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# Supercharging in electrospray ionization: effects on signal and charge

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**Abstract**

Multiply charged ions are ideally suited to tandem mass spectrometry, where fragmentation efficiency and pathways are typically a strong function of charge. Addition of either of two compounds, *m*-nitrobenzyl alcohol (*m*-NBA) or glycerol, to electrospray solutions results in an increase in the number of charges that can be added to gas-phase protein cations. Overall electrospray ionization (ESI) signal is not adversely affected by adding these compounds. Thus, these charge enhancers work by increasing the absolute number of higher charge state ions. This ability to enhance charge appears to be related to the high surface tensions of these compounds. Electrospray droplets consisting of solvents with higher surface tension require additional charging at the droplet surface in order to undergo Rayleigh fission. During the ion formation process, the higher density of charge at the droplet surface translates into higher charge states of the gas-phase analyte ion. Addition of *m*-NBA also enhances formation of high charge states of negative ions. For the synthetic polymer, poly(ethylene glycol) (PEG), addition of *m*-NBA results in an increase in the charge states by increasing the cationization of the polymer. In contrast, addition of glycerol results in a decrease in the charge states, presumably because it competes for sodium ions due to its high sodium affinity. Addition of 1–20% *m*-NBA to electrospray solutions can enhance the formation of higher charge state ions with no reduction in overall electrospray signal for a wide variety of analyte ions. This appears to be an ideal compound for enhancing high charge states for MS/MS and other experiments for which high charge states are desired. (Int J Mass Spectrom 219 (2002) 63–72)


*Keywords:* Mass spectrometry; Electrospray ionization; *m*-NBA; Supercharging

## 1. Introduction

Mass spectrometry is an indispensable method for making high accuracy molecular weight measurements of biological macromolecules [1], non-covalent complexes [2], and synthetic polymers [3]. Tandem mass spectrometry (MS/MS) of biomolecules can provide information about location and identification of post-translational modifications [4–7], and sequence [8]. For synthetic polymers, MS/MS can provide information about the starting and end group identity [9], and can be used to differentiate between linear and cyclic structures [10]. For these types of measurements, electrospray ionization (ESI) has the advantage that multiply charged ions of large molecules are typically produced. This enables virtually any type of mass spectrometer to be used for measuring the molecular weight of large molecules. The multiply charged ions produced by ESI can be more readily dissociated than singly charged ions, making them ideal for MS/MS experiments [11]. The physical properties of ions are often strongly dependent

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on charge state. This includes the proton transfer [12] and ion–ion reactivity [13], and fragmentation pathways and efficiency [14–16]. For example, electron capture cross-sections in electron capture dissociation (ECD) increase quadratically with charge [16], making ECD much more efficient with increasing charge state. ECD of more highly charged ions typically provides more extensive fragmentation from which more sequence information can be obtained [16].

The number of charges on gas-phase ions formed by ESI depends on several factors, including analyte conformation in solution [17–23], competition for charge between the analyte and other species [24–27], instrumental factors [28,29], etc. For example, large biomolecules that are denatured in solution carry away more charges in ESI than when they have more compact solution-phase conformations. Because of this, ESI can be used to monitor the pH-[17,18], heat-[19], and solvent-induced [20,21] denaturation of proteins, and to assess the relative conformational stability of homologous proteins [22,23]. There are many methods that can remove charge from ions formed by electrospray. For example, addition of compounds with high gas-phase basicities, either as liquids added into electrospray solutions [24–26] or as gases introduced into the electrospray interface [26,27], shifts the charge state distribution of protonated peptide and protein ions towards lower charge (higher *m/z*) due to proton transfer, with the degree of charge reduction correlating with the gas-phase basicity of the additive [24–26]. Ion–ion reactions, pioneered by McLuckey and his co-workers [30], can reduce highly charged ions down to a single (or even no) charge. Similarly, ion–electron interactions reduce overall charge [16,31]. Analyte charge reduction can also be promoted by increasing the energy of collisions in the electrospray interface [28], and by prolonging the time ions spend in high-pressure regions of the electrospray interface/mass spectrometer, e.g., an octupole or hexapole used for external ion accumulation prior to injection into a FT-ICR cell [29].

