Reversed phase liquid chromatography hyphenated to continuous flow–extractive desorption electrospray ionization–mass spectrometry for analysis and charge state manipulation of undigested proteins

Li Li,a Samuel H. Yang,a Veronika Vidova,b Elisa M. Rice,a Aruna B. Wijeratne,a Vladimír Havlíčekb,c and Kevin A. Schuga*

aDepartment of Chemistry & Biochemistry, University of Texas at Arlington, Arlington, TX, USA. E-mail: kschug@uta.edu
bInstitute of Microbiology, v.v.i., Academy of Sciences of the Czech Republic, Prague, Czech Republic
cRegional Centre of Advanced Technologies and Materials, Department of Analytical Chemistry, Faculty of Science, Palacky University, 17 listopadu 12, 771 46 Olomouc, Czech Republic

The application of continuous flow–extractive desorption electrospray ionization (CF–EDESI), an ambient ionization source demonstrated previously for use with intact protein analysis, is expanded here for the coupling of reversed phase protein separations to mass spectrometry. This configuration allows the introduction of charging additives to enhance detection without affecting the chromatographic separation mechanism. Two demonstrations of the advantages of CF–EDESI are presented in this work. First, a proof-of-principle is presented to demonstrate the applicability of hyphenation of liquid chromatography (LC) to CF–EDESI. LC-CF-EDESI-MS has good sensitivity compared to LC–electrospray ionization (ESI)–mass spectrometry. Second, the supercharging mechanism investigated in CF–EDESI provides an insight into a highly debated supercharging process in ESI. The results indicate that the mechanism of protein charging seen in HPLC-CF-EDESI is different from supercharging phenomena in conventional ESI. The surface tension mechanism and binding mechanism may both contribute to protein supercharging in ESI.

Keywords: protein chromatography, ambient ionization, charge-state manipulation, response factor, ionization mechanism

Introduction

Electrospray ionization–mass spectrometry (ESI-MS) is a powerful analytical tool that has facilitated a wide range of analytical applications.1–5 The versatility of soft ionization analysis was further enhanced with the development of new ambient ionization and atmospheric pressure ionization techniques.6–9 Two hallmark ambient ionization techniques, desorption electrospray ionization (DESI)6 and direct analysis in real time (DART),10 demonstrated in a wealth of literature that ambient ionization techniques are capable of analysis of a wide variety of compounds directly from different sample matrices. Since the introduction of DESI and DART, new ambient ionization techniques have been presented, each occupying their own niche in the analytical field.11–16 The coupling of ambient ionization to separation techniques offers even greater advantages. For example, complex mixtures that involve multiple analytes of interest can be
isolated prior to ambient ionization for accurate and sensitive analysis without the use of a high-resolution mass analyzer capable of high mass accuracy. In addition, ambient ionization techniques allow sensitive analysis from nonpolar solvents, which are used in normal phase liquid chromatography. Nonvolatile additives and buffer salts, such as phosphoric acid/phosphate or citrate, which are commonly used to improve chromatography, can be coupled to MS detection without their associated ion suppression effects. These advantages are particularly useful in overcoming challenges encountered when hyphenating conventional ESI to high-performance liquid chromatography (HPLC). However, there is a limited number of applications featuring the combination of ambient ionization with HPLC. DESI and DART, the most developed techniques, have clearly demonstrated viable examples of separations with ambient ionization. DESI has been applied to a number of different separation techniques including capillary electrophoresis, solid phase extraction (or micro-extraction) and thin layer chromatography, in addition to its coupling with HPLC. Thus, it would be greatly beneficial to explore the hyphenation of separation techniques such as HPLC with other ambient ionization approaches for the creation of methods with expanded analytical applications.

Recently, our group has introduced a new ambient ionization technique called continuous flow–extractive desorption electrospray ionization (CF–EDESI) [Figure 1(A)]. Following principles similar to other ambient ionization techniques, CF–EDESI features a dual sample source, which allows for separate introduction of sample analyte from the ESI source. The fixed 180° angle between the ESI source and MS inlet is a particularly attractive feature of CF–EDESI, in that it circumvents a significant need for optimization of the source/inlet.
geometry, which is often required for other ambient ionization techniques.

