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RESEARCH ARTICLE

Microdroplet Fusion Chemistry for Charge State Reduction of Synthetic Polymers via Bipolar Dual Spray with Anionic Reagents

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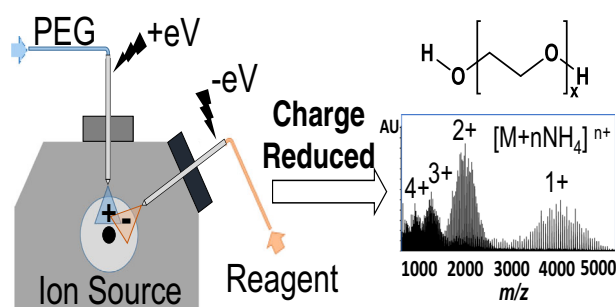
Abstract. Microdroplet fusion chemistry is an emerging area of analyte manipulation that utilizes the ion source region of a mass spectrometer to covalently derivatize or manipulate the charge state distribution. This technique utilizes two electrospray emitters in close proximity, where the droplets from each electrospray plume fuse and undergo the subsequent chemistry. In this study, microdroplet fusion chemistry via bipolar dual spray has demonstrated the ability to reduce the average charge state of polyethylene glycol (PEG) cations using anionic reagents. Bipolar dual spray was implemented on a commercial mass spectrometer with limited hardware modifications to the ion source. Reagents including ammonium hydroxide, formic acid, and lithium chloride showed dramatic shifts in the average charge state of PEG 3.8 K cations (e.g., 5.0+ to 2.5+) along with the emergence of newly detected charge states. An organic base, tributylamine, had no effect on the charge state distribution of PEG 3.8 K cations. These results were consistent with an ion-pairing mechanism, where reagent anions destabilized ammonium cation interactions with PEG 3.8 K upon droplet fusion from the negative and positive emitters. Additional bipolar dual spray experiments with PEG 12.6 K demonstrated the ability to transform uninterpretable mass information into distinct charge states ranging from $[M+8NH_4]^+$ to $[M+3NH_4]^+$. Overall, this study provides insight into the nature of dual spray chemistry and will aid future experimental design in microdroplet covalent chemistry.

Keywords: Charge reduction, Microdroplet fusion chemistry, Dual spray, Droplet chemistry, Synthetic polymers

Introduction

The essential steps in a mass spectrometry (MS) analysis involve the ionization of molecules and subsequent transmission and detection of ions in vacuo. Electrospray ionization (ESI) is a common ionization technique that generates ions under soft conditions (i.e., intact ionization of the parent molecule). ESI has a propensity to generate multiply charged ions in the ionization process especially as the molecular weight and polarity of the analyte increase [1]. As a result of multiply charging, complex mixtures including polydisperse and heterogenous analytes (e.g., polyols, oligomers, post-translationally modified proteins) can be difficult to interpret as significant ion overlap can occur from the presence of these distributions.

One way to decrease the complexity from multiply charging involves the transformation of multiply charged ions into lower-charged ions, i.e., charge reduction (CR). This process involves the transfer or charge stripping from an analyte ion to

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another species at ambient or vacuum conditions. Transformation affords decompression of the mass spectrum and resolution of similar *m/z* ions with different molecular masses. Following this chemistry, mass spectral interpretation becomes similar to MALDI mass spectra and in silico deconvoluted ESI mass spectra. Numerous chemical approaches to reduce the charge state of multiply charged ions have been developed including ion/ion reactions [2–4], ion/molecule reactions [5, 6], ion/electron reactions [7], leak-in chemistry [8, 9], solution additives [10, 11], and [210]Po radioactive emitters [12–19].

An emerging area of analyte manipulation, microdroplet chemistry, utilizes the ion source region to chemically derivatize analytes during the electrospray ionization process. Microdroplet chemistry typically utilizes one electrospray emitter and infusion of the bulk reaction mixture. Here, bimolecular reactions rely on accelerated chemistry within the shrinking secondary electrospray droplets or by ion/molecule reactions prior to entering the mass spectrometer [20]. Numerous classic organic reactions have demonstrated rapid reaction kinetics [21], e.g., imine bond formation [22], Hantzsch synthesis [23], and isoquinoline synthesis [24]. In another approach, microdroplet fusion chemistry utilizes two electrospray emitters in close proximity, where the droplets from each electrospray plume fuse and undergo subsequent chemistry. Examples in the literature include charge state manipulation and chemical derivatization [25–28]. In the work of Lee et al., application of positive potentials to both of the electrospray emitters determined the kinetics of acid-induced cytochrome c unfolding and observed a subsequent increase in the charge state distribution [25]. Alternatively, applying opposite potentials to each electrospray emitter has demonstrated the ability to charge reduce multiply charged cations [26]. In the charge reduction experiments, immunoglobulin g (IgG) cations were charge reduced using triethylammonium formate from [M+15H][15+] to singly charged IgG using a bipolar electrospray chamber and differential mobility analysis. Applying opposite potentials has also demonstrated chemical derivatization in a high-throughput workflow, where Schiff base chemistry was applied to proteomic work flows (i.e., derivatization of the peptide primary amines) and ultimately increased sequence coverage with the use of ultraviolet photodissociation [27].