The production of highly charged gas-phase analyte ions is promoted by using conditions in which the analyte is in an elongated conformation in solution, using solvents that are minimally competitive for charge, and minimizing the number and energy of collisions experienced by analyte ions as they are transferred into the low-pressure region of the mass spectrometer. Although there are several methods for reducing the charge of ions formed by ESI [16,24–31], few methods for further promoting the formation of highly charged analyte ions have been developed. Multiply protonated even-electron ions ($MH_n^{n+}$) of peptides and proteins can be further ionized in the gas-phase to radical cations ($MH_n{}^{(n+1)+\bullet}$) via electron impact [32] and high-energy collisions with molecular oxygen [33]. We recently reported on several solvents [26,34] that when added into electrospray solutions, cause the charge state distributions of proteins and peptides to dramatically shift towards higher charge, with the most dramatic enhancement obtained with glycerol and *m*-nitrobenzyl alcohol (*m*-NBA) [34]. Here, we evaluate the effect of these two charge-enhancing compounds on overall ESI signal and their applicability to enhancing charge on a synthetic polymer and on negative ions.

## 2. Experimental

Experiments are performed on a quadrupole mass spectrometer with an in-house built electrospray source. This instrument is described elsewhere [35]. Ions are generated by nanoelectrospray using 1.0 mm o.d./0.78 mm i.d. borosilicate capillaries that are pulled to a tip with an i.d. of ∼4 μm using a Flaming/Brown micropipette puller (Model P-87, Sutter Instruments, Novato, CA). The electrospray is initiated by applying a potential of ∼1000 V to a Pt wire (0.127 mm diameter, Aldrich, Milwaukee, WI) which is inserted into the nanoelectrospray needle to within ∼2 mm of the tip. The wire and nanoelectrospray needle are held in place with a patch clamp holder (WPI Instruments, Sarasota, FL). The solution flow rates are between 60 and 200 nL/min. The ions generated by electrospray are sampled from atmospheric pressure through a 12 cm long stainless steel capillary (0.50 mm i.d.) which is heated to 195 °C. This

temperature is higher than that of the gas which passes through the capillary. The voltages on the heated metal capillary, the first and second skimmers are 12, 15, and 7 V, respectively.

Equine cytochrome *c* (>95%), and insulin chain A (oxidized) were purchased from Sigma (St. Louis, MO) and were used without further purification. Solutions of cytochrome *c* were prepared with analyte concentrations of ∼10 μM. The reported solution compositions are on a v/v basis. All cytochrome *c*-containing electrospray solutions used in this study contain 3% acetic acid. Addition of small amounts of acid to water/methanol solutions results in higher charge states due to denaturation of proteins in solution [17]. Insulin chain A (oxidized)-containing electrospray solutions contained 3% diethylamine to improve the electrospray signal for negative ions [36]. Electrospray solutions of PEG 1500 were prepared with analyte concentrations of ∼0.1% w/v, and contained 28 mM sodium acetate to aid cationization of PEG. Methanol (99.99%) was obtained from EM Science (Gibbstown, NJ). Sodium acetate monohydrate and poly(ethylene glycol) (PEG) with an average molecular weight of 1500 Da were obtained from Aldrich (Milwaukee, WI).

Surface tension measurements were performed using a Krüss (Hamburg, Germany) Drop Shape Analysis System, Model DSA10, with 30 mL glass sample tubes. The instrument was controlled using DropImage software (Finn Knut Hansen, University of Oslo, Norway), using sessile droplet mode. Water was used for surface tension calibration.

The abundances of the charge states are reported relative to the most abundant charge state in the mass spectrum. One parameter used to describe a given charge state distribution is the average charge state ($q_{\text{average}}$). This parameter is computed as follows:

$$q_{\text{average}} = \frac{\sum_i^N q_i w_i}{\sum_i^N w_i}$$

where $N$ is the number of observed analyte charge states in a given mass spectrum, $q_i$ the net charge of the $i$th charge state, and $w_i$ is the signal intensity of the $i$th charge state. For the case of PEGs, which are polydisperse, $w_i$ is the sum of the intensities of the various PEG oligomers of the $i$th charge state. The average molecular weight of PEG was calculated as a weighted average, with the molecular weight of each oligomer weighted by its mass spectral abundance.

