.9 Da higher than that of the precursor peptide P2 (932.5 Da). This indicates that AGCK and TFTSC are the two chains of P2, and P2 has one disulfide bond bridging these two chains. According to the sequence information of each chain revealed by CID as mentioned above, it is therefore clear that the disulfide bond linkage in P2 is the one bridging Cys³ of chain A and Cys⁵ of chain B (Table 1).

These results confirmed that the EC/DESI-MS can be used for reduction of disulfide bonds of peptides in enzymatic digest mixtures. Such an experiment is versatile. One utility is the fast recognition of disulfide-containing peptides from the digest,

Table 2. Relative Abundance Changes After the Electrolysis

proteins/peptides	peptide ions	<i>m/z</i>	relative abundances when cell was turned off (RA_{off})	relative abundances when cell was turned on (RA_{on})	$(RA_{\text{on}} - RA_{\text{off}}) / RA_{\text{off}} \times 100\%$
Somatostatin 1–14	$[P2 + H]^+$	933.3	18	5	−72.2
	$[P2 + Na]^+$	955.3	4	1	−75.0
	$[P2 + 2H]^{2+}$	467.3	22	9	−59.1
	$[P2 + 3H]^{3+}$	312.3	6	0	−100.0
	$[NFFWK + H]^+$	741.3	100	100	0.0
	$[NFFWK + Na]^+$	763.3	10	10.5	+5.0
	$[NFFWK + 2H]^{2+}$	371.4	42	43	+2.4
Insulin	$[P3 + 2H]^{2+}$	1037.5	70	38	−45.7
	$[P3 + 3H]^{3+}$	692.2	96	59	−38.5
	$[P4 + 2H]^{2+}$	1282.5	20	8	−60.0
	$[P4 + 3H]^{3+}$	855.1	62	40	−35.5
	$[P5 + 2H]^{2+}$	1082.5	38	25	−34.2
	$[P5 + 3H]^{3+}$	722.1	74	44	−40.5
	$[P6 + 2H]^{2+}$	770.0	26	16	−38.5
	$[P7 + 3H]^{3+}$	641.3	26	14	−46.2
	$[YTPKA + 2H]^{2+}$	290.6	38	37.5	−1.3
	$[ALY + H]^+$	366.4	54	54	0.0
	$[GIVE + H]^+$	417.4	98	100	+2.0
	$[VEAL + H]^+$	431.4	58	56	−3.5
	$[FVNQ + H]^+$	507.5	96	94	−2.1
	$[YQLE + H]^+$	552.5	78	76	−2.6
	$[YTPKA + H]^+$	579.6	100	100	0.0
$[QLENY + H]^+$	666.5	25	25	0.0	

based on the abrupt changes of the relative ion abundances before and after electrolysis. The MS and MS/MS analysis following electrolytic reduction further provide the sequence information of the peptides and the connectivity of the disulfide linkage.

c. Pepsin Digested Insulin. The success in the peptide digest analysis encouraged us to test the method for protein digests. Bovine pancreas insulin (MW 5733.5 Da, sequence shown in Table 1) is known to have A and B chains linked by two disulfide bonds, and the chain A of insulin has an additional intrapeptide disulfide bond. After pepsin digestion, five disulfide-containing peptides denoted as P3, P4, P5, P6, and P7 along with seven additional peptides ALY, GIVE, VEAL, FVNQ, YQLE, YTPKA, and QLENY were obtained (Table 1). The reason to use pepsin in this case is because that pepsin digestion occurs in acidic environment, in which the possible disulfide bond rearrangement can be precluded.² Figure 3a shows the DESI-MS spectrum acquired when a solution of pepsin digested insulin (10 μM) in methanol/water (1:1 by volume) containing 1% acetic acid flowed through the thin-layer flow cell without any potential applied to the cell. The triply and doubly charged ions of P3 (m/z 692.2, 1037.5), P4 (m/z 855.1, 1281.5), and P5 (m/z 722.1 and 1082.5) were seen in the spectrum. Also, the doubly charged P6 and triply charged P7 were detected at m/z 770.0 and 641.3, respectively. In addition, ions of other peptides in the peptic digest, including $[YTPKA + 2H]^{2+}$ (m/z 290.6), $[ALY + H]^+$ (m/z 366.4), $[GIVE + H]^+$ (m/z 417.4), $[VEAL + H]^+$ (m/z 431.4), $[FVNQ + H]^+$ (m/z 507.5), $[YQLE + H]^+$ (m/z 552.5), $[YTPKA + H]^+$ (m/z 579.6), and $[QLENY + H]^+$ (m/z 666.5) were observed and are listed in Figure 3a inset. As expected, when a -1.6 V potential was applied to the cell (spectrum shown in Figure 3b), the relative abundances for the

ions of disulfide-containing peptides P3–P7 decreased significantly by 34–60% (detailed abundance change information is given in Table 2). On the other hand, the other seven peptides without disulfide bonds are not sensitive to electrochemical reduction and their relative ion abundances have marginal changes (the maximum decrease is only by 3.5%, Table 2). This result emphasizes that the disulfide-containing peptides can be distinguished from others based on their responses to electrolytic reduction, even in a complex mixture of protein digest.

In addition, when the electrochemical cell was turned on, new peaks of m/z 384.3, 514.8, 628.5, 656.5, 766.6, 880.6, 913.4, 1027.5, 1254.6, and 1311.5 were observed, corresponding to $[HLCGSHL + 2H]^{2+}$, $[LVCGERGFF + 2H]^{2+}$, $[FVNQHLCGSHL + 2H]^{2+}$, $[GIVEQCCASVCSL + 2H]^{2+}$, $[HLCGSHL + H]^+$, $[LVCGERGF + H]^+$, $[QCCASVCSL + H]^+$, $[LVCGERGFF + H]^+$, $[FVNQHLCGSHL + H]^+$, and $[GIVEQCCASVCSL + H]^+$, respectively (Figure 3b). The appearance of these new peaks shows that six free peptide chains, HLCGSHL, LVCGERGFF, FVNQHLCGSHL, GIVEQCCASVCSL, LVCGERGF, and QCCASVCSL generated from the electrolytic reduction of the pepsin digested insulin, were detected. On the basis of the measured MWs (Table 1), we could assign these peptide chains to their precursor peptides. As shown in the Supplementary Table 1 (Supporting Information), among 15 possible combinations of the six detected peptide chains, only the sum of the MWs of two chains GIVEQCCASVCSL and HLCGSHL (2076.9 Da) is fairly close to the MW of P3 (2073.3 Da), which differ by 3.6 Da in mass. This suggests that P3 consists of two chains, GIVEQCCASVCSL and HLCGSHL, and the mass difference of 3.6 Da indicates that P3 contains two disulfide bonds. In a similar way, we also can relate the chain pairs of

