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Desalting Protein Ions in Native Mass Spectrometry Using Supercharging Reagents

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1

Abstract

Effects of the supercharging reagents *m*-NBA and sulfolane on sodium ion adduction to protein ions formed using native mass spectrometry were investigated. There is extensive sodium adduction on protein ions formed by electrospray ionization from aqueous solutions containing millimolar concentrations of NaCl, which can lower sensitivity by distributing the signal of a given charge state over multiple adducted ions and can reduce mass measuring accuracy for large proteins and non-covalent complexes for which individual adducts cannot be resolved. The average number of sodium ions adducted to the most abundant ion formed from ten small (8.6-29 kDa) proteins for which adducts can be resolved is reduced by 58% or 80% on average, respectively, when 1.5% *m*-NBA or 2.5% sulfolane are added to aqueous solutions containing sodium compared to without the supercharging reagent. Sulfolane is more effective than *m*-NBA at reducing sodium ion adduction and at preserving non-covalent protein-ligand and protein-protein interactions. Desalting with 2.5% sulfolane enables detection of several glycosylated forms of 79.7 kDa holo-transferrin and NADH bound to the 146 kDa homotetramer LDH, which are otherwise unresolved due to peak broadening from extensive sodium adduction. Although sulfolane is more effective than *m*-NBA at protein ion desalting, *m*-NBA reduces salt clusters at high *m/z* and can increase the signal-to-noise ratios of protein ions by reducing chemical noise. Desalting is likely a result of these supercharging reagents binding sodium ions in solution, thereby reducing the sodium available to adduct to protein ions.

Introduction

The effects that different salts can have on protein stability have been known since the late 1800's, when Franz Hofmeister reported an ordering of cations and anions based on their

ability to salt in or salt out proteins,[1] and both non-specific and specific salt-protein interactions

can strongly influence protein structure and function.[1-8] Phosphate and Tris buffers as well as

sodium chloride are often added to aqueous protein solutions in order to stabilize native or

native-like protein structure by mimicking the environment inside the cell, which has an ionic

strength of ~150 mM.[9] Specific salts and other small molecules are essential in the enzyme-

cofactor or protein-ligand interactions in such varied and vital processes as electron transport,[10, 11]

ion pumping across cell membranes,[12, 13] and drug interactions.[14, 15]

Some protein structural methods, such as X-ray crystallography and NMR, are not

adversely affected by high salt concentrations, whereas high salt concentrations can be

detrimental to the performance of electrospray ionization (ESI) and to a lesser extent matrix-

assisted laser desorption ionization (MALDI) mass spectrometry (MS). Salts can increase

baseline noise due to the formation of ionic clusters and can cause ion suppression.[16-21] For

example, alcohol dehydrogenase tetramer ions formed by nanoelectrospray ionization (nESI)

from 50 mM ammonium acetate can be measured with excellent signal-to-noise ratios (S/N),

whereas the tetramer is undetectable from the same solution with 10 mM Tris or HEPES

buffers.[16] Similarly, addition of 10 mM CsCl to solutions of lysozyme in 1:1 methanol:water

decreases the total ion abundance for the protein by 130-fold.[17] Salt adduction to protein ions

distributes the protein signal over multiple adducted ions, reducing the S/N of each protein ion,

and broadens mass spectral peaks of large proteins and protein complexes for which individual

adducts cannot be resolved.[19, 21-25] Peak broadening decreases mass measurement accuracy for

these large proteins and can also inhibit the identification of covalent (glycosylations,

phosphorylation, or other post-translational modifications) or non-covalent (specific ion or

ligand binding) protein modifications.[23] McLuckey and coworkers found that the extent of

sodium ion adduction on a protein ion formed by ESI is related to the protein pI, solution pH, and charge state.[18, 26] There is more sodium ion adduction to low charge states,[18, 21, 25-27] but there is more adduction of trivalent metal ions to high charge states.[28]