## 3. Results and discussion

### 3.1. ESI signal and charge enhancement

The addition of either glycerol or *m*-NBA, even at small levels for the latter, to "denaturing" solutions containing cytochrome *c* (47% water/50% methanol/3% acetic acid) results in large shifts in the ESI mass spectral charge state distributions to higher charge state. For example, adding 1% *m*-NBA to this solution results in an increase in the maximum charge state from 21+ to 24+ and an increase in the average charge state from 17.3+ to 20.8+ (Fig. 1c). Similarly, addition of 43% glycerol to water/acetic acid solutions produces a maximum and average charge state of 22+ and 18.3+, respectively (Fig. 1b). To determine how these charge-enhancing compounds effect overall ESI signal, replicate measurements were done both with and without these compounds added. For each measurement, a new nanoelectrospray tip was used. This was done to avoid any contamination of the electrospray tips and to avoid any damage to the needles upon changing solutions. Although both of these problems are eliminated, the use of a new tip for each measurement adds to the variability of signal with each tip, and this introduces some error.

To evaluate the effects of *m*-NBA on analyte signal, 10 spectra were collected for each of two solutions of cytochrome *c* ($10^{-5}$ M), the first of which contained 47% water/50% methanol/3% acetic acid, and the second of which contained 46.5% water/49.5% methanol/1% *m*-NBA/3% acetic acid (one representative spectrum of each shown in Fig. 1a and c, respectively). Similarly, to evaluate the effects of glycerol, 10 spectra were collected for each of two solutions of cytochrome *c* ($10^{-5}$ M), the first of which contained 47%

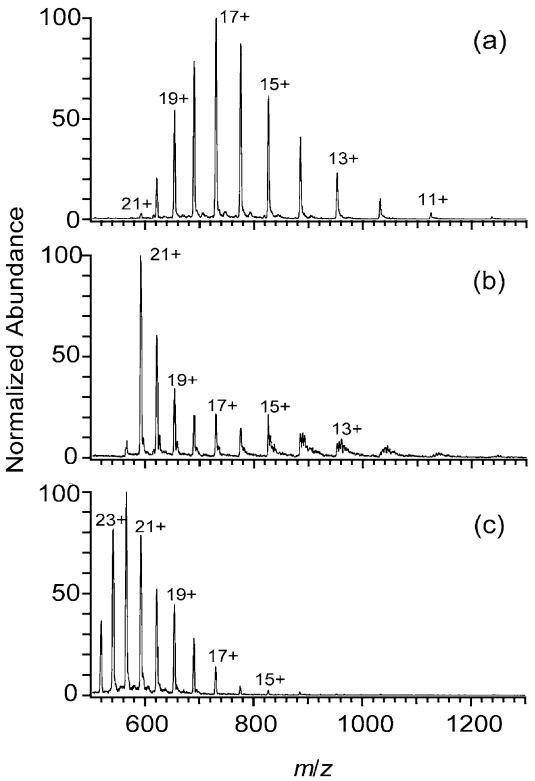


Fig. 1. Representative mass spectra from 10 replicate measurements of cytochrome *c* ($10^{-5}$ M) from (a) 47% water/50% methanol/3% acetic acid; (b) 43% glycerol/54% water/3% acetic acid; and (c) 46.5% water/49.5% methanol/1% *m*-NBA/3% acetic acid.

water/50% methanol/3% acetic acid, and the second of which contained 43% glycerol/54% water/3% acetic acid (one representative spectrum shown in Fig. 1b). Table 1 (*m*-NBA) and Table 2 (glycerol) list both the maximum and average charge states as well as the

Table 1
Effects of adding *m*-NBA to electrospray solutions of cytochrome *c* ($10^{-5}$ M)

| | 0% *m*-NBA | 1% *m*-NBA |
|---|---|---|
| Maximum charge state | 21(+) | 24(+) |
| Average charge state | 17.3 ± 0.2(+) | 20.8 ± 0.5(+) |
| Total analyte abundance | 58000 ± 14000 | 68000 ± 30000 |
| Analyte base peak abundance | 16000 ± 3000 | 15000 ± 7800 |

Base solution is 47% water/50% methanol/3% acetic acid. Errors correspond to one standard deviation from 10 replicate measurements.