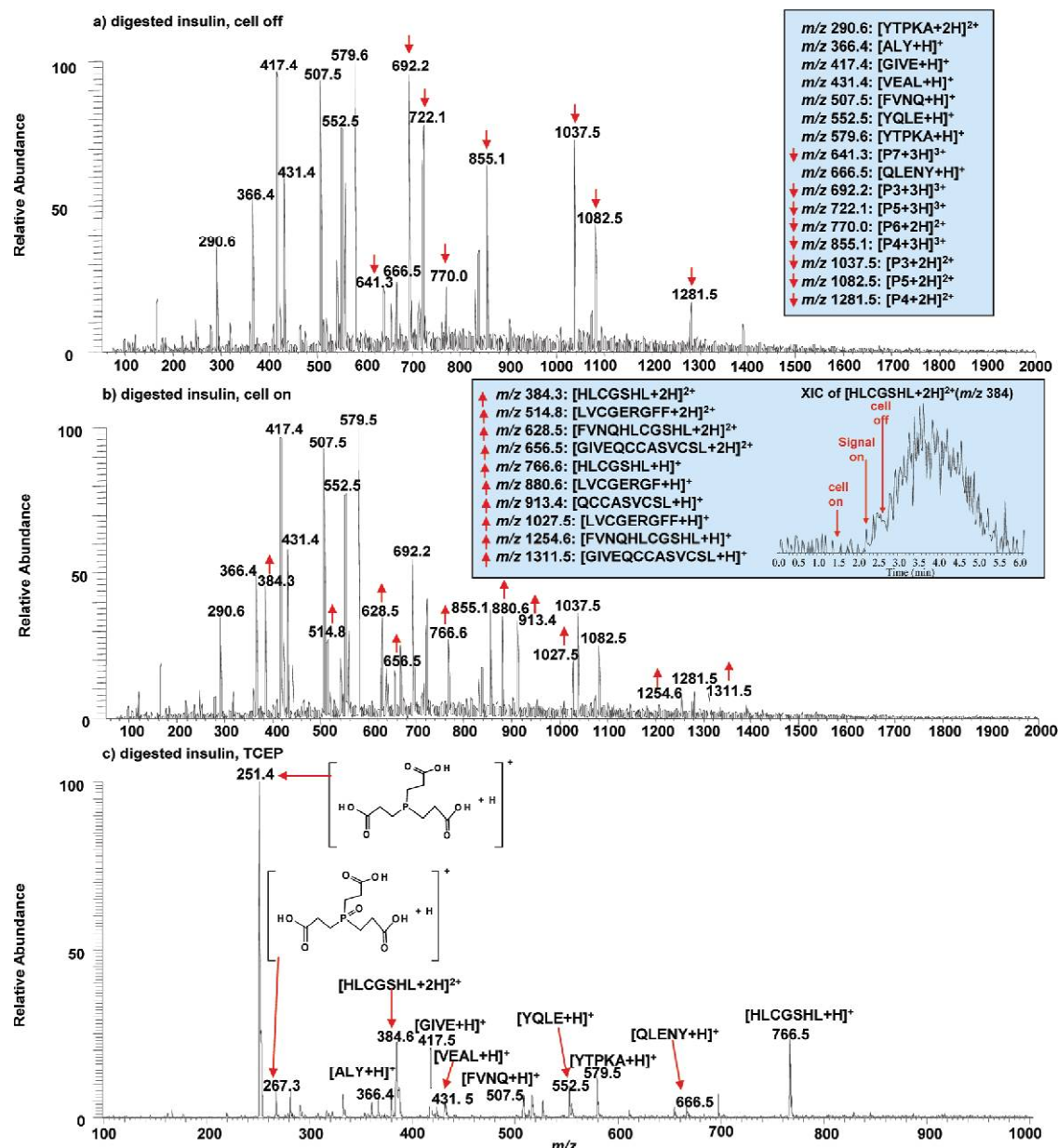


Figure 3. DESI-MS spectra acquired when a solution of pepsin digested insulin (10 μ M) in methanol/water (1:1 by volume) containing 1% acetic acid flowed through the thin-layer electrochemical cell with an applied potential of (a) 0.0 V and (b) -1.6 V (the extracted ion chromatogram (XIC) of [HLCGSHL + 2H]²⁺ (m/z 384) is shown in the inset); (c) ESI-MS spectrum of peptic digested insulin (10 μ M) reduced by TCEP in methanol/water (1:1 by volume) containing 1% acetic acid. The m/z 251.4 is the protonated TECP and the ion of its oxidized form appears at m/z 267.3.

GIVEQCCASVCSL/FVNQHLCGSHL and QCCASVCSL/FVNQHLCGSHL to P4 and P5, respectively, which are both bridged via two disulfide bonds as well. Among these three precursor peptides, P4 is the largest one in the insulin digest. However, as the A chain ions of P6 and P7 are missing in the DESI-MS spectrum (Figure 3b, only B chains LVCGERGF and LVCGERGF were detected), it is not possible to identify the chain compositions of P6 and P7. The reason that both A chain ions of P6 and P7 can not be observed after reduction is probably because these chains lack the basic amino acid residues and thereby have low ionization efficiencies. Similar phenomena of missing chain A in the spectra of reduced insulin samples was noticed before.^{33,43}

