Published in final edited form as:

Anal Chem. 2017 October 03; 89(19): 10188–10193. doi:10.1021/acs.analchem.7b01225.

Corona Discharge Suppression in Negative Ion Mode Nanoelectrospray Ionization via Trifluoroethanol Addition

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Abstract

Negative ion mode nanoelectrospray ionization (nESI) is often utilized to analyze acidic compounds, from small molecules to proteins, with mass spectrometry (MS). Under high aqueous solvent conditions corona discharge is commonly observed at emitter tips, resulting in low ion abundances and reduced nESI needle lifetimes. We have successfully reduced corona discharge in negative ion mode by trace addition of trifluoroethanol (TFE) to aqueous samples. The addition of as little as 0.2% TFE increases aqueous spray stability not only in nESI direct infusion, but also in nanoflow liquid chromatography (nLC)/MS experiments. Negative ion mode spray stability with 0.2% TFE is approximately six times higher than for strictly aqueous samples. Upon addition of 0.2% TFE to the mobile phase of nLC/MS experiments, tryptic peptide identifications increased from 93 to 111 peptides, resulting in an average protein sequence coverage increase of 18%.

Graphical abstract

Mass Spectrometry

Negative Ion Mode; Electrospray; Corona Discharge; Nanoflow Liquid Chromatography

Introduction

Nanoelectrospray ionization (nESI) has gained popularity as a “soft” ionization source due to several advantages compared with conventional electrospray ionization (ESI). Due to the

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Supplementary Material

Example images of nESI needles before and after corona discharge, graphs examining onset of corona discharge versus nESI voltage, example spectra of BSA, ubiquitin, and the 5 peptide mixture with increasing organic solvent composition, sample “native” myoglobin spectra with TFE, and the list of all LC/MS identified tryptic peptides observed

smaller capillary diameters employed in nESI, initially formed droplets have nanometer size, whereas ESI produces micrometer-sized droplets.^{1–4} The reduction in initial droplet size lowers the deposition of ionization energy and increases the likelihood of gas-phase ions being generated from evaporating progeny droplets, resulting in higher ion counts and lower limits of detection.^{3,5–9} For example, consumption of only zeptomoles of peptides was recently reported with microfabricated nESI needles.¹⁰ The lower deposition of ionization energy in nESI compared with ESI is often utilized to maintain native proteins and complexes into the gas phase, allowing for the examination of tertiary and quaternary structure with ion mobility and/or mass spectrometric measurements.^{11–13} Nanoflow liquid chromatography (nLC) separations (nL/min) are ideally suited for coupling with nESI, resulting in robust and stable positive ion mode ionization and online detection with modern mass spectrometers.^{14–17}

While positive ion mode nESI is suitable for the detection of basic and neutral compounds, typically acidic molecules are not ionized effectively.^{18,19} Many proteomes have been predicted to exhibit multimodal pI distributions with a minimum at pI 7.^{20,21} However, in conventional positive ion mode nLC/MS analysis, the majority of detected peptides have pIs greater than 5.^{22,23} By including a second dimension of peptide separation prior to reverse phase elution, such as strong cation exchange (SCX) or isoelectric focusing (IEF), acidic peptides can be identified with positive polarity nESI.^{24–26} However, Yates and co-workers reported an absence of peptides with pIs < 4 in a combined protein fractionation/multidimensional protein identification technology (MudPIT) approach; still excluding a majority of the low pI region.²⁴ Several acidic post-translational modifications, such as sulfation, phosphorylation, and sialylation, greatly reduce overall analyte pI. Utilization of negative polarity nESI can improve the detection of acidic molecules due to the inherent tendency to form negative charges. For example, negative ion mode LC/mass spectrometry (MS) is a major analytical approach in the field of metabolomics.^{27–31} Coon and co-workers demonstrated the use of alkaline separations and negative ion mode detection as an effective method to probe the acidic proteome.^{23,32} However, in contrast to positive ion mode, nESI unfortunately does not typically perform robustly in negative ion mode.