Table 2
Effects of adding glycerol to electrospray solutions of cytochrome *c* ($10^{-5}$ M)

| | 0% Glycerol | 43% Glycerol |
|---|---|---|
| Maximum charge state | 21(+) | 22(+) |
| Average charge state | 16.4 ± 0.1(+) | 18.3 ± 0.3(+) |
| Total analyte abundance | 75000 ± 29000 | 38800 ± 13000 |
| Analyte base peak abundance | 15000 ± 5000 | 10000 ± 4000 |

For "0% glycerol," the solution matrix is 47% water/50% methanol/3% acetic acid, and for "43% glycerol" the solution matrix is 43% glycerol/54% water/3% acetic acid. Errors correspond to one standard deviation from 10 replicate measurements.

standard deviations for the signal intensity resulting from these replicate measurements. For both *m*-NBA and glycerol, a clear and reproducible enhancement in both the maximum and average charge is obtained. However, there are clearly large fluctuations in overall analyte signal. Addition of 1% *m*-NBA does not change the base peak abundance or total analyte signal at the 95% confidence level. Addition of 43% glycerol does appear to reduce the analyte signal at the 95% confidence level. However, the large fluctuation in signal likely includes factors that are not random, complicating the comparison of signals. For example, both the signal and standard deviation for the same solution (0% glycerol, Table 2, and 0% *m*-NBA, Table 1) measured on two different days, are significantly different. We attribute this primarily to subtle changes in the pulled electrospray tips. Over the course of many such experiments, it appears that the differences in analyte signal induced by glycerol are not very significant. This shows that the charge enhancement comes about due to more charges available to the ion, not solely by the reduction of lower charge states. Thus, addition of either of these charge-enhancing compounds results in an increase in the absolute abundances of higher charge states.

It is interesting to note that the spectra of cytochrome *c* generated from a glycerol-containing solution (Fig. 1b) have a slight, but reproducible bimodal distribution of charge states. The dominant distribution is centered on the 21+ charge state, and a very minor distribution is centered on the 15+ charge

state. Polyols, such as glycerol, are known to stabilize folded conformations of proteins [37], which give rise to charge state distributions centered at lower charge (higher *m/z*) than unfolded conformations [17,38]. Thus, the occurrence of bimodal charge distributions may be a consequence of glycerol stabilizing folded states of the protein to some extent.

### 3.2. Anions

Negative ion ESI spectra of insulin chain A (oxidized) (2.5 kDa) from base solutions containing 47% water/50% methanol/3% diethylamine with 0–4.5% *m*-NBA and 0–10% glycerol are shown in Figs. 2 and 3, respectively. With no glycerol or *m*-NBA, the base peak is $(M - 5H)^{5-}$ (Figs. 2a and 3a). With 4.5% *m*-NBA, the base peak increases to $(M - 6H)^{6-}$, and the average charge state increases from 5.03− to

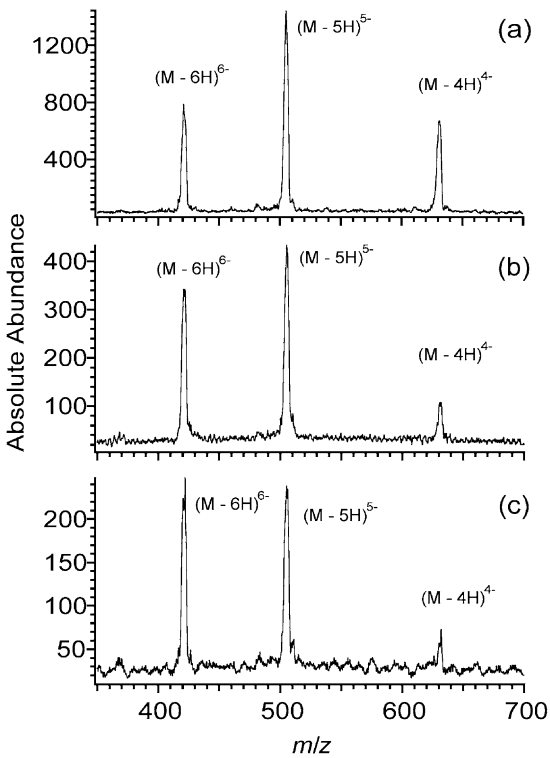


Fig. 2. Negative ion electrospray ionization mass spectra of insulin chain A (oxidized), from 47% water/50% methanol/3% diethylamine solutions with (a) 0%; (b) 2.5%; and (c) 4.5% *m*-NBA.