In addition, the connectivities of disulfide bonds in this case can be possibly assigned based on information acquired by tandem MS analysis. For instance, the doubly charged ion (m/z 628) of the chain B of P4 generated from electrolysis gives rise to fragment ions of b_4 , $b_4\text{-NH}_3$, b_5 , $b_5\text{-NH}_3$, b_6 , b_7 , $b_7\text{-NH}_3$, b_8 , b_9 , b_{10}^{2+} , y_4 , y_5 , y_7 , $y_8\text{-NH}_3$, ($y_9\text{-NH}_3$)²⁺, y_9 , y_9^{2+} and y_{10}^{2+} upon CID (Supplementary Figure 1e, Supporting Information). This set of fragment ions originates from the cleavage of all of the backbone amide bonds of the chain B ion and determines that the chain sequence is FVNQHLCGSHL with one cysteine residue located at the seventh amino acid site. Since the P4 contains two pairs of disulfide bonds, it is therefore clear that its chain A has three cysteines, of which two of them are paired up and the other

one is linked with the Cys⁷ of the B chain FVNQHLGSHL. CID spectrum of the singly charged A of P4 (m/z 913) was further examined (Supplementary Figure 1d, Supporting Information), and its fragment ions of b_7 , b_8 , b_9 , b_{10} , b_{11} , b_{12} , y_{11} -NH₃, y_{12} and y_{13} -NH₃ suggests that the chain A sequence is GIXXXXASVCSL (X represents an unknown residue). As the CID of triply charged P4 ion (m/z 856, Supplementary Figure 1b, Supporting Information) produces B1-11/A(y_8^{2+}), B1-11/A(y_9^{2+}), B1-11/A(y_{10}^{2+}), and B1-11/A(y_{11}^{2+}) via the loss of GIVEQ, GIVE, GIV and GI fragments from the chain A, it can be further inferred that the chain A sequence is GIVEQX-XASVCSL. Because chain A is known to have three cysteines, it sequence should be GIVEQCCASVCSL, which agrees with its measured MW (Table 1). Also, it can be further inferred that the Cys⁶ in the chain A is paired up with Cys¹¹ of the chain to form an intrapeptide bond, since the backbone cleavage between these two sites is missing in the CID of triply charged P4 ion (Supplementary Figure 1b, Supporting Information). The conservation of the same amino acid residue region during the CID of both P3 and P5 ions (Supplementary Figure 1a and c, Supporting Information) was also noticed, confirming such a disulfide linkage assignment. Thus, in P4, the Cys⁷ of chain A forms one interpeptide bond with Cys⁷ of chain B. It is therefore evident that both the connectivities of disulfide bonds and the sequence of P4 (Table 1) can be determined using the information acquired from this EC/DESI-MS method. In comparison to the previous electrochemical detection of thiol- and disulfide-containing peptides,^{19,20} our method provides much more chemical information as a result of the online mass spectrometric detection such as the connectivities of disulfide bonds and the sequence information.

We also tested the analysis of the pepsin digested insulin using chemical reduction with TCEP reagent, a traditional protocol to break a disulfide bond. As shown in Figure 3c, in addition to some noncysteine containing peptide ions, only +1 and +2 ions of free chain HLCGSHL was observed at m/z 766.5 and 384.6, respectively. The other five cysteine-containing peptides that were detected in the DESI-MS spectrum (Figure 3b) were not seen in Figure 3c. It is likely that these ion signals were suppressed by the excess amount of TCEP used in the chemical reduction. In our EC/DESI-MS analysis, this issue is avoided because the method does not involve chemical reductants. Also, electrolytic reduction of disulfide is much faster than chemical reduction which usually requires 30 min to 2 h for completion. For example, in the case of insulin digest, the electrochemical cell was turned on only for about 1 min (Figure 3b inset shows the time when the cell was turned on and off) and a good DESI-MS spectrum was acquired. In most cases of the EC/DESI-MS experiments, it is noticed that reduced products could be detected even when the reduction potential was applied for only 30 s. The short reduction time required and online DESI-MS detection thereby expedite the analysis process.

2. Reduction of Peptides Containing Intrapeptide or Symmetric Interpeptide Disulfide Bonds

In the analysis described above, all disulfide-containing peptides resulting from enzymatic digestion contain asymmetric chains with different MWs. However, biologically active peptides containing an intrapeptide disulfide bond (e.g., oxytocin) or symmetric chains linked with an interpeptide disulfide bond (e.g., glutathione) also occur. For these peptides, the peak of reduced peptide ion might overlap with the ³⁴S isotopic peak of the intact

peptide ion in the acquired MS spectrum. To overcome this problem, our strategy is to adopt fast and selective derivatization of the reduced peptides, using selenamide reagents such as ebselen and *N*-(phenylseleno)phthalimide,⁴¹ a new protein/peptide thiol labeling method recently developed in our laboratory. Compared to other reactions to derivatize thiols such as thiol exchange and Michael-addition reactions, selenamide reagents can selectively and rapidly derivatize protein/peptide thiols in seconds,⁴¹ satisfying the requirement for the recognition of reduced peptides with free thiol groups via online derivatization.