In this study, we demonstrate the ability of microdroplet fusion chemistry using bipolar dual spray to reduce the charge state distribution of ammoniated synthetic polymers on a commercial mass spectrometer with limited hardware modification. Charge-reduced analytes include polyethylene glycol (PEG) 3.8 K and PEG 12.6 K. In addition, the nature of charge reduction has been explored utilizing various solvents, acids, bases, and ionic salts as the reagent, where the results strongly suggest an ion-pairing mechanism between reagent anions and analyte cations. Overall, this work demonstrates the ability to quickly modify an ion source and charge-reduced analytes with simple reagent solutions.

Experimental Section

Materials

LC/MS grade methanol and 99.9% toluene were purchased from Fisher Scientific (Pittsburgh, PA). Thirty percent ammonium hydroxide (aq) was purchased from Avantor (Radnor, PA). Ultrapure 18-MΩ water was generated by a Milli-Q water purification system (EMD Millipore, Billerica, MA). Tetrabutylamine and formic acid were purchased from Sigma-Aldrich (St. Louis, MO). Poly (ethylene glycol) 3800 amu was purchased from Agilent Technologies (Santa Clara, CA). Poly (ethylene glycol) 12,600 amu was purchased from Polymer Laboratories (Church Stretton, UK).

Microdroplet Fusion Chemistry

Experiments were performed on a commercially available Agilent 6560 Ion Mobility (IM) quadrupole time-of-flight (Q-TOF) mass spectrometer (Santa Clara, CA). Overall, the experimental setup was based on the Agilent design of their dual ESI ion source. In order to implement microdroplet fusion chemistry, a dual ESI Agilent source was modified to electrically isolate the reference mass ESI emitter (i.e., lock mass) from the ESI-inducing voltage on the MS front plate. The Agilent dual ESI source (Figure 1) has two ESI emitters oriented orthogonal to the inlet (approximately 10-mm distance from the inlet) and a 30° angle between the emitters. The analyte emitter ionizes analyte effluent from a liquid chromatograph or infusion pump, while the reference mass emitter ionizes a calibrant ion from an internal instrument pump to ensure mass accuracy. In normal operation, the front plate of the mass spectrometer has an applied voltage to induce electrospray
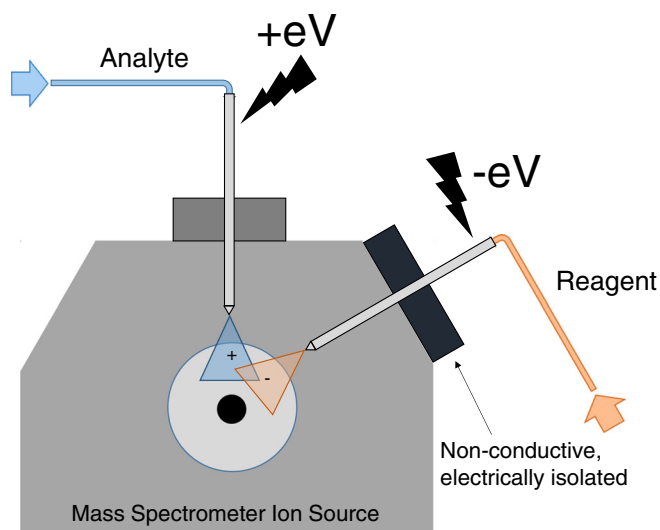

Figure 1. Schematic of the modified dual ESI commercial ion source. The conductive ESI emitter guide has been replaced with a non-conductive material for the reagent emitter. The tips of the ESI emitters were located approximately 10 mm axially from the inlet, while a 30° angle was present between the emitters

ionization in both of the ESI emitters. In order to apply opposite polarities on the emitters, the reference mass ESI emitter was electrically isolated from the front plate by removing the metal ESI emitter mounting guide and re-placing it with a polyether ketone (PEEK)–fabricated mounting guide ($4 \times 4 \times 0.5$ cm). A Bertan 205B-10R high voltage power supply was connected to the reference mass emitter and a shared ground with alligator clips, which allowed the application of negative voltage (i.e., opposite to the positive analyte voltage). The reference mass emitter will be referred as the reagent emitter since this emitter was exclusively used to introduce reagents to effect the analyte droplets. It is also noteworthy to mention that performing microdroplet fusion chemistry on a commercial ion source presented some experimental restrictions, e.g., limited control of the distance from the emitters to inlet and angle between reference mass and analyte emitters.

Microdroplet fusion chemistry was performed by simulta-neously infusing the reagent and analyte solutions and over-lapping the bipolar ESI plumes. Reagent solutions were infused at a flow rate of 10 μl/min via a syringe pump, where a negative voltage was applied directly to the reagent emitter. As higher negative voltages increased the extent of charge reduction, the highest possible negative voltage was applied that did not induce an electrical discharge. Applied negative voltages were approximately − 8 kV. For the analyte emitter, a syringe pump infused analytes with a flow rate of 5 μl/min, where the instru-ment power supply provided the ESI-inducing positive voltage (approximately 3 kV). It is noted that changes in flow rate did not substantially effect charge reduction or signal intensity as long as the reagent flow rate was equal to or greater than the analyte flow rate. Successful charge reduction was observed at 100 μl/min and 200 μl/min, reagent and analyte flow rate, respectively (S1). For the PEG 3.8 K and PEG 12.6 K analytes, PEG solutions were prepared in 1 g/L ammonium formate in methanol at a concentration of 200 ppm. Reagents were pre-pared as stated below. In addition, microdroplet fusion chem-istry was performed with the application of positive potentials on both emitters; whereupon, mass spectra were dominated by salt clusters while PEG analyte ions were suppressed and no longer observed.