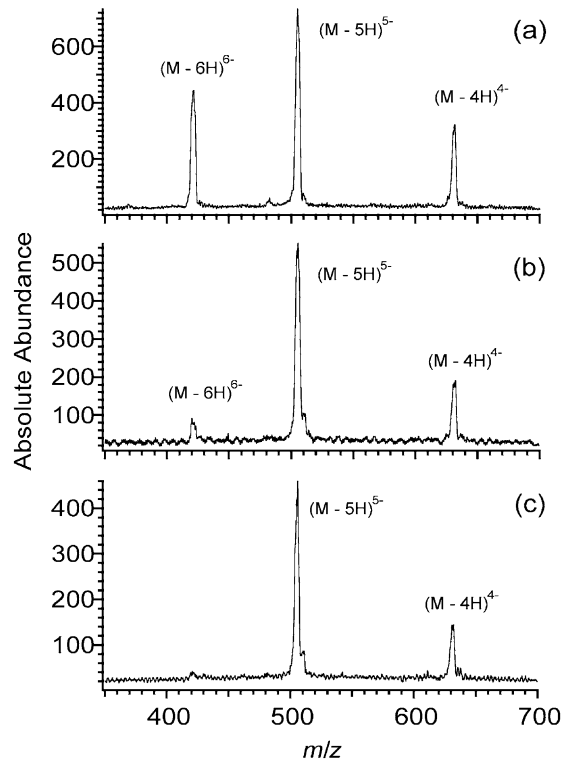


Fig. 3. Negative ion electrospray ionization mass spectra of insulin chain A (oxidized), from 47% water/50% methanol/3% diethylamine solutions with (a) 0%; (b) 1%; and (c) 10% glycerol.

5.32− (the standard deviation in average charge state for three replicate measurements of insulin chain A (oxidized) electrosprayed from the solution with 0% *m*-NBA is 0.07). Although the average charge state increases with *m*-NBA, the total analyte signal is lower. The reason for this is under further investigation. Unlike with *m*-NBA, addition of glycerol reduces the average charge state (Fig. 3). With 1% glycerol, the average charge state decreases from 5.03− to 4.90−. It is possible that glycerol stabilizes a more compact structure of this peptide, causing the observed shift towards lower charge.

### 3.3. Synthetic polymer

To determine if the charge-enhancing properties of the additives are also observed for polymers that are

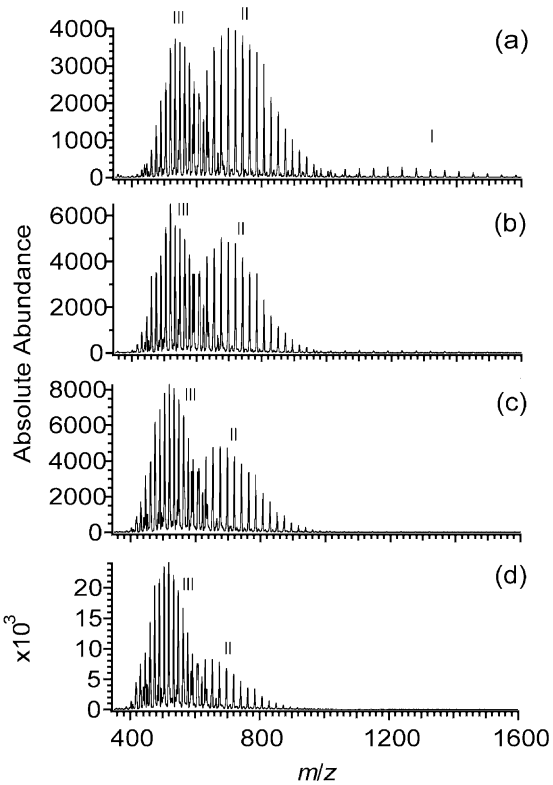


Fig. 4. Electrospray mass spectra of PEG 1500 (0.1%) from methanol solutions with 28 mM sodium acetate containing (a) 0%; (b) 1%; (c) 5%; and (d) 20% *m*-NBA: $(M+Na)^+$ (I), $(M+2Na)^{2+}$ (II), and $(M+3Na)^{3+}$ (III).