Initial work presented an application using CF-EDESI in order to perform manipulation of protein charge states. Increases in the protein charge state distributions (CSDs) were achieved by taking advantage of the dual sample source of CF-EDESI, where separation of the sample protein from the charging additive prior to analysis was possible. Increasing the protein charge states offers a number of advantages. The availability of increased charge states facilitates more efficient electron capture dissociation and electron transfer dissociation by quadratically increasing the electron capture cross-section of the protein, which greatly benefits investigations of post-translational modifications. Also, increasing CSDs lower the m/z of high molecular weight proteins within the limited ranges of certain mass analyzers. As a result, much research effort has been devoted towards finding new ways of manipulating protein CSDs using an extensive list of different charging additives.

A primary limitation of supercharging in conventional ESI-MS is that the supercharging agents, which generally are also protein denaturants, must be directly incorporated in solution and infused together with the proteins of interest. With the extensive list of viable charging additives, only a limited number of ESI-friendly solution systems are suitable for mass spectrometric analysis of proteins in their folded, native form. Recently, new efforts have been devoted towards achieving the same enhanced protein charge states with the added feature of maintaining the native protein structural conformation. Preserving the protein folded structure has its merits as it allows characterization of noncovalent protein-protein and protein-substrate interactions.

Even with the exponential growth of protein studies in ESI, the mechanism of formed and altered protein CSDs is not completely understood. It is believed that the protein ionization mechanism in ESI is dominated by a charged residue model-controlled process. The gas-phase ions are formed after the remaining solvent molecules evaporate, which leaves the analyte with the charges that the droplet carried. Many papers have reported that the CSDs of proteins in ESI-MS are controlled by the surface tension of the least volatile solvent component. However, this is not conclusive when compared between acetic acid and HCl solutions. Other studies have also shown that surface tension may not be the reason for supercharging. Instead, the binding of the supercharging agent to the protein at certain sites may be a primary cause for observed changes in CSDs.

In this work, we present applications of CF-EDESI that feature the advantages of using this ambient ionization technique. In addition to protein charging and conformational studies, we also report a proof-of-principle for the hyphenation of CF-EDESI to reversed phase liquid chromatography (RP-LC) (Figure 1(B)). A RP-LC method was developed to resolve seven protein analytes using conventional ESI. The method was then adapted onto the CF-EDESI platform to compare the two techniques. Chromatographic effluent from the column was directed through a hypodermic needle into the path of the electrospray solvent. Different concentrations of charging additives were introduced through the electrospray source to show that manipulation of protein charge states can be performed simultaneously with chromatographic separation. In the meantime, we investigated the effect of solvent surface tension on protein CSDs by means of introducing protein and supercharging agent through different combinations in the CF-EDESI setup.

### Experimental and materials

#### Chemicals and materials

Cytochrome C, lysozyme, transferrin, myoglobin, hemoglobin, lactalbumin, bovine serum albumin, sulfolane, m-nitrobenzyl alcohol (mNBA) and ammonium acetate were purchased from Sigma-Aldrich (St Louis, MO, USA). LC-MS grade water, acetonitrile and methanol were supplied by Burdick and Jackson (Muskegon, MI, USA). Formic acid and acetic acid were obtained from J.T. Baker (Phillipsburg, NJ, USA).

#### CF-EDESI mass spectrometry

A Thermo Scientific LCQ Deca XP (San Jose, CA) ion trap mass spectrometer with a custom-mounted on-axis ESI source was used for the CF-EDESI analysis. Instrumental settings were as follows. ESI was performed in the positive ionization mode with a spray capillary voltage of 5.0 kV. The nitrogen sheath gas was set at a flow rate of 60 arbitrary units for direct infusion measurements, while it was adjusted to 80 arbitrary units to handle the faster flow rates of the chromatographic separations. The mass spectrometer capillary inlet temperature was set at 200°C. Scans over a two-minute time period were taken per measurement with a scan range of 800–2600 m/z on the high mass range setting of the instrument with 3 mscans and 200 ms scan times. Data analysis was performed using Thermo Xcalibur Data Analysis software (v. 1.5). The instrumental configuration of the CF-EDESI apparatus has been described previously. As in previous work, the distances between the electrospray source needle and the CF needle (1.5 mm), and between the electrospray source and the inlet of the mass spectrometer (8 mm), were optimized with in-line injections of a standard solution of 10 μg progesterone and 100 μM bradykinin through the CF needle to obtain the most abundant signal response from the system. For direct infusion experiments, the electrospray flow rate was maintained at 10 μL min⁻¹ while the sample analyte through the CF needle was also set at 10 μL min⁻¹ using two syringe pumps.