Data Analysis

Analysis of mass spectra was performed using MassHunter software (Version B.07.00, Agilent Technologies). The
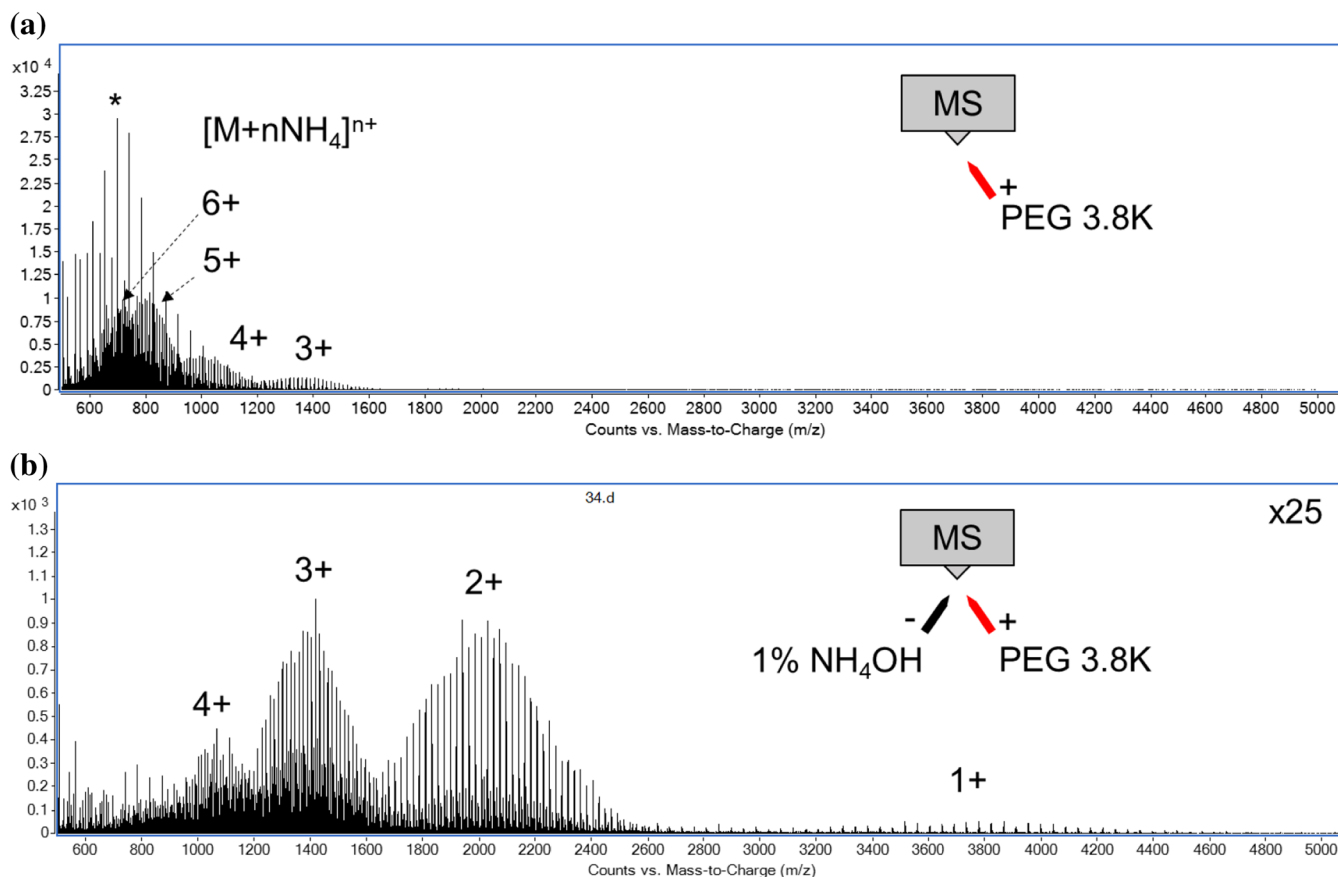
(a)

(b)

Figure 2. (a) Positive mode direct infusion of PEG 3.8 K. The asterisk (*) symbol represents a low molecular weight PEG impurity and will be used throughout additional figures to represent an impurity. (b) Dual spray of positive mode direct infusion of PEG 3.8 K and negative mode direct infusion of 1% NH$_4$OH in methanol. The × 25 represents the y-axis scale compared with panel a. The lower intensity distribution ranging from the 2+ to the 4+ charge state is consistent with neutral ammonium formate adducts and is observed throughout all of the charge reduction data

observed average charge state for the analyte ions was determined from mass spectral data using Eq. (1). Ion signal for $[M+nNH4]^{n+}$, above the signal to noise ratio of 3, was summed to generate total ion current for a specific charge state (e.g., TIC (CS_1) for charge state 1+). The summation was performed for each charge state in the mass spectrum. The total ion current for each charge state was multiplied by the appropriate charge state to produce the numerator in Eq. (1). The denominator in Eq. (1) was calculated by summing the total ion currents for each of the identified charge states.

$$\text{Average charge state} = \frac{\Sigma_n^{\infty}[n*\text{TIC}(CS_n)]}{\Sigma_n^{\infty}[\text{TIC}(CS_n)]} \qquad (1)$$

Results and Discussion

Charge reduction mass spectrometry has demonstrated the ability to simplify the interpretation of complex mass spectra. A variety of ambient and vacuum reactions have demonstrated the ability to reduce the overall charge state in the context of a mass spectrometry experiment. Here, we investigate the ability

and the factors that lead to charge reduction via microdroplet fusion chemistry using a bipolar dual spray setup.