typically cationized with alkali metal ions, a methanol solution of PEG 1500 (0.1% w/v) containing 28 mM sodium acetate was prepared. The resulting ESI mass spectrum contains three charge states: $(M + Na)^+$, $(M + 2Na)^{2+}$, and $(M + 3Na)^{3+}$ (Fig. 4a). Adding up to 20% *m*-NBA results in a clear shift in the ESI signal towards higher charge state (Fig. 4b–d). With 20% *m*-NBA, the relative abundance of $(M + Na)^+$ decreases from 7% without *m*-NBA to <1% (below the detection limit). Similarly, the relative abundance of $(M + 2Na)^{2+}$ decreases from 100 to 43%. The highest charge state $(M + 3Na)^{3+}$, increases from 93 to 100%. In addition to charge enhancement, the addition of *m*-NBA at levels as high as 20% also results in an increase in overall analyte signal. Adding *m*-NBA at levels above 20% (50 and 90%) did not produce further enhancements in charge or analyte signal.

Table 3
Effects of adding *m*-NBA to methanol solutions of PEG 1500 (0.1%) on the absolute ion abundances of PEG charge states

| | 0% *m*-NBA | 20% *m*-NBA |
|---|---|---|
| $(M + Na)^+$ | 5700 ± 1900 | <50 |
| $(M + 2Na)^{2+}$ | 39700 ± 5800 | 20800 ± 5400 |
| $(M + 3Na)^{3+}$ | 38700 ± 10800 | 80500 ± 21300 |
| Total analyte abundance | 84100 ± 15000 | 101300 ± 25000 |

For "0% *m*-NBA," the solution matrix is methanol, and for "20% *m*-NBA," the solution matrix is 80% methanol/20% *m*-NBA. Both solutions contain 28 mM sodium acetate. Errors correspond to one standard deviation from three replicate measurements.

To evaluate the effects of *m*-NBA on the absolute abundances of the different charge states, three replicate measurements were performed for each of two of the above PEG 1500 solutions: 0 and 20% *m*-NBA. With 20% *m*-NBA, the absolute abundance of $(M + 3Na)^{3+}$ is more than doubled, the abundance of $(M + 2Na)^{2+}$ is reduced almost by half, and the abundance of $(M + Na)^+$ is reduced to a value below our detection limit (Table 3). The total analyte ion signal is slightly higher, but not significantly. Because the total analyte abundance is approximately the same with 20% *m*-NBA added, the decrease in the abundances of the lower charge states $(M + 2Na)^{2+}$ and $(M + Na)^+$, is translated into an increase in the abundance of the highest observed charge state $(M + 3Na)^{3+}$.

Another effect of adding *m*-NBA is that the mass spectral molecular weight distributions calculated for these spectra shift slightly, but reproducibly, towards lower molecular weight (Table 4). Without *m*-NBA,

Table 4
Effects of adding *m*-NBA to electrospray solutions of PEG 1500 (0.1%) from methanol solutions with 28 mM sodium acetate on the average molecular weights of PEG calculated from different charge states

| | 0% *m*-NBA (Da) | 20% *m*-NBA (Da) |
|---|---|---|
| $(M + Na)^+$ | 1106 ± 10 | N/A |
| $(M + 2Na)^{2+}$ | 1433 ± 38 | 1263 ± 3 |
| $(M + 3Na)^{3+}$ | 1594 ± 3 | 1498 ± 10 |

Errors correspond to one standard deviation from three replicate measurements.

the calculated average molecular weight depends on charge state, with the value calculated from the abundance of $(M + 3Na)^{3+}$ greater than that for $(M + 2Na)^{2+}$. This is presumably due to the greater ability of the larger polymer ions to solvate and stabilize charge. The same is true for the 20% *m*-NBA solutions, but the calculated average molecular weight, averaged over both the 3+ and 2+ ions, is shifted to *lower mass*. This indicates that the lower molecular weight species can be preferentially detected with the increased charging provided by *m*-NBA. The dependence of the observed average molecular weight of fluorinated polymers on electrospray solvent composition has been noted previously by Cole and co-workers [39]. This observation indicates that solution composition, as well as instrumental factors [40], need to be taken into account when developing methods for characterizing polydisperse samples by ESI-MS.