[Arg⁸]-vasopressin (MW: 1084.2 Da), a peptide containing one intrapeptide disulfide bond was chosen for investigation first. When a solution of 0.1 mM [Arg⁸]-vasopressin doped with *N*-(phenylseleno)phthalimide flowed through the thin-layer electrochemical cell with no potential applied, the singly and doubly charged ions of [Arg⁸]-vasopressin were detected at m/z 1083.7 and 543.1, respectively. Also, m/z 637.0 and 723.9 from the background were observed (Figure 4b). When a -2.0 V potential was applied, the intensities of m/z 1083.7 and 543.1 decreased. On the other hand, one new peak at m/z 699.5 corresponding to the doubly charged ion of the reduced peptide CYFQNCPRG labeled with two selenium tags was clearly observed (Figure 4c), which is well separated from the doubly charged intact peptide ion m/z 543.1 (without derivatization, the resulting doubly charged ion of the reduced peptide would appear at m/z 544.1 and overlap with the S³⁴ isotopic peak of m/z 543.1). The double addition of the selenium tags with the reduced peptide (the reaction equation is shown in Figure 4a) indicates that the precursor peptide [Arg⁸]-vasopressin has one intrapeptide disulfide bond. After reduction, the reduced peptide carries two free cysteines that allow double addition of selenium tags. As expected, upon CID, the derivatized reduced peptide ion of m/z 699 (Figure 4d) shows a series of backbone cleavages, giving rise to a series of *b* and *y* ions (b_2 , b_3 , b_5 , y_2 , y_3 , y_4 , y_5 , y_6 , and y_7) plus a_2 , a_3 -NH₃, and a_4 -NH₃. In contrast, the doubly charged intact [Arg⁸]-vasopressin ion (m/z 543.1) (Figure 4e) dissociates into fewer fragment ions upon CID owing to the presence of the disulfide bond linkage. Again, this data demonstrates that increased structural information can be obtained via electrolytic reduction of disulfide bonds. Another ion arising from the reduction was observed at m/z 643.1, corresponding to the reduced peptides labeled with one selenium tag and one mercury atom. The CID spectrum of m/z 643 (Supplementary Figure 2, Supporting Information) shows that the mercury is covalently attached to the peptide at the first cysteine residue. The origin for this mercury-thiolate product remains unclear and we did not see the similar products for other peptides examined in this study. It is likely that the mercury-thiolate product was formed via anodic oxidation of thiol product in the presence of mercury⁴⁴ or via disulfide bond reduction on mercury surface as an intermediate.¹⁵ In addition, similar EC/DESI-MS results were obtained for oxytocin (MW: 1007.2 Da), another biologically active peptide containing one intrapeptide disulfide bond. The electrolytic reduction cleaved its disulfide bond and reduced oxytocin with two free cysteines underwent the double addition with ebselen, another selenamide reagent, which was detected by DESI-MS. The spectra and reaction equation are shown in the Supplementary Figure 3 (Supporting Information).

Glutathione disulfide (GSSG) (MW: 612.6 Da), the oxidized form of the cysteine-containing tripeptide glutathione (GSH), contains two symmetric chains linked with one interpeptide disulfide bond. It is known from our previous study²⁹ that the

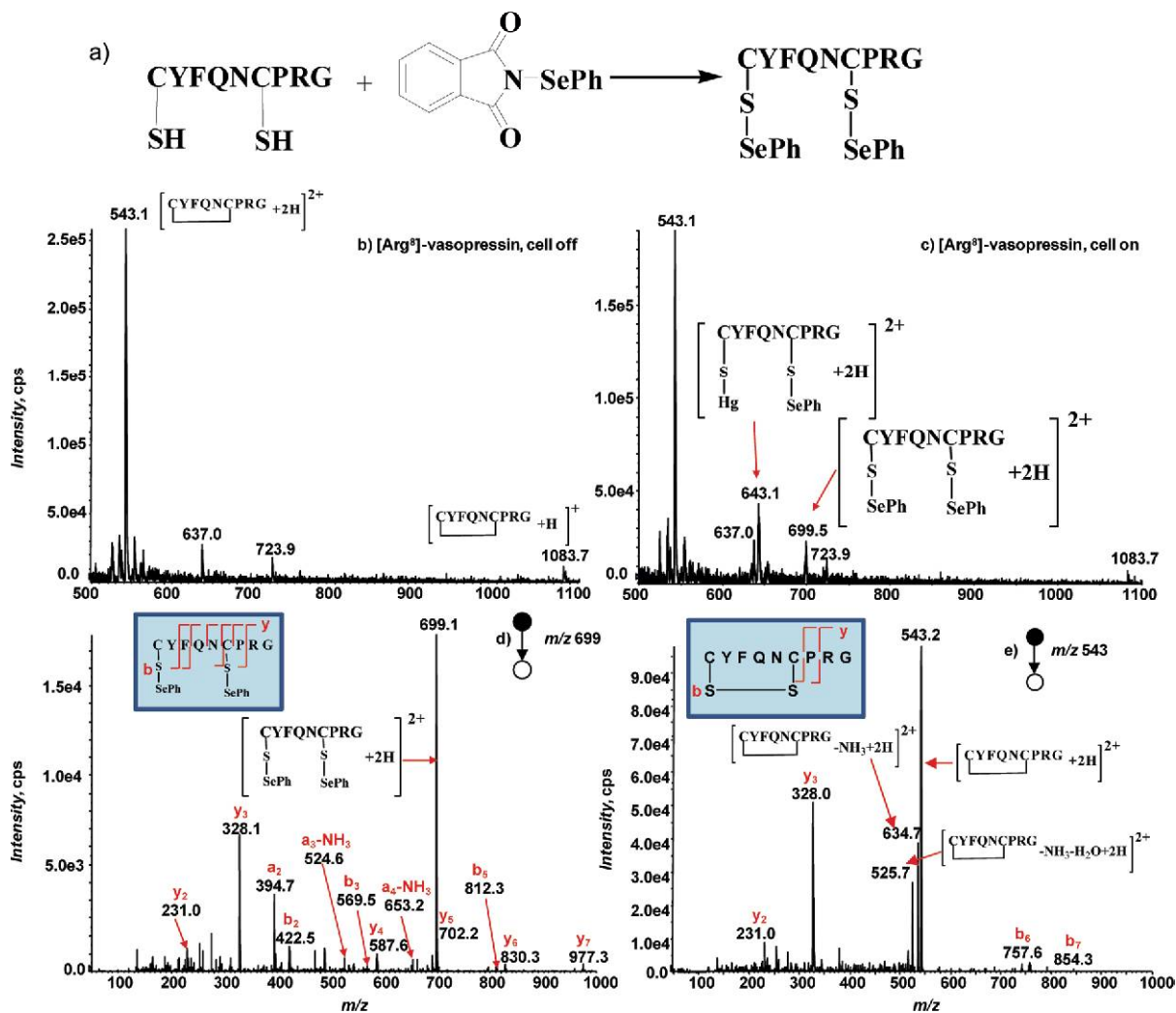


Figure 4. (a) Scheme showing the derivatization of reduced $[\text{Arg}^8]$ -vasopressin (sequence CYFQNCPRG) by *N*-(phenylseleno)phthalimide; DESI-MS spectra acquired when a solution of $[\text{Arg}^8]$ -vasopressin acetate salt (0.1 mM) and *N*-(phenylseleno)phthalimide (0.5 mM) in acetonitrile/water (1:1 by volume) containing 1% acetic acid flowed through the thin-layer electrochemical cell with an applied potential of (b) 0.0 V and (c) -2.0 V; CID MS² spectra of (d) the doubly charged ion of the reduced $[\text{Arg}^8]$ -vasopressin with two phenylselenenyl tags (m/z 699) and (e) the doubly charged ion of intact $[\text{Arg}^8]$ -vasopressin (m/z 543).