Positive mode direct infusion ESI of PEG 3.8 K generated a charge state distribution (CSD) ranging from $[M+6NH_4]^{6+}$ to $[M+2NH_4]^{2+}$ (Figure 2a), where the average charge state was 5.0+. A singly charged, low m/z distribution of PEG ions was observed from m/z 500 to 1000, which was consistent with impurities and not fragment ions (i.e., determined by accurate mass LC/MS analysis, data not shown). These ions are labeled with an asterisk symbol and were not included in the average charge state calculation. In the center of Figure 2a, a graphical representation of the MS and the number and polarity of the ESI emitters can be observed to provide quick insight into the instrumental conditions. In Figure 2b, the PEG 3.8 K analyte spray (i.e., infused into the positively charged analyte emitter) was fused with droplets from negative electrospray of 1% ammonium hydroxide in methanol (i.e., reagent emitter). The CSD of PEG cations shifted to predominantly $[M+3NH_4]^{3+}$ and $[M+2NH_4]^{2+}$ with the emergence of singly ammoniated PEG cations, decreasing the average charge state from 5.0+ to 2.7+. Low abundance neutral adducts of ammonium formate were observed as minor components in the 2+, 3+, and 4+ charge states compared with the more intense $[M+nNH_4]^{n+}$ ions. These ammonium formate neutral adducts were also present in the PEG 3.8 K control mass spectrum (Figure 2a) as low abundance
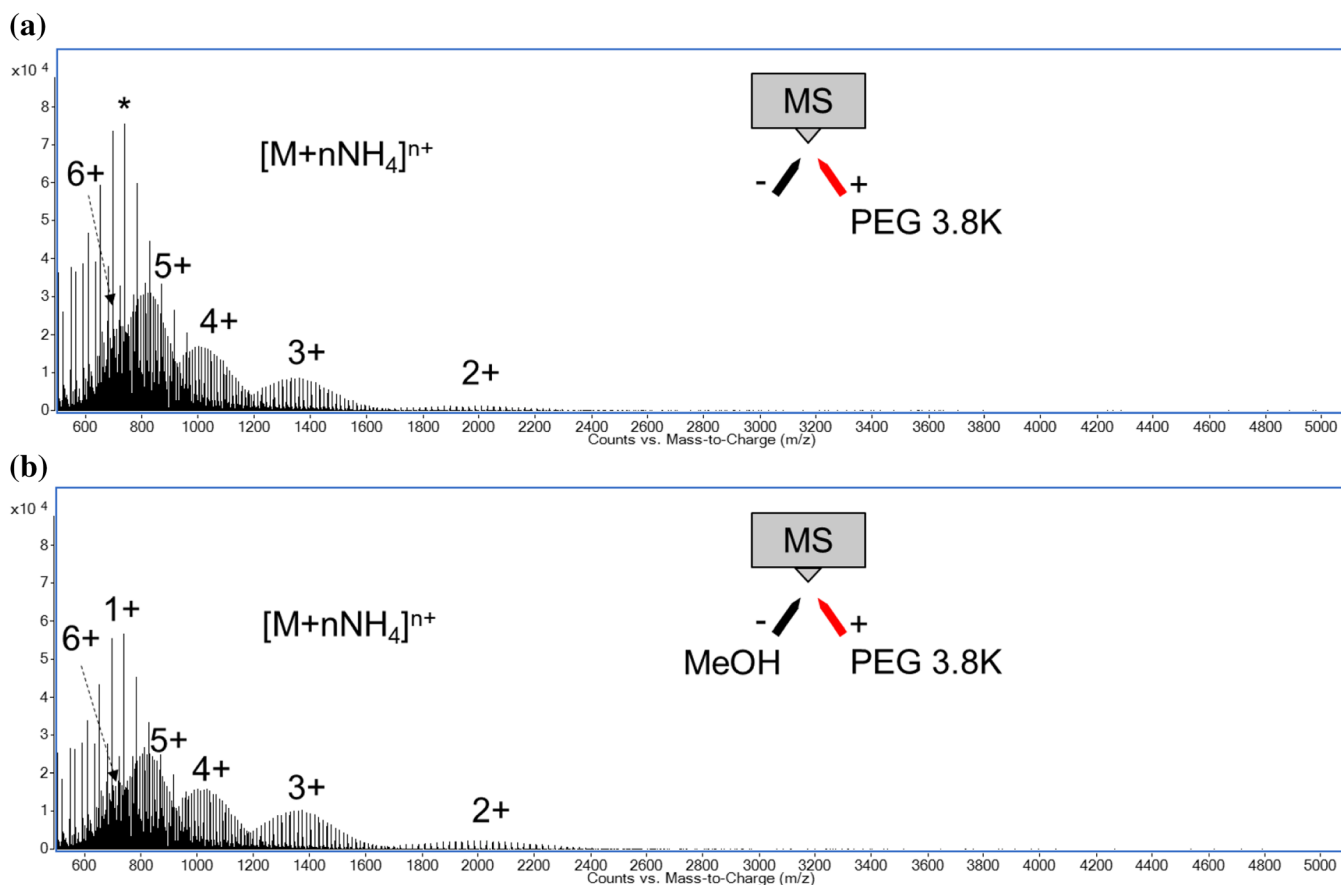
(a)