In contrast to the results obtained with *m*-NBA, addition of glycerol into methanol electrospray solutions of PEG 1500 results in a reduction in charge (Fig. 5). The most likely explanation for this result is that glycerol likely has a high sodium affinity and that glycerol is competing with PEG for the sodium ions, resulting in a reduction in cationization of PEG.

The ESI spectrum of PEG 1500 from aqueous solutions results in even higher average charge than obtained from methanol solutions (Fig. 6a). The addition of *m*-NBA into aqueous solutions of PEG results in a slight reduction in charge (Fig. 6b). This result provides some insight into the mechanism for the charge enhancement typically observed for these compounds.

### 3.4. Mechanism of supercharging

The results for PEG presented here, and other results,[1] strongly indicate that the charge-enhancing ability of these compounds is directly related to their high surface tensions. The surface tensions of water, *m*-NBA, glycerol, acetic acid, and methanol are 72, 50 ± 5, 63, 27, and 22 mN/m, respectively.[2] During the ESI process, droplets undergo Rayleigh fission [41]. In order for this to occur, a higher surface charge density is required for droplets consisting of higher surface tension solvents. During ion formation, this higher charge density translates into higher charge state gas-phase analyte ions being formed. Water has the highest surface tension of any of these solvents, so one might expect that the highest charge states should be produced out of pure aqueous solutions. This is in fact what is observed for PEG 1500. Addition of

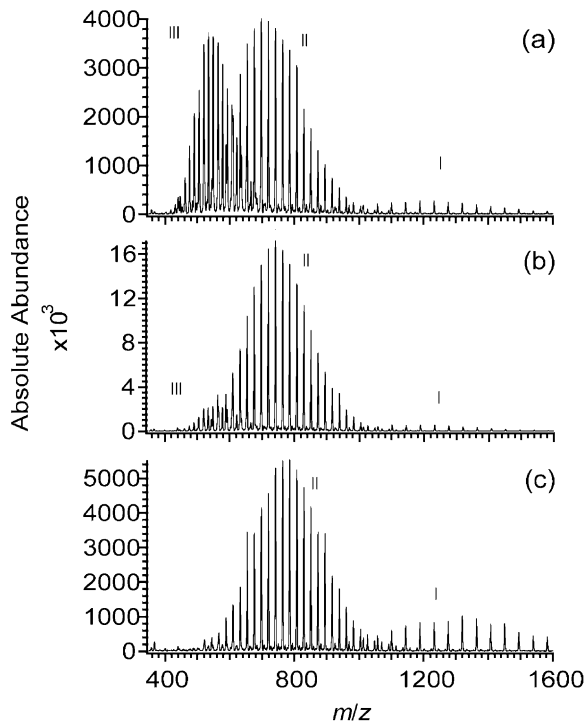


Fig. 5. Electrospray mass spectra of PEG 1500 from methanol solutions with 28 mM sodium acetate containing (a) 0%; (b) 1%; and (c) 10% glycerol: $(M + Na)^{+}$ (I), $(M + 2Na)^{2+}$ (II), and $(M + 3Na)^{3+}$ (III).

[1] Unpublished results.

[2] The values for the surface tensions of water, glycerol, acetic acid, and methanol are from T.E. Daubert, R.P. Danner, Physical and Thermodynamic Properties of Pure Chemicals Data Compilation, Hemisphere Publishing Group, New York, 1989. The value reported for *m*-NBA is for 98% pure material and was measured by A.T.I. at the UC Berkeley Department of Chemical Engineering as described in the Section 2.