peak of singly charged GSH (m/z 308) generated by electrolytic reduction of GSSG overlaps with the ³⁴S isotopic peak of the doubly charged GSSG ion (m/z 307). In this study, this issue can be overcome using online rapid derivatization of the product GSH by ebselen. In the experiment, the solution of glutathione disulfide (GSSG) was doped with ebselen and injected through the electrochemical flow cell. The singly and doubly charged GSSG ions at m/z 613.0 and 307.0, respectively, were detected (Supplementary Figure 4a, Supporting Information). When -2.0 V potential was applied to the working electrode of the cell, a new peak m/z 582.9 appears (Supplementary Figure 4b, Supporting Information), which corresponds to the protonated ion of the derivatized GSH with one ebselen tag and clearly separated from the GSSG peaks. This assignment was verified by its CID MS² spectrum which shows major fragment ions of the protonated ebselen and GSH. Also, the intensities of $[\text{GSSG} + \text{H}]^+$ and $[\text{GSSG} + 2\text{H}]^{2+}$ decreased, indicating the consumption of GSSG during electrolysis. Unlike $[\text{Arg}^8]$ -vasopressin and oxytocin which contain an intradisulfide bond, the reduced GSSG product contains

one free cysteine residue so that only the single addition of ebselen to GSH is possible. This feature is useful in determining whether or not the disulfide bond of the examined peptide is in inter- or intrapeptide form. Further, the number of selenium tags on the reduced peptides points out the number of their free cysteine residues.

3. Reduction of Intact Proteins Containing Disulfide Bonds

We further examined the protein α -lactalbumin (MW: 14178.0 Da) from bovine milk for the EC/DESI-MS. This protein (sequence is shown in Figure 5a inset) has 123 amino acid residues and contains four disulfide cross-links (Cys⁶-Cys¹²⁰, Cys²⁸-Cys¹¹¹, Cys⁶¹-Cys⁷⁷, and Cys⁷³-Cys⁹¹) to maintain and stabilize its three-dimensional structure in solution.⁴⁵ To investigate the electrolytic reduction of the disulfide bonds in bovine α -lactalbumin, we also adopted the selenamide derivatization strategy as it is helpful to recognize the multiply charged reduced protein ions from those intact protein ions using our ion trap mass spectrometer with unit mass resolution. Figure 5a illustrates the DESI-MS spectrum acquired when a solution of α -lactalbumin

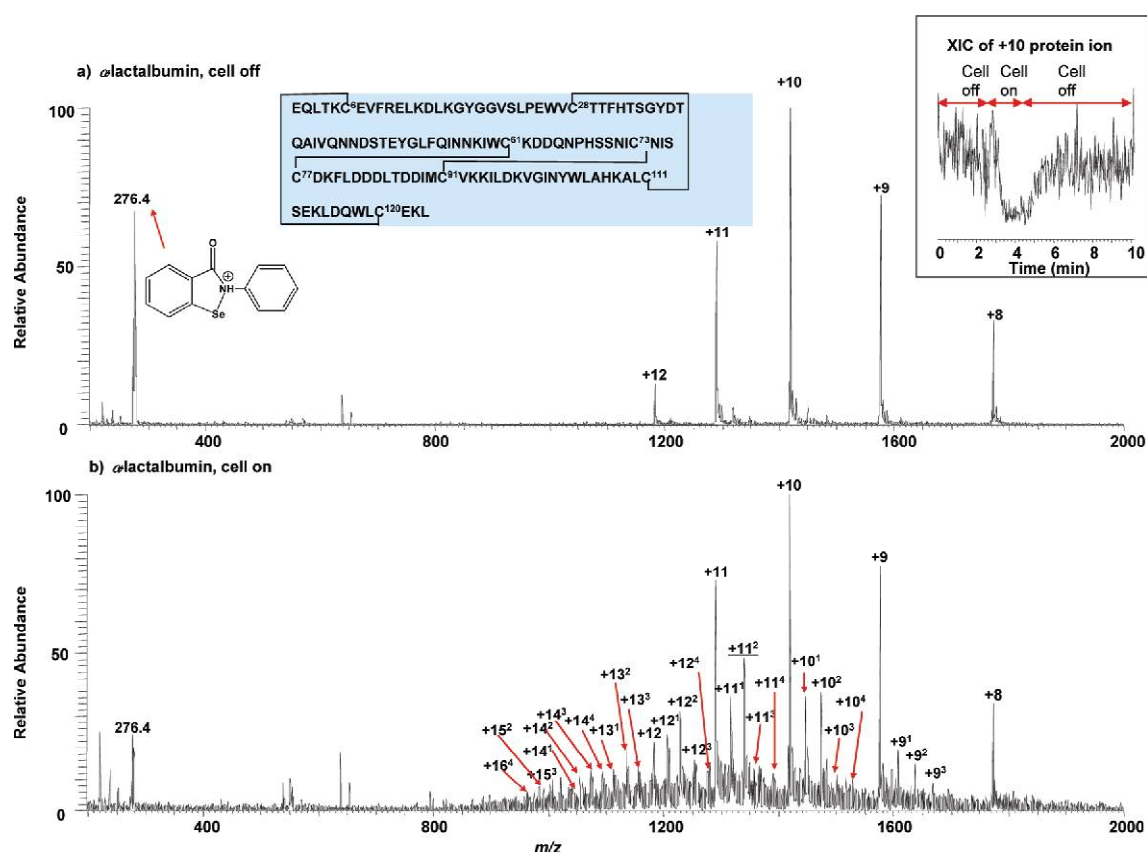


Figure 5. DESI-MS spectra acquired when a solution of α -lactalbumin ($20 \mu\text{M}$) and ebselen (0.2 mM) in acetonitrile/water (1:1 by volume) containing 1% acetic acid flowed through the thin-layer electrochemical cell with an applied potential of (a) 0.0 V (the insets show the intact protein sequence and the XIC of the +10 protein ion) and (b) -2.1 V (the superscripts on the charge numbers labeled in the spectrum indicates the number of the added ebselen tags to the reduced protein ions).