(b)

Figure 3. (a) Positive mode direct infusion of PEG 3.8 K and a negative voltage applied to the reagent emitter without a solution present. (b) Positive mode direct infusion of PEG 3.8 K and negative mode direct infusion of neat methanol

ions, so this was not expected to be a contributor to the charge reduction process. In addition, the total ion current was reduced approximately 25-fold from the PEG 3.8 K control to the charge-reduced mass spectrum. Several studies utilizing solution phase additives to perform charge reduction on analyte ions observed similar results [10, 11]. The reduced signal was attributed to intense low m/z clustering of the highly concentrated additives, which in turn, suppressed the analyte ion signal.

To demonstrate that the drastic transformation of the charge state distribution was the result of reagent additives to the negative reagent emitter, several control experiments were performed. In the first control, a negative potential was applied to the reagent emitter without any solution being infused, while the PEG 3.8 K solution was electrosprayed from the positive, analyte emitter (Figure 3a). The resulting mass spectrum exhibited a similar CSD as the direct infusion of PEG 3.8 K (Figure 2a). The average charge state was 4.8+, further confirming that the presence of negative potential in the ion source does not significantly affect the charge state distribution. A slight change in the average charge state may be attributed to changes in ion sampling, where the higher charged cations are sampled less efficiently with the presence of an additional electrically attractive element in the ion source. Next, a neat solution of methanol was utilized in a bipolar dual spray

experiment with PEG 3.8 K (Figure 3b). The resulting mass spectrum exhibited a similar charge state distribution as the direct infusion of PEG 3.8 K (Figure 2a) and the previous dual spray control, where $[M+6NH_4]^{6+}$ to $[M+2NH_4]^{2+}$ were observed. The average charge state was calculated to be 4.7+ and ultimately demonstrated that methanol was not a major contributor to charge reduction in the previous experiments.

Two different solution-type reagents (i.e., ionic salt, acidic) were utilized to test and observe the effects on the CSD in the context of a bipolar dual spray experiment. In Figure 4a, the PEG 3.8 K analyte electrospray was fused with droplets from negative electrospray of 1% formic acid in methanol. The CSD of PEG cations shifted to predominantly $[M+3NH_4]^{3+}$ and $[M+2NH_4]^{2+}$, again with the emergence of singly ammoniated PEG cations. Similar to the 1% ammonium hydroxide results (Figure 2b), the average charge state of PEG was shifted from 5.0+ to 2.7+ and had the presence of neutral ammonium formate adducts. Next, 1% LiCl in methanol was subjected to droplet fusion with the PEG 3.8 K analyte, and the average PEG charge state shifted from an average of 5.0+ to 2.6+ (Figure 4b). The charge-reduced mass spectrum using 1% LiCl showed similar adducts as the previous dual spray experiments (i.e., neutral ammonium formate), which further suggested that these adducts were arising from the presence of ammonium
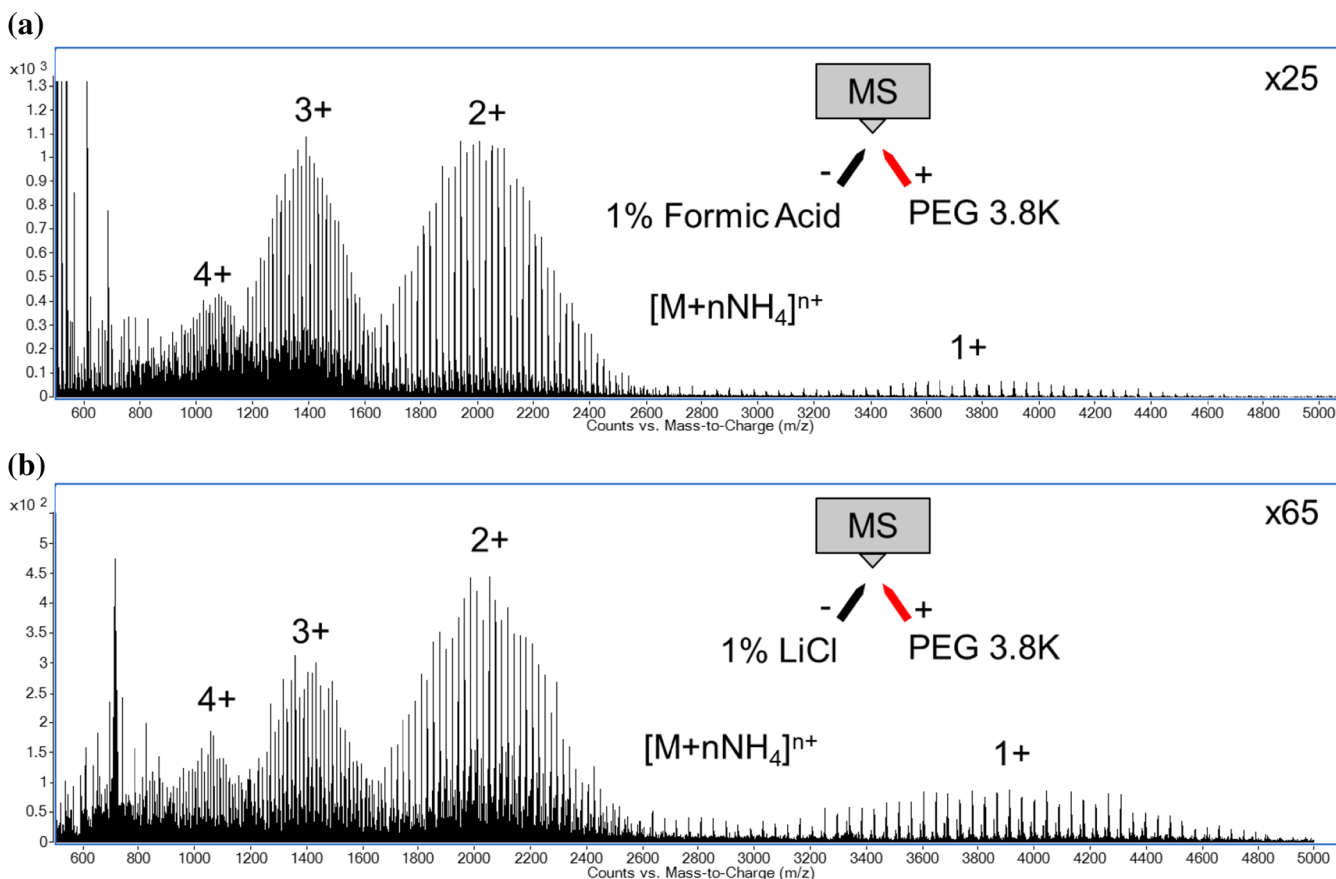