#### Sample preparation

All protein stock standards, except hemoglobin, were dissolved in 100% water at 1 mM stock concentrations; the samples were then diluted to a working concentration of 100 μM. Hemoglobin was dissolved in 5 mM NH₄OAc in 100% water to prevent precipitation before being diluted to a working concentration of 100 μM. In protein chromatography, mixtures of all proteins were dissolved in 100% water at 1 mM stock concentrations; the samples were then diluted to a working concentration of 100 μM.
seven proteins were prepared from 100μM stock solutions. Electro spray solvents were prepared in MeOH/H₂O 50:50 (v/v) with volume adjustments made to accommodate the varying concentrations of charging additive. Acetic acid solutions were prepared in concentrations of 0.10%, 2.0% and 10.0% (v/v); sulfolane solutions were prepared in concentrations of 0.24%, 0.47% and 0.95% (v/v); and mNBA solutions were prepared in concentrations of 0.10%, 0.40% and 1.0% (v/v).

**Results and discussion**

**RPLC coupled to CF-EDESI**

Enhanced capabilities for protein analysis were extended by demonstrating the hyphenation of RPLC to CF-EDESI. Method development was performed using conventional HPLC-ESI-MS to establish a chromatographic separation of a mixture of seven protein standards. A representative chromatogram is shown in Figure 2(A). Eight analyte peaks were monitored during the separation due to the fact that hemoglobin separates into two forms when denatured during HPLC separations, denoted form A (earlier eluting) and form B (later eluting) in this work. The forms could be identified based on their individual CSs. The developed method was then imported into the HPLC-CF-EDESI-MS platform (Figure 1(B)). A 5:2 split ratio was used after the column to yield an approximately 0.120 mL min⁻¹ flow rate eluting from the CF needle. The electrospray flow rate and sheath gas flow rate were adjusted to accommodate this increased flow rate from the CF needle, relative to protein charge state manipulation experiments. A representative chromatogram for the HPLC-CF-EDESI-MS analysis is shown in Figure 2(B). From a comparison of the two chromatograms, good agreement is seen between the two techniques. As expected, all eight protein analytes were detected in CF-EDESI as they were in conventional ESI with the same elution order. Differences in absolute retention time are due to the slightly lower flow rate used for the HPLC separation in the CF-EDESI experiment.

To further elaborate on the advantages of LC-CF-EDESI, a series of charging experiments were conducted using increasing concentrations of acetic acid to manipulate protein charge states simultaneously with the chromatographic separation. Similar to previous charging experiments, the protein CSD of cytochrome C was monitored with increasing acetic acid composition in the electrospray solvent. It is important to note that previous discussions of softer ionization do not apply to HPLC-CF-EDESI-MS, as proteins are effectively denatured when subjected to the denaturing conditions of an HPLC separation. Regardless, increased charging was still observed. The cytochrome C CSs were shifted towards higher states with increasing amount of charging additive. With the presented method, complex mixtures of protein analytes can be rapidly separated with liquid chromatography and then immediately subjected to protein charging to facilitate detection and tandem mass spectrometry interrogation.

Additional experiments were also conducted in order to investigate the effects of sulfolane and mNBA on the chromatographic method. The same additive concentrations of sulfolane and mNBA that were used in the myoglobin experiments were also used for the HPLC-CF-EDESI experiments. The results, however, were significantly different from those observed for the experiments conducted with varying concentrations of acetic acid using the same chromatographic conditions. The addition of either sulfolane or mNBA did not show any enhancement of sensitivity and instead seemed to contribute towards higher background noise and lower sensitivity during the analysis. Also, the addition of sulfolane and mNBA did not facilitate significant protein charge state manipulation with the developed chromatographic method. The results seem to indicate that the mechanism of protein charging seen in HPLC-CF-EDESI is different from that which
is generally referred to for supercharging phenomena in conventional ESI.

In HPLC-CF-EDESI, significant increases to solvent flow rates, which decrease interaction or mixing time of the electrospray droplet with the protein sample, and changes in the mobile phase composition under chromatographic conditions reduce the effectiveness of charging additives, such as sulfolane and mNBA, in increasing the surface tension of the electrospray droplets. The high flow rates of the HPLC-CF-EDESI method may also explain the increased background noise seen in the method. However, charging by acetic acid is still achievable with HPLC-CF-EDESI. In the electrospray process, protein molecules are believed to migrate to the droplet surface before being formed into gas-phase ions.\(^{25,32}\) We propose that acetic acid is capable of increasing protein charge states in HPLC-CF-EDESI because it follows a different charging mechanism. The high concentration of acid simply provides a greater number of protons to charge proteins that will be preferentially assimilated onto the surface of ESI droplets following the extractive desorption process.