To reduce the adverse effects of many salts on MS performance, salts are often removed by dialysis,[29, 30] ion exchange chromatography,[16, 31] or diafiltration[32-34] prior to analysis by MS, and a myriad of products for fast filtration and online chromatographic desalting of protein solutions have been developed. However, removing even low concentrations of salts can significantly change the structure of some proteins and protein complexes. For example, NtrC4 (a σ activator protein from *Aquifex aeolicus*) requires millimolar concentrations of certain salts, e.g., Mg^{2+}, BeF_3^-, and ADP, in order to assemble into an active hexamer.[35, 36] Several techniques for reducing sodium ion adduction to proteins in ESI have been developed that do not require removal of the salts from solution prior to ion formation. Buffer loading,[21, 22] in which high concentrations of a volatile buffer, typically ammonium acetate, is added to solution, reduces sodium ion adduction to proteins and reduces the number of salt ion clusters formed. The addition of 7 M ammonium acetate to aqueous solutions containing 20 mM NaCl results in an increase in the S/N of the most abundant ion of cytochrome *c* and ubiquitin by more than 6-fold and 11-fold, respectively, compared to solutions without the buffer added.[21] Buffer loading also works well for proteins that require high concentrations of salt to function or assemble, like concanavalin A.[22] Other ammonium buffer salts can also effectively reduce sodium ion adduction to proteins, and can do so at much lower concentrations. For example, 25 mM ammonium bromide added to an aqueous 1 mM NaCl solution containing ubiquitin decreases the average number of sodium ions adducted to the most abundant charge state of the protein from 6.0 to 0.4 and increases the S/N of this ion by a factor of 66.[25, 27] The ability of different salts to

desalt proteins in the ESI droplet is related to the proton affinities of the anions, where anions with low proton affinities lead to less sodium adduction.[27] However, anions with low proton affinities also tend to adduct to the protein as an acid molecule and form ion clusters,[27] which can decrease the protein ion signal. Konermann and coworkers suggested that some salts, such as citrate and tartrate, may chelate ions in solution, such as calcium, that can non-specifically adduct to proteins.[37]

Supercharging reagents can be used to produce high charge-state ions by ESI from both denaturing[38-45] and native solutions.[46-55] Supercharging reagents are high-boiling point compounds that are typically added in small amounts (1-2%) to sample solutions prior to ESI.[38-40, 45-55] For aqueous solutions, the low concentration of the supercharging reagents does not significantly affect the protein structure prior to ESI.[50-52] However, the concentration of supercharging reagent increases in the droplet as solvent evaporation occurs, and the native structure of the protein can be chemically/thermally destabilized in the electrospray droplet, resulting in partial or extensive loss of folded structure with a concomitant increase in the number of charges on the gaseous protein ions that are formed.[48-53] Protein-protein or protein-ligand complex dissociation may also occur as a result of this protein destabilization.[46, 48, 50, 51, 53] Supercharging reagents can also increase protein charging from denaturing solutions in which the protein is initially unfolded,[38-45] a result attributable to many factors, including the high surface tension of supercharging reagents compared to water-methanol-acid solutions.[40] Droplets with higher surface tension can support more charge, and this high charge density can result in the formation of high charge-state ions. Loo and coworkers showed that the presence of *m*-NBA or sulfolane in the spray solvent in desorption electrospray ionization (DESI) experiments performed on HPLC column effluents containing trifluoroacetic acid (TFA)

decreases TFA cluster ion intensity and increases protein S/N compared to when no

supercharging reagent is in the DESI solvent.[20] The authors suggested that this result is likely

due to the supercharging reagent binding to TFA anions, thereby inhibiting TFA cluster

formation, or preventing TFA dissociation into TFA anion and a free proton in solution, reducing

the amount of free TFA anion in solution.[20]

Here, we show that *m*-NBA and sulfolane, two of the most common supercharging

reagents, reduce sodium adduction to protein ions formed by nESI from native solutions.

Sulfolane is more effective than *m*-NBA at reducing sodium ion adduction while still preserving

non-covalent protein-protein and protein-ligand interactions. However, the use of *m*-NBA to

desalt protein ions can lead to up to a 7-fold increase in protein ion S/N as a result of fewer

clusters and lower chemical noise. This new method for desalting protein ions in nESI can

improve the mass measuring accuracy for large proteins and protein complexes and can be used

to resolve different glycoforms or ligand-adducted forms of proteins that are otherwise obscured

by peak broadening resulting from extensive sodium adduction. *m*-NBA and sulfolane bind to

sodium ions, suggesting that sodium sequestration by supercharging reagents may be the origin

of the desalting effect of these supercharging reagents.

Experimental

Experiments were performed using a Waters Quadrupole-Time-of-Flight (Q-TOF)

Premier (Waters, Milford, MA) mass spectrometer. Protein ions were formed by

nanoelectrospray from borosilicate capillaries (1.0 mm o.d./0.78 mm i.d., Sutter Instruments,

Novato, CA, USA) that were pulled to a tip i.d. of ~1 μm with a Flaming/Brown micropipette

puller (Model P-87, Sutter Instruments, Novato, CA, USA) and positioned ~2 mm from the

capillary inlet to the Q-TOF instrument. Nanoelectrospray was initiated by applying a potential

of about +1.0 kV to a 0.127 mm diameter platinum wire inserted into the capillary and in contact

with the sample solution. The nanoelectrospray potential was adjusted to optimize protein ion

S/N for each tip. Values of the average number of sodium ions adducted are from four replicate

measurements using four different nanospray emitters to account for tip-to-tip variation in

adduction levels. All comparative S/N measurements were made using the same

nanoelectrospray capillary to eliminate tip-to-tip variability, and the capillary was washed with

methanol and water between each solution. All proteins were purchased as lyophilized powders

from Sigma except for barnase and barstar, which were expressed in *E. coli* and purified as

described previously.[56] Ammonium bicarbonate, sodium chloride, platinum wire, and

supercharging reagents were obtained from Sigma (St. Louis, MO, USA).