(a)

(b)

Figure 4. Dual spray of (**a**) positive mode direct infusion of PEG 3.8 K and negative mode direct infusion of 1% formic acid in methanol, (**b**) positive mode direct infusion of PEG 3.8 K and negative mode direct infusion of 1% LiCl in methanol. The × 25 and × 65 represent the y-axis scale compared with Figure 2a

formate in the analyte solution. In addition to the neutral ammonium formate adducts, charged adducts of Li^+ (e.g., $[M+Li]^+$ and $[M+2Li]^{2+}$) were observed in the mass spectrum (S2). This observation further demonstrates that oppositely charged droplets are fusing, where Li^+ ions from the negative emitter (initially as Li_xCl_{x+1} clusters) are in competition with ammonium cations and eventually adduct to the PEG molecules. When comparing the ionic salt and acidic solution results with the basic solution results, the similar ability and extent of charge reduction suggested that the initial pH of the reagent solution did not influence the charge state distribution. These

observations are distinctly different from acid-induced unfolding of biological polymers via monopolar microdroplet fusion chemistry, where pH, ion composition, and subsequent conformational changes in cytochrome c led to increases in charge state [25].

To further demonstrate the role of anions in the charge reduction process, an organic base without an anionic counter ion was investigated. In this set of experiments, toluene was utilized as the solvent due to the aprotic characteristic and inability to deprotonate in the presence of a stronger weak base. Bipolar dual spray of PEG 3.8 K and neat toluene exhibited an
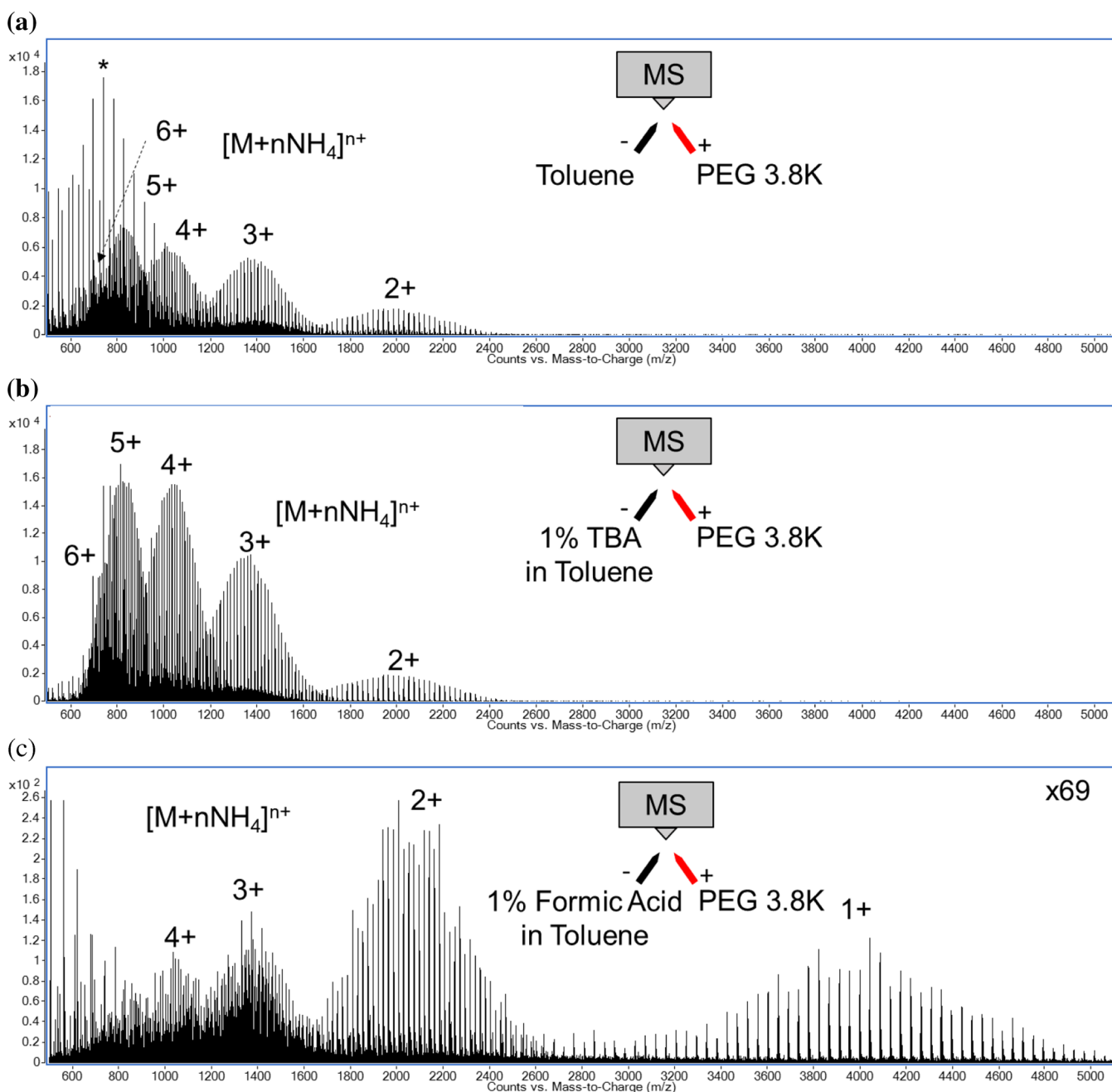