($20 \mu\text{M}$) and ebselen (0.2 mM) in acetonitrile/water (1:1 by volume) containing 1% acetic acid flowed through the thin-layer electrochemical cell, the multiply charged ions of intact α -lactalbumin with a charge distribution of $+8 \sim +12$ centered at +10 were detected. When a -2.1 V potential was applied to trigger protein reduction, the abundance of α -lactalbumin ions decreased as exemplified by the extracted ion chromatogram (XIC) of the +10 protein ion (m/z 1418.9, Figure 5a inset). Besides, the multiply charged ions of α -lactalbumin carrying one, two, three and four ebselen tags arose (Figure 5b), suggesting that the protein underwent electrolytic reduction and the subsequent chemical reactions with ebselen (an EC mechanism). Also, based on the maximum number of the ebselen tags, it is indicative that the protein underwent two disulfide bond cleavages, leading to totally four free cysteine residues for derivatization. The partial reduction of disulfide bonds in α -lactalbumin suggests that the remaining two disulfide bonds are sterically hindered in the interior area of the protein while the other two are accessible for the electrolytic cleavage on the amalgam surface. Indeed, according to the literature report,⁴⁶ Cys⁶-Cys¹²⁰ is the easiest one to be reduced and Cys²⁸-Cys¹¹¹ is the second easiest one to be cleaved. Our experimental results show that α -lactalbumin protein can be electrochemically reduced and the resulting reduced proteins were detected online by MS. It is very likely that these partially reduced α -lactalbumin ions can be interrogated by tandem mass spectrometry to provide more structurally informative fragment ions than the intact one, which

was not further explored in our experiment as a result of the low resolving power of the ion trap used.

In addition, examination of Figure 5b also reveals the role of disulfide bonds on maintenance of the protein conformational structures. As mentioned above, liquid sample DESI offers a convenient way to probe protein conformational changes in solution since the sample can be ionized directly without sample pretreatment.³¹ A simple but effective way to detect the conformational change of proteins is to record the shift of charge state distribution (CSD) of protein ions.^{47–49} Typically, when a protein is in the folded structure, a narrow CSD in low charge states is observed while CSD is broadened and shifted to high charge states after unfolding. This is probably because that, in comparison to a folded protein, the unfolded one has a greater capacity to accommodate a significantly large number of charges on its surface. In this experiment, electrochemical reduction can serve as a good stimulant to trigger protein conformational changes simply via electrolytic reduction processes, which can be online monitored with DESI-MS. Prior to electrolysis, the intact α -lactalbumin exhibits the CSD of $+8 \sim +12$ with the most abundant peak located at +10 ion. These charge numbers are relatively low, even under the denaturing solvent environment (containing acetonitrile and 1% acetic acid) in this experiment, probably owing to the presence of four disulfide bonds which limits the extent of the protein unfolding. After electrolysis, the CSD of the reduced α -lactalbumin ions with ebselen tags has shifted to higher charge states ($+9 \sim +16$) and the most abundant

charge state centered at +11 (indicated with underlined charge number). This CSD change could stem from the addition of selenium tags or the conformational changes of the protein as a result of the disulfide bond cleavages. The former speculation is less unlikely because the addition of selenium tags in the peptide cases discussed above (e.g., oxytocin and glutathione) does not help to increase the charges of derivatized peptide ions. No higher charge state was observed in the case of protein β -lactoglobulin A after derivatization by ebselen in our previous experiment.²⁹ It is more likely that the observed CSD shift is a result of the cleavage of disulfide bonds in the protein. In this experiment with α -lactalbumin, the electrolytic cleavage of two disulfide bonds results in protein unfolding so that the protein can carry more charges. This phenomenon is in agreement with the early ESI report that increased charge occurred to the detected protein ions after the cleavage of their disulfide bonds.⁴⁸ It is also consistent with the previous report⁴⁵ that the two-disulfide form of α -lactalbumin retains only half the secondary and tertiary structures of the intact α -lactalbumin. Indeed, once the α -lactalbumin was completely reduced by TCEP and then ionized by ESSI, a variant form of ESI,⁵⁰ a further CSD shift to +19 was noticed (Supplementary Figure 5, Supporting Information). These results suggest that, like the first two disulfide bonds reduced in the electrolysis, the two remaining disulfide bonds are also responsible in keeping protein folded. Thus, it can be seen that the EC/DESI-MS can provide unique insights into the role of disulfide bonds of a protein in maintaining its conformation.

CONCLUSIONS

EC/DESI-MS is a powerful tool for the structural elucidation of different types of disulfide bond containing peptides and proteins and for mixture analysis of protein/peptide digests. Based on the relative ion abundance change during electrolysis, one can identify the disulfide-containing peptides from others in enzymatic digest mixtures. In conjunction with mass mapping and tandem mass analysis, peptide sequencing and location of the disulfide linkages is possible. Adoption of selective selenamide derivatization reactions facilitates the analysis of intra- and interpeptide disulfide containing peptides by the EC/DESI-MS. The effect of disulfide bonds on maintaining protein conformational structures can also be investigated based on the charge state distribution (CSD) shifts of protein ions after reduction. The reported electrochemical mass spectrometry for disulfide analysis is fast, simple and controllable by switching on/off the electrolysis potential. No chemical reductant is involved so that direct ionization after reduction is feasible. In addition, unlike the gas-phase ion activation methods used for the cleavage of disulfide bond which could lead to backbone dissociation, our electrochemical reduction takes place in solution and is very selective to disulfide bonds. As peptides from protein digests and intact proteins can be reduced, this method should find important applications in both top-down and bottom-up approaches in protein analysis. Furthermore, for proteins containing multiple disulfide bonds, there is a possibility to achieve selective cleavage of certain disulfide bonds by using controlled reduction potentials;⁵¹ such an investigation is under way. In this study, we used different potentials to reduce different disulfide-containing proteins/peptides in order to obtain the optimum reduction yield, which suggests the feasibility of such an investigation. As disulfide bonds play an

important role in protein conformations and functions, this electrochemical mass spectrometric method would find its valuable utilities in proteomics research.