Under negative polarity, corona discharge is often observed at the tip of nESI emitters.^{23,33} Corona discharge results in degradation of spray stability and needle tip geometry, ultimately leading to loss of signal. This phenomenon was observed as early as by Fenn and co-workers' first experiments with negative ion mode ESI.^{18,34} Several methods have been implemented to suppress the observed plasma. The introduction of high dielectric nebulizing gas, such as sulfur hexafluoride or dry oxygen, has been employed to increase the local dielectric constant at the ESI needle tip as well as to promote stable generation of analyte droplets for negative ion generation.^{34–36} Also, pressurization (2–6 bar) of the mass spectrometer's inlet region has been shown to afford an increased dielectric constant, reducing corona discharge.^{37,38} In addition, organic solvents, such as methanol (MeOH), isopropyl alcohol, fluorosolvents, and chlorosolvents can be added to samples to reduce the onset spray voltage below the dielectric breakdown of air.^{39,40} Chlorosolvents have previously also been utilized as electron scavengers for the reduction of corona discharge events, but are immiscible with aqueous samples.^{39,41,42} Fluorosolvent addition has also been utilized extensively as a method to retain and improve ionization of hydrophilic small

molecules and oligonucleotides through ion-pairing on reverse-phase resins and improved ESI droplet desolvation.^{43–46} Furthermore, small needle-tip geometry has been reported to decrease minimum spray voltage below ambient atmosphere dielectric limits.⁴⁷ While these approaches have been effective, instrument modification, dilution of precious sample, and analyte solubility are still of concern, particularly with regards to online separations and “native” mass spectrometry experiments.⁴⁸

Herein we report the stabilization of negative ion mode nESI and suppression of corona discharge via trifluoroethanol (TFE) addition to aqueous sample matrices. A series of small molecules, peptides, and proteins were examined with varying amounts of TFE modifier. The utility of TFE as a mobile phase additive was examined via nanoflow separation and detection of tryptic peptides at elevated pH in negative ion mode. With the addition of trace TFE, negative ion mode nESI analysis can be used to effectively characterize acidic molecules present in many complex aqueous matrices analyzed with mass spectrometry.

Materials and Methods

Chemicals

Water, MeOH, ethanol (EtOH), 2,2,2-trifluoroethanol, ammonium bicarbonate, ammonium acetate, 48% hydrofluoric acid (HF), and formic acid were purchased from Fisher Scientific (Pittsburgh, PA). Equine myoglobin (Myo), bovine ubiquitin, bovine serum albumin (BSA), bovine α -casein, bovine β -casein, 5 M ammonium hydroxide, triethylamine (TEA), dithiothreitol, maltohexanose (MH), tetrahydrocortisone-(3- β -D-glucuronide) (TC), paromomycin (PA), angiotensin I (AngI), and [Arg⁸]-vasopressin (Vas), were obtained from Sigma-Aldrich (St. Louis, MO). Phosphatidylserine 16:0-18:2 (PS) was purchased from Avanti Polar Lipids (Alabaster, AL). Tyrosine phosphopeptide (TSTEPQpYQPGEN) (pYpep) was purchased from EMD Millipore (Billerica, MA). Sulfated cholecystokinin (CCKS) was purchased from Abcam (Cambridge, MA). The synthetic peptide (YFYLIPILEYLK) (SynPep) was purchased from GenicBio (Shanghai, China). Modified sequencing grade trypsin was obtained from Promega (Madison, WI). Water, MeOH, and EtOH were HPLC grade. TFE was peptide synthesis grade. All compounds were used as purchased.

Direct Infusion nESI Mass Spectrometry

All direct infusion mass spectra were collected on a Waters Micromass LCT Premier equipped with a Z-Spray nESI source. Borosilicate nESI needles were pulled to approximately 3–8 μ m tip i.d. on a Sutter Instruments P-97 micropipette puller and gold coated using a Quorum Technologies SC7620 sputter coater as previously described.¹¹ Each acquisition was performed with the emitter tip approximately 6 mm away from the sample cone. To each solution, unless otherwise noted, 0.2% ammonium hydroxide was added to promote analyte deprotonation. Sample flow rates were estimated to be 20–50 nL/min for direct infusion experiments.¹¹ Each protein was examined at 10 μ M. Peptide and small molecule concentration in their respective solutions were 5 μ M. Spray voltage was applied at – (1.25–1.35) kV and remained constant for each experiment performed. The source region was heated to 65°C for all experiments.