Figure 5. Dual spray of **(a)** positive mode direct infusion of PEG 3.8 K and negative mode direct infusion of neat toluene, **(b)** positive mode direct infusion of PEG 3.8 K and negative mode direct infusion of 1% tetrabutylamine in toluene, **(c)** positive mode direct infusion of PEG 3.8 K and negative mode direct infusion of 1% formic acid in toluene

average charge state of 4.3+ (Figure 5a), which was lower than the PEG 3.8 K control and methanol dual spray experiment, 5.0+ and 4.7+, respectively. The decreased charge state in this control is likely attributed to low-level impurities in toluene that can generate anions upon ionization (i.e., the label indicates 99.9% purity with water and sulfur impurities). Nevertheless, a 1% tributylamine in toluene solution was subjected to bipolar dual spray with the PEG 3.8 K analyte (Figure 5b). The average charge state following dual spray experiments was determined to be 4.4+, exhibiting little difference to the toluene control. This observation demonstrates that bases do not have a role in the charge reduction of cations in the context of bipolar dual spray experiment and further supports the role of anions. Next, 1% formic acid in toluene was subjected to droplet fusion with PEG 3.8 K; whereupon, the average charge state was shifted to 2.5+ (Figure 5c). Overall, these experiments and observations demonstrate the important role of anions in the charge reduction process of positively charged ions.

Based on these observations along with the controls, it is hypothesized that the reagent anions generated in the negative electrospray emitter are undergoing ion-pairing interactions with ammonium cations and/or ammoniated PEG cations in the fused electrospray droplets and as a result are responsible for the decrease in observed charge state. Several studies have demonstrated the ability of anionic additives,