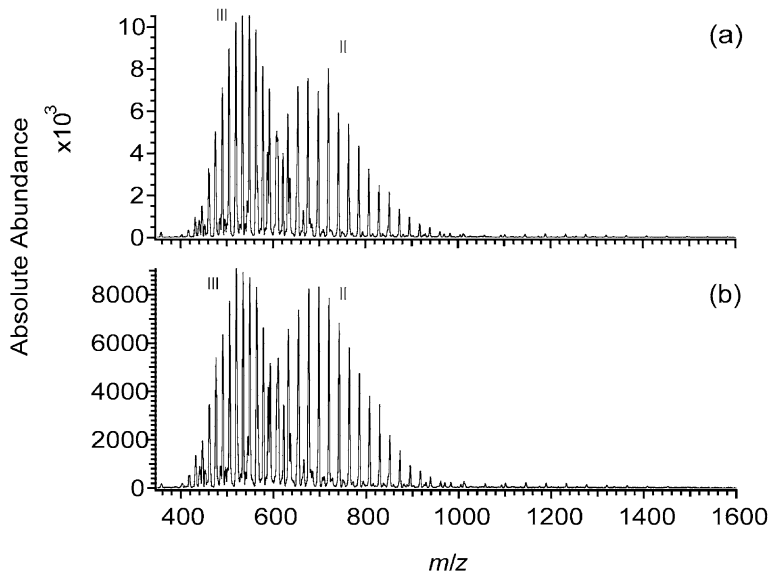


Fig. 6. Electrospray mass spectra of PEG 1500 from aqueous solutions with 28 mM sodium acetate containing (a) 0% and (b) 1% *m*-NBA: $(M + Na)^{+}$ (I), $(M + 2Na)^{2+}$ (II), and $(M + 3Na)^{3+}$ (III).

*m*-NBA to aqueous solutions actually reduces the overall charge (Fig. 6). This does not occur, however, for proteins. Proteins in pure aqueous solution typically adopt compact conformations. These compact structures typically carry away fewer charges than elongated structures due to their smaller cross-sections, which expose the protein to fewer charges for a given surface charge density [42,43]. Acetic acid is typically added to electrospray solutions to denature a protein. This results in more highly charged ions being produced despite the fact that acetic acid has a lower surface tension than water. Addition of *m*-NBA or glycerol to these solutions can enhance the charging because they increase the surface tension of the droplet. Glycerol, acetic acid, and *m*-NBA all have lower vapor pressures than water and methanol, so the concentrations of these compounds are expected to be enhanced during solvent evaporation from the droplet.

Models [42,43] have been proposed to account for the charging of macromolecules based on the molecular size and charge density on the electrospray droplet surface. Although our results agree qualitatively with these models, it is very difficult to make a meaningful quantitative comparison, due to the uncertainty in the solvent composition and temperature of the electrospray droplet at the moment of ion formation. Both of these factors affect the surface tension and hence the charge density at the droplet surface. Another uncertainty is the analyte conformation. Future studies into the mechanism of charge enhancement will utilize systems in which solution composition and analyte conformation are fixed. This should make possible quantitative evaluation of these models.

## 4. Conclusions

Addition of either *m*-NBA or glycerol to electrospray solutions of proteins results in a significant increase in the number of charges on the gas-phase analyte ions. Addition of these compounds at levels necessary to significantly enhance charge does not measurably affect the ESI signal. Thus, addition of these compounds produces an increase in the absolute number of ions with higher charge states.

The charge-enhancing abilities of these two compounds appear to be due to their high surface tensions. Droplets that have higher surface tensions require more charges to undergo Rayleigh fission. During the process of ion formation, the higher charge density on the surface of the droplet translates into higher charge states of the analyte being formed.

For the synthetic polymer, PEG, addition of *m*-NBA also enhances the formation of higher charge states (except from aqueous solutions) corresponding to sodium cationized molecular ions. In contrast, addition of glycerol results in a decrease in cationization, presumably due to its high sodium affinity, which results in glycerol competing for the cations.

Being able to generate higher charge state ions by ESI is important for MS/MS studies in which dissociation efficiency and information from fragmentation often increases with increasing charge. For all the work done to date, it appears that *m*-NBA is an ideal charge-enhancing compound. Addition of *m*-NBA to electrospray solutions at levels between 1 and 20% can produce significant charge enhancement and it does not adversely affect the overall electrospray signal.

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