Nanoflow On-Line Liquid Chromatography/Mass Spectrometry

BSA, α -casein, and β -casein were mixed in a 1:1:1 ratio at 75 μ M each. This mixture was subjected to reduction, alkylation, and tryptic digestion at a 1:50 protease:protein ratio. High pH tolerant trap and analytical columns were slurry packed with Waters Xbridge C18, 5 μ m particles as previously described.^{23,49} Trap column and analytical column dimensions were 75 μ m i.d. $\times$ 5 cm and 75 μ m i.d. $\times$ 17 cm, respectively. Fused silica nESI emitters were pulled on a Sutter Instruments P-2000 laser micropipette puller and HF etched as previously described to approximately 4–6 μ m tip i.d.⁵⁰ Mobile phase solvent A (95% H₂O, 5% MeOH) and B (5% H₂O, 95% MeOH) were buffered with 0.2% TEA, formic acid, and ammonium hydroxide to pH 11.⁵¹ TFE was added to both mobile phases at 0.2% where noted. Sample loading was completed at 2 μ L/min, placing a total of 500 fmol of each tryptically digested protein on column. Gradient elution was completed at 250 nL/min with the following gradient: 2–75% B; 0–85 min. Negative ion mode mass spectra were collected at 1M data points on a Bruker 7 T Solarix Fourier transform ion cyclotron resonance mass spectrometer with 0.1 s ion accumulation time. Nanospray voltage was held at 1.25 kV and the drying gas temperature was set to 150°. Mass spectra were externally calibrated to < 2 ppm error. Following triplicate data collection, peptide matches were determined using an in-house Excel macro. Peptides were only assigned if error was < 10 ppm. Alkylation of cysteine was set as a fixed modification. Variable modifications were set at two missed tryptic cleavages, and methionine oxidation.

Results and Discussion

Negative Ion Nanoelectrospray Stability with Different Solvent Modifiers

A five peptide mixture of angiotensin I, sulfated cholecystokinin, [Arg⁸]-vasopressin, synthetic peptide (YFYLIPLEYLK), and tyrosine phosphopeptide was subjected to direct infusion MS analysis for a total of 30 min to assess negative ion mode nESI stability, defined here as one standard deviation of the sample's total ion counts for the duration of the experiment. Figure 1 shows the corresponding results from experiments with three different solvent modifiers. A completely aqueous solution resulted in an average of $0.78 \pm 0.14 \times 10^6$ counts. By adding 0.2% MeOH, the observed average counts approximately doubled to $1.54 \pm 0.09 \times 10^6$ with the signal variability decreasing as indicated. However, at approximately 12 min, corona discharge occurred, degrading the needle's conductive coating and resulting in lower ion counts with increased fluctuation. Sporadic corona discharge reoccurred for the remainder of the experiment. Example images of emitters before and after corona discharge can be seen in the supporting information. (Figure S-1). With 0.2% EtOH or 0.2% TFE solutions, average ion counts of $1.48 \pm 0.04 \times 10^6$ and $1.54 \pm 0.02 \times 10^6$ were observed, respectively, both corresponding to lower signal variability than for MeOH addition. However, upon trace addition of TFE, spray current is approximately twice as stable as with EtOH addition and four times more stable than with MeOH addition under identical conditions. Overall, the addition of TFE resulted in similar ion counts as for EtOH and MeOH, but resulted in suppression of corona discharge events for more robust anion generation. The plasma at the nESI needle tip, visible to the naked eye at –1.25 kV, also was absent following TFE addition. When the nESI voltage applied to a TFE laden solution is steadily increased, the onset of corona discharge is ~100 V higher compared with other

organic modifiers, indicating that the local dielectric constant at the needle tip is increased (Figure S-2).