whether through dual spray or single electrospray, to interact with analyte cations while in droplets to manipulate charge or covalently modify upon activation and rearrangement in the gas-phase [10, 27, 28]. With the introduction of excess anion downstream in the electrospray process (i.e., dual spray experiment) and subsequent ion-pairing, two potential mechanisms may be occurring in this study. First, reagent anions interact with an ammoniated PEG cation, where dissociation of the ion pair from the PEG analyte occurs during desolvation or in the gas phase. Cotham, Shaw, and Brodbelt demonstrated the ability to generate stable ion pairs (i.e., non-covalent) between sulfonates and protonated basic amino acids that survived into the gas phase using dual spray reactions [27]. In our experiments, the reagents were not highly polarizable (i.e., sulfonate groups) and the PEG analytes did not contain basic sites to anchor the anion; therefore, it was not expected to observe stabilized ion pairs in our experiments. Since no stabilized ion pairs between reagent anions and ammoniated PEG cations were detected in these experiments, the underlying mechanism for this system remains ambiguous. In contrast to the mechanism proposed above, one could also hypothesize an interaction between reagent anions and free ammonium cations in the fused droplet, resulting in a competition between PEG 3.8 K and reagent anions for the ammonium cations.
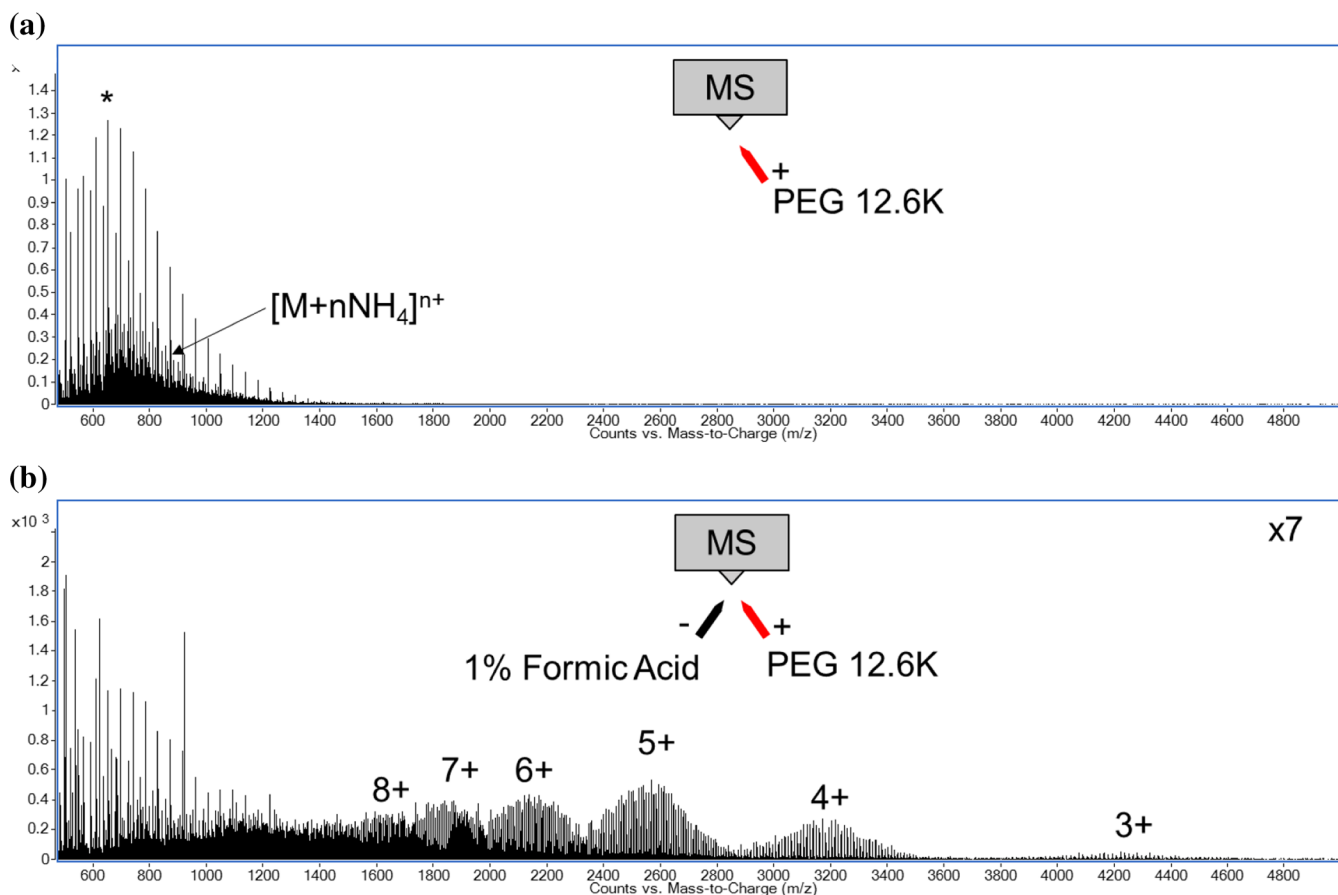

Figure 6. (a) Positive mode direct infusion of PEG 12.6 K. (b) Dual spray microdroplet fusion of positive mode direct infusion of PEG 12.6 K and negative mode direct infusion of 1% formic acid in methanol

In addition to PEG 3.8 K, a higher molecular weight PEG molecule was subjected to charge reduction via bipolar dual spray to demonstrate the drastic shift in charge state. In Figure 6a, the mass spectrum resulting from the positive mode electrospray of PEG 12.6 K is illustrated. A complicated mass spectrum is observed ranging from m/z 500–1200, and individual charge states cannot be discerned like PEG 3.8 K. Bipolar dual spray experiments between PEG 12.6 K and 1% formic acid exhibited a decrease in observed PEG charge state, where ammoniated PEG cations ranging from 8+ to 3+ were easily determined. While the PEG 3.8 K experiments were able to demonstrate the production of one new charge state (i.e., 1+), charge reduction of PEG 12.6 K was able to generate several new charge states not observed in the control and overall generated a further extent of reduction.

Conclusions

Microdroplet fusion chemistry via bipolar dual spray has demonstrated the ability to reduce the average charge state of PEG cations using anionic reagents. Bipolar dual spray was implemented on a commercial mass spectrometer with limited hardware modifications to the ion source. Reagents including ammonium hydroxide, formic acid, and lithium chloride showed dramatic shifts in the PEG 3.8 K average charge state, e.g., 5.0+ to 2.5+. An organic base, tributylamine, had no effect on the charge state distribution of PEG 3.8 K. These results were consistent with an ion-pairing mechanism, where reagent anions destabilized ammonium cation interactions with PEG 3.8 K upon droplet fusion from the negative and positive emitters. This study provides insight into the nature of dual spray chemistry and will aid future experimental design in microdroplet covalent chemistry.

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