ASSOCIATED CONTENT

Supporting Information

Additional tables and mass spectra are provided. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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ACKNOWLEDGMENT

The financial support of this work by NSF (CHE-0911160) is gratefully acknowledged. We are grateful for assistance from Mr. Jiwen Li and Dr. Kehua Xu. In particular, we thank Antec Leyden company and Mr. Martin Eysberg very much for their generous support in this electrochemical mass spectrometry study.

REFERENCES

- (1) Bilusich, D.; Bowie, J. H. Fragmentations of $(M-H)^-$ anions of underivatized peptides. Part 2: characteristic cleavages of Ser and Cys and of disulfides and other post-translational modifications, together with unusual internal processes. *Mass Spectrom. Rev.* **2009**, *28*, 20–34.
- (2) Gorman, J. J.; Wallis, T. P.; Pitt, J. J. Protein disulfide bond determination by mass spectrometry. *Mass Spectrom. Rev.* **2002**, *21*, 183–216.
- (3) Sun, Y.; Andrews, P. C.; Smith, D. L. Identification of disulfide-containing peptides in endocrine tissue extracts by HPLC-electrochemical detection and mass spectrometry. *J. Protein Chem.* **1990**, *9*, 151–157.
- (4) 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.
- (5) Yang, H.; Liu, N.; Qiu, X.; Liu, S. A new method for analysis of disulfide-containing proteins by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry. *J. Am. Soc. Mass Spectrom.* **2009**, *20*, 2284–2293.
- (6) Eva-Fung, Y. M.; Kjeldsen, F.; Silivra, O. A.; Dominic Chan, T. W.; Zubarev, R. A. Facile disulfide bond cleavage in gaseous peptide and protein cations by ultraviolet photodissociation at 157 nm. *Angew. Chem., Int. Ed.* **2005**, *44*, 6399–6403.
- (7) Andreazza, H. J.; Bowie, J. H. The application of negative ion electrospray mass spectrometry for the sequencing of underivatized disulfide-containing proteins: insulin and lysozyme. *Phys. Chem. Chem. Phys.* **2010**, *12*, 13400–13407.
- (8) Zhang, M.; Kaltashov, I. A. Mapping of protein disulfide bonds using negative ion fragmentation with a broadband precursor selection. *Anal. Chem.* **2006**, *78*, 4820–4829.
- (9) Zubarev, R. A.; Kruger, N. A.; Fridriksson, E. K.; Lewis, M. A.; Horn, D. M.; Carpenter, B. K.; McLafferty, F. W. 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) Gunawardena, H. P.; Gorenstein, L.; Erickson, D. E.; Xia, Y.; McLuckey, S. A. Electron transfer dissociation of multiply protonated and fixed charge disulfide linked polypeptides. *Int. J. Mass Spectrom.* **2007**, *265*, 130–138.