We hypothesize the mechanism of corona discharge suppression in negative ion mode via TFE addition is due to the unique electronic properties of the gaseous TFE molecule. As modified aqueous solvent undergoes nanoelectrospray, the higher vapor pressure of TFE compared with water implies preferential TFE evaporation from desolvating analyte droplets.⁵² The gas phase TFE can act as electron scavenger due to the highly electronegative fluorine atoms, suppressing plasma formation in a manner similar to SF₆ gas.³⁶ These properties result in a higher local dielectric constant at the tip of the nESI emitter, thus decreasing corona discharge events. To further confirm whether the observed corona discharge suppression is a gas-phase phenomenon, TFE in MeOH was orthogonally nanoelectrosprayed via a separate lockspray emitter at a ~6 mm offset when compared to the analyte nESI emitter. As seen in Figure 2, the total ion current for the protein myoglobin nanoelectrosprayed in negative ion mode from an aqueous solution with 0.2% NH₄OH is both increased and stabilized when TFE is present in the orthogonal lockspray solution as compared to MeOH alone although direct addition to the sample solution is most effective. These results support the hypothesis that TFE corona discharge suppression is likely linked to an increase in the gas-phase dielectric constant at emitter tips.

Negative Ion Charge State Effects with Solvent Modifiers

While the addition of TFE increases nESI stability in negative ion mode, it is possible that the presence of TFE may alter observed analyte charge states, similar to the supercharging reagents m-nitrobenzyl alcohol and sulfolane.⁵³ By altering analyte charging, the mechanisms of tandem mass spectrometry activation techniques such as electron detachment dissociation (EDD), negative ion electron capture dissociation (niECD), negative electron transfer dissociation (NETD) and negative ultraviolet photodissociation (NUVPD) can be altered, resulting in either increased or reduced information content.^{32,54–56} Furthermore, shifts in analyte charge can place a compound outside the optimal detection range of some mass analyzers, including quadrupoles. Finally, the maintenance of native protein structure into the gas phase is dependent on the charge state of the protein, thus alterations in the overall protein charge can cause Coulomb-induced unfolding events.⁵³

We examined the average charge states (Z_{avg}) and ionization behaviors of three proteins upon negative ion nESI as a function of organic modifier percentage. As seen in Figure 3 (left), neither EtOH nor TFE resulted in significantly altered myoglobin charge states although a [Heme + TFE][–] adduct was observed for TFE laden solutions. At TFE concentrations greater than 1.0% (139 mM), TFE appears to disrupt Heme hydrogen bonding, resulting in slightly higher Apo to Holo myoglobin ratios as compared with the EtOH laden solvent. For BSA and ubiquitin, no significant charge state alteration was observed up to 1.0% TFE (Figure S-3). Z_{avg} was determined following triplicate analysis of ubiquitin, myoglobin, and BSA with and without solvent modifier. For these three proteins, observed charge states for equivalent organic modifier percentages were within error of each modifier type (Figure 4). Thus, the relatively low concentration of organic modifier in each solution does not affect Z_{avg} observed under primarily aqueous conditions. We further

examined the addition of TFE to “native” buffers to improve negative ion mode spray stability: identical myoglobin charge state distributions were observed in 100 mM ammonium acetate solution at pH 7.2 with and without 1.0% TFE addition (Figure S-4). The charge states observed under these conditions have been previously reported as indicative of the native gas phase confirmation of myoglobin.⁵³

Mixtures of five peptides and four small molecules, respectively, were also examined as a function of organic modifier percentage. As seen in Figure 3 (right) and Figure S-5, increasing the percentage of EtOH or TFE did not significantly alter observed charge states of the peptides or small molecules studied. In both model systems, as organic modifier percentage is increased, the signal observed for all molecules is slightly improved. These results indicate that, with trace addition of TFE to negative ion mode nESI analysis, corona discharge can be mitigated while not altering charging of proteins, peptides, and small molecules.