**Supercharging mechanism study**

With the first reports of supercharging agents (sulfolane and mNBA), a mechanism was proposed based on a rationale involving the charge residue model and the Rayleigh equation.\(^{25}\) It was believed that the increased surface tension caused by those high boiling point agents would directly attribute to protein unfolding or conformational change. But this proposal was not widely accepted.\(^{25,32,33}\) Several recent studies also demonstrated the preservation of noncovalent complexes in ESI-MS upon supercharging.\(^{34}\) However, other mechanisms related to binding\(^{29}\) and the Brønsted acidities of additives\(^{37}\) have also been reported.

Here, we provide additional explanation of protein supercharging through CF-EDESI experiments, using cytochrome C. In CF-EDESI, the charging agent is not present in the electrospray droplet to augment the surface tension and facilitate increased charging, as it does in conventional ESI.\(^{25,32,33}\) Charging agents such as sulfolane and mNBA did not show an obvious impact in increasing charge states in CF-EDESI; characteristic charge state for native-state cytochrome C centered at \(m/z +8\) was observed in most of the results for cytochrome C experiments. However, at high concentration (1%) of sulfolane, adduct peaks caused by sulfolane itself completely suppressed the signal from protein in experiment 1 (normal CF-EDESI); no protein and charging agent binding peak was observed. This result was different from that produced in conventional ESI. Since the supercharging agent did not appear in sample droplets in our case, the surface tension mechanism for supercharging protein sounds reasonable based on our results. However, we cannot rule out completely the possibility of a binding mechanism, since the contact of supercharging agent and protein was possible in CF-EDESI.

When using acetic acid as the supercharging agent, an identical result was obtained with the introduction of protein solution through different lines as shown in experiments 1 and 2.
However, when the protein and acetic acid were premixed in experiments 3 and 4, different spectra were observed when introducing the premix through different lines. In experiment 3, two envelopes of CSs were observed, as shown in figure 3(C). The first envelope centered at +14 indicated the denatured form of cytochrome C. The second was calculated as the dimer form of cytochrome C which was also centered about the +14 charge state. The aggregation either happened before the protein was ionized, or it was a result of the shrinking droplet process. The former is more likely given the unique result obtained in this experiment (i.e. the dimer was not observed in other experiments). The capability for supercharging is more likely to depend on the contact time (for cytochrome C and its dimer, the contact times are the same), not on the surface area or the binding site availability. Thus, either cytochrome C or its dimer form has the same chances to acquire the same amount of charges. This conclusion was confirmed in experiment 4. When protein and acid were premixed and introduced through the electrospray, the observed spectrum had only one CS envelope (not dimer), as shown in figure 3(D). From this result, we could propose that the contact time of protein mix and electrospray is critical for the protein to be sufficiently charged.

From these CF-EDESI experiments, some insight was provided for the argument of the supercharging mechanism. The surface tension mechanism and binding mechanism may both contribute to protein supercharging. However, the process is still complicated by the combination of several factors such as protein properties, additive properties, solution ionization transfer to gas phase, thermodynamics and charge equilibriums, among others.

Conclusions

CF-EDESI-MS has been demonstrated to provide additional flexibility for reagent addition to manipulate protein supercharging. CF-EDESI also provides a unique method by which the way charging additives affect droplet dynamics and protein charge states can be studied, using unique experimental configurations. Yet, the mechanism for supercharging appears to be different from that observed through conventional ESI.

Combined with HPLC, protein separations can be achieved and supercharging additives used and evaluated without changing the chromatographic separation mechanism, or having the need of post-column addition. The need for flow splitting is a disadvantage from a hardware perspective, but it does ensure that sensitivity relative to ESI is well maintained. Further studies are needed to understand whether the analysis of proteins from more complex sample matrices using LC-CF-EDESI-MS holds any significant advantage from a matrix effect standpoint; many other ambient ionization methods have shown enhanced robustness when the sample and ESI generation are segregated, as they are in CF-EDESI.
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References