- (11) Xia, Y.; Cooks, R. G. Plasma induced oxidative cleavage of disulfide bonds in polypeptides during nano-electrospray ionization. *Anal. Chem.* **2010**, *82*, 2856–2864.
- (12) Peng, I. X.; Ogorzalek Loo, R. R.; Shiea, J.; Loo, J. A. Reactive-electrospray-assisted laser desorption/ionization for characterization of peptides and proteins. *Anal. Chem.* **2008**, *80*, 6995–7003.
- (13) Gunawardena, H. P.; O'Hair, R. A. J.; McLuckey, S. A. 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.
- (14) Kim, H. I.; Beauchamp, J. L. Mapping disulfide bonds in insulin with the route 66 method: selective cleavage of S-C bonds using alkali and alkaline earth metal enolate complexes. *J. Am. Soc. Mass Spectrom.* **2009**, *20*, 157–166.
- (15) Honeychurch, M. J. The reduction of disulfide bonds in proteins at mercury electrodes. *Bioelectrochem. Bioenerg.* **1997**, *44*, 13–21.
- (16) Allison, L. A.; Shoup, R. E. Dual electrode liquid chromatography detector for thiols and disulfides. *Anal. Chem.* **1983**, *55*, 8–12.
- (17) Lazurea, C.; Rochemonta, J.; Seidaha, N. G.; Chrétien, M. Novel approach to rapid and sensitive localization of protein disulfide bridges by high-performance liquid chromatography and electrochemical detection. *J. Chromatogr.* **1985**, *326*, 339–348.
- (18) Garvie, C. T.; Straub, K. M.; Lynn, R. K. Quantitative liquid chromatographic determination of disulfide-containing peptide analogues of vasopressin with dual Hg/Au electrochemical detection. *J. Chromatogr.* **1987**, *413*, 43–52.
- (19) Sun, Y.; Smith, D. L.; Shoup, R. E. Simultaneous detection of thiol-containing and disulfide-containing peptides by electrochemical high-performance liquid chromatography with identification by mass spectrometry. *Anal. Biochem.* **1991**, *197*, 69–76.
- (20) Zhong, M.; Lunte, S. M. Tubular-wire dual electrode for detection of thiols and disulfides by capillary electrophoresis/electrochemistry. *Anal. Chem.* **1999**, *71*, 251–255.
- (21) Takats, Z.; Wiseman, J. M.; Gologan, B.; Cooks, R. G. Mass spectrometry sampling under ambient conditions with desorption electrospray ionization. *Science* **2004**, *306*, 471–473.
- (22) Wiseman, J. M.; Puolitaival, S. M.; Takats, Z.; Cooks, R. G.; Caprioli, R. M. Mass spectrometric profiling of intact biological tissue by using desorption electrospray ionization. *Angew. Chem., Int. Ed.* **2005**, *44*, 7094–7097.
- (23) Cooks, R. G.; Ouyang, Z.; Takats, Z.; Wiseman, J. M. Ambient mass spectrometry. *Science* **2006**, *311*, 1566–1570.
- (24) Venter, A.; Nefliu, M.; Cooks, R. G. Ambient desorption ionization mass spectrometry. *Trends Anal. Chem.* **2008**, *27*, 284–290.
- (25) 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.
- (26) Zhang, Y.; Chen, H. Detection of saccharides by reactive desorption electrospray ionization (DESI) using modified phenylboronic acids. *Int. J. Mass Spectrom.* **2010**, *289*, 98–107.
- (27) Sun, X.; Miao, Z.; Yuan, Z.; Harrington, P. B.; Colla, J.; Chen, H. Coupling of single-droplet micro-extraction with desorption electrospray ionization mass spectrometry. *Int. J. Mass Spectrom.* **2010**, in press.
- (28) Groenewold, G. S.; Appelhans, A. D.; McIlwain, M. E.; Gresham, G. L. Characterization of coordination complexes by desorption electrospray mass spectrometry with a capillary target. *Int. J. Mass Spectrom.* **2010**, in press.
- (29) Li, J.; Dewald, H. D.; Chen, H. Online coupling of electrochemical reactions with liquid sample desorption electrospray-mass spectrometry (DESI-MS). *Anal. Chem.* **2009**, *81*, 9716–9722.
- (30) Ma, X.; Zhao, M.; Lin, Z.; Zhang, S.; Yang, C.; Zhang, X. Versatile platform employing desorption electrospray ionization mass spectrometry for high-throughput analysis. *Anal. Chem.* **2008**, *80*, 6131–6136.
- (31) 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.
- (32) Bruckenstein, S.; Gadde, R. R. Use of a porous electrode for in situ mass spectrometric determination of volatile electrode reaction products. *J. Am. Chem. Soc.* **1971**, *93*, 793–794.
- (33) Dayon, L.; Roussel, C.; Girault, H. H. Probing cysteine reactivity in proteins by mass spectrometric EC-tagging. *J. Proteome Res.* **2006**, *5*, 793–800.
- (34) Permentier, H. P.; Jurva, U.; Barroso, B.; Bruins, A. P. Electrochemical oxidation and cleavage of peptides analyzed with on-line mass spectrometric detection. *Rapid Commun. Mass Spectrom.* **2003**, *17*, 1585–1592.
- (35) Zhou, F.; Van Berkel, G. J. Electrochemistry combined on-line with electrospray mass spectrometry. *Anal. Chem.* **1995**, *67*, 3643–3649.
- (36) Xu, X.; Lu, W.; Cole, R. B. On-line probe for fast electrochemistry/electrospray mass spectrometry. Investigation of polycyclic aromatic hydrocarbons. *Anal. Chem.* **1996**, *68*, 4244–4253.
- (37) Bond, A. M.; Colton, R.; D'Agostino, A.; Downard, A. J.; Traeger, J. C. A role for electrospray mass spectrometry in electrochemical studies. *Anal. Chem.* **1995**, *67*, 1691–1695.
- (38) Permentier, H. P.; Jurva, U.; Barroso, B.; Bruins, A. P. Electrochemical oxidation and cleavage of peptides analyzed with on-line mass spectrometric detection. *Rapid Commun. Mass Spectrom.* **2003**, *17*, 1585–1592.
- (39) Permentier, H. P.; Bruins, A. P. Electrochemical oxidation and cleavage of proteins with on-line mass spectrometric detection: development of an instrumental alternative to enzymatic protein digestion. *J. Am. Soc. Mass Spectrom.* **2004**, *15*, 1707–1716.
- (40) Markus, G. Electrolytic reduction of the disulfide of insulin. *J. Biol. Chem.* **1964**, *239*, 4163–4170.
- (41) Xu, K.; Zhang, Y.; Tang, B.; Laskin, J.; Roach, P. J.; Chen, H. Study of highly selective and efficient thiol derivatization using selenium reagents by mass spectrometry. *Anal. Chem.* **2010**, *82*, 6926–6932.
- (42) Hogan, J. M.; Pitteri, S. J.; McLuckey, S. A. Phosphorylation site identification via ion trap tandem mass spectrometry of whole protein and peptide ions: bovine α -Crystallin A chain. *Anal. Chem.* **2003**, *75*, 6509–6516.
- (43) Stephenson, J. L.; Cargile, B. J.; McLuckey, S. A. Ion trap collisional activation of disulfide linkage intact and reduced multiply protonated polypeptides. *Rapid Commun. Mass Spectrom.* **1999**, *13*, 2040–2048.
- (44) Stricks, W.; Kolthoff, I. M. Polarography of glutathione. *J. Am. Chem. Soc.* **1952**, *74*, 4646–4653.
- (45) Hendrix, T. M.; Griko, Y.; Privalov, P. Energetics of structural domains in α -lactalbumin. *Protein Sci.* **1996**, *5*, 923–931.
- (46) Kuwajima, K.; Ikeguchi, M.; Sugawara, T.; Hiraoka, Y.; Sugai, S. Kinetics of disulfide bond reduction in α -lactalbumin by dithiothreitol and molecular basis of superreactivity of the Cys6-Cys120 disulfide bond. *Biochemistry* **1990**, *29*, 8240–8249.
- (47) Katta, V.; Chait, B. T. Observation of the heme-globin complex in native myoglobin by electrospray-ionization mass spectrometry. *J. Am. Chem. Soc.* **1991**, *113*, 8534–8535.
- (48) Loo, J. A.; Edmonds, C. G.; Udseth, H. R.; Smith, R. D. Effect of reducing disulfide-containing proteins on electrospray ionization mass spectra. *Anal. Chem.* **1990**, *62*, 693–698.
- (49) Kaltashov, I. A.; Abzalimov, R. R. Do ionic charges in ESI MS provide useful information on macromolecular structure?. *J. Am. Soc. Mass Spectrom.* **2008**, *19*, 1239–1246.
- (50) Takáts, Z.; Wiseman, J. M.; Gologan, B.; Cooks, R. G. Electrosonic spray ionization. A gentle technique for generating folded proteins and protein complexes in the gas phase and studying ion–molecule reactions at atmospheric pressure. *Anal. Chem.* **2004**, *76*, 4050–4058.
- (51) Cecil, R.; Weitzman, P. D. J. The electroreduction of the disulfide bonds of insulin and other proteins. *Biochem. J.* **1964**, *93*, 1–11.