Negative Ion On-Line nLC/MS of Tryptic Peptides

The addition of TFE to mobile phase solvents was also examined as a method of stabilizing nESI at high aqueous percentages commonly encountered at the beginning of liquid chromatography gradients. Figure 5a shows base peak chromatograms, demonstrating the effect of adding 0.2% TFE to a negative ion mode nLC/MS experiment. From 7–27 min, increased peptide signal is clearly observed upon TFE addition. In Figure 5b, sample extracted ion chromatograms demonstrate how commonly identified peptides are altered by TFE addition in the early elution region of analysis. Sequence coverage observed for α -casein, β -casein, and BSA without TFE present was $53 \pm 8\%$, $24 \pm 7\%$, and $70 \pm 7\%$, respectively. When 0.2% TFE was included in the mobile phases, the sequence coverage for α -casein, β -casein, and BSA increased to $80 \pm 3\%$, $38 \pm 7\%$, and $81 \pm 3\%$, respectively. A total of 111 unique peptides were detected when TFE was added, compared with 93 unique peptides without TFE. A list of all detected peptides can be found in the supporting information (Tables S-1 & S-2).

Detected peptides were classified into three categories based on mobile phase composition; early eluting (2–26% B), moderate eluting (27–51% B), and late eluting (> 52% B). Based on this classification, without TFE 17 peptides are classified as early eluting, 55 as moderate eluting, and 21 as late eluting. Upon addition of 0.2% TFE to the mobile phase, 61 peptides are classified as early eluting, 34 as moderate eluting, and 16 as late eluting. Peptides that were found in both nLC/MS experiments were examined for retention time shifts upon the addition of 0.2% TFE. Without TFE addition, the average peptide elution time was 42.0 min, whereas after TFE addition the average retention time was 34.7 min. The addition of TFE to mobile phase A caused a reduction in stationary phase retention due to the increased hydrophobicity of the eluent. Despite an approximately 7 min shift in peptide retention, TFE addition and subsequent corona discharge suppression allows for robust detection of peptides eluting in a mostly aqueous solvent and an overall increase in total ion current and peptide identification by 30% and 18% respectively.

Conclusion

Trace addition of TFE to aqueous samples for negative ion mode nESI analysis results in significant suppression of corona discharge at nESI emitter tips. This suppression, in turn, results in stabilized signal and increased longevity of nESI needles under negative polarity. All samples examined in this study benefited in signal abundance and stability without altering observed analyte charge states upon TFE addition. Furthermore, TFE addition to negative ion nLC/MS experiments improved sequence coverage and only slightly altered chromatography. To further improve separations in the future, mobile phase A composition of 97.8% H₂O, 2% MeOH, and 0.2% TFE will be used to reduce peptide elution shifts. Additional experiments to elucidate the mechanism of TFE corona discharge suppression are currently in progress.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank Dr. Brandon Ruotolo for generous use of the Waters Micromass LCT Premier TOF, Dr. Robert Kennedy and Dr. Jenny Wong for use of the Sutter Instruments P-2000, and the NIH (R01 GM107148) for funding.

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Figure 1. Negative ion nESI stability observed with a five peptide mixture and various solvent modifiers at -1.25 kV.

Figure 2.

Negative ion nESI stability for 10 μ M myoglobin in 99.8% H_2O and 0.2% NH_4OH solution examined with an ~ 6 mm offset lockspray concomitantly spraying 100% MeOH (red) or 2% TFE in MeOH (purple). When 0.2% TFE is only included in the analyte solution (black) stability is maximized.

Figure 3.

Negative ion nESI spectra of myoglobin and small molecule mix (TC = tetrahydrocortisone-(3- β -D-glucuronide), PA = paromomycin, PS = phosphatidylserine 16:0-18:2, MH = maltohexanose) in A & D) 100% H₂O, B & E) 0.2% modifier, and C & F) 1.0% modifier, respectively. Spectra are offset by 15 m/z for clarity.

Figure 4. Average charge states observed for A) ubiquitin, B) myoglobin, C) BSA as a function of organic modifier percentage. Error bars are one standard deviation from the mean.

Figure 5.

A) Base peak chromatograms of early elution region from negative ion mode nLC/MS analysis of tryptic peptides without (top) and with (bottom) TFE present in the mobile phase. B) Example extracted ion chromatograms of early eluting tryptic peptides observed in both experiments.