Results and Discussion

      Effects of supercharging reagents on protein charge and sodium ion adduction. The

average charge of ubiquitin ions formed by nESI from 5 μM ubiquitin in 10 mM ammonium

bicarbonate and 1 mM sodium chloride (pH 7.8) is $5.33 \pm 0.01+$ (Figure 1a). There is extensive

sodium ion adduction to the 5+ - 7+ charge states, with more adduction on lower charge states.

The average number of sodium ions adducted to these charge states are 3.5 ± 0.1, 3.5 ± 0.1, and

2.0 ± 0.4, respectively (Table 1 and Table S-1). Mass spectra obtained from the same solution

with either 1.5% *m*-NBA or 2.5% sulfolane are shown in Figures 1b and 1c, respectively. The

average charge of ubiquitin formed from these respective solutions is $7.1 \pm 0.1+$ and $6.3 \pm 0.1+$.

The average number of sodium ions adducted to the 5+ - 7+ charge states is significantly lower

when either supercharging reagent is present. The average number of sodium ions adducted to

the most abundant charge state decreases from 3.5 ± 0.1 in the spectrum obtained without a reagent to 1.2 ± 0.2 with m-NBA and 0.44 ± 0.03 with sulfolane. m-NBA is not soluble in aqueous solution above ~1.5%, and at the concentration used for both supercharging reagents, there should be little effect on the protein structure in solution prior to droplet formation by nESI.[46, 51, 52] The presence of the 1 mM sodium chloride in these protein solutions has only a small effect on the charge of the protein ions formed with supercharging reagents. For example, the average charge of ubiquitin ions produced by nESI from solutions containing both added sodium chloride and m-NBA is only about one charge lower compared to that with the same amount of m-NBA but without sodium chloride added. The slightly lower charging with sodium chloride is likely due to stabilization of folded protein structure in the ESI droplet as a result of the higher ionic strength.

The same experiments were performed with nine other proteins for which sodium adducts could be resolved. The results are given in Table 1 for the most abundant charge state in each spectrum and in Table S-1 for every charge state. The presence of m-NBA or sulfolane in aqueous solutions containing sodium decreases the average number of sodium ions adducted to both the most abundant charge state and any given charge state in the mass spectra for *all* proteins. For these ten proteins, the number of sodium ions adducted to the most abundant charge state decreases by an average of 58% with m-NBA, and by 80% with sulfolane compared to solutions without supercharging reagents. Thus, adding small quantities of supercharging reagents is an effective way to desalt protein ions in aqueous solutions containing sodium and also increases the average charge state of protein ions formed from these native solutions. Sulfolane at 2.5% is more effective than 1.5% m-NBA at reducing sodium adduction to protein ions. The effectiveness of m-NBA and sulfolane at reducing sodium adduction to protein ions in

nESI is protein-dependent. However, the extent of adduct reduction with supercharging reagents

does not correlate well with any of several protein characteristics, such as protein size, pI, or

number of disulfide bonds (Table 1).

With 2.5% sulfolane, the average charge of the proteins increases by an average of 1.0+

from solutions containing no supercharging reagent, whereas with 1.5% *m*-NBA, the average

charge increases by 2.5+. This result is consistent with previous results that showed that

sulfolane, on a per volume basis, is a less effective supercharging reagent than *m*-NBA, and

therefore likely disrupts native protein structure to a lesser extent than *m*-NBA.[51]

The effectiveness of sulfolane at protein ion desalting without extensively perturbing

protein structure is important for desalting non-covalent protein-protein or protein-ligand

complexes. Nanoelectrospray of myoglobin (Figure 2, Table 1), which contains a non-

covalently bound heme group, from an aqueous solution with 10 mM ammonium bicarbonate

and 1 mM NaCl results in 100% holo-myoglobin ions. Only 48% of the ion population is holo-

myoglobin with 1.5% *m*-NBA, and the charge-state distribution is bimodal, indicating that some

of the myoglobin is unfolded in the nESI droplet. In contrast, holo-myoglobin is 83% of the ion

population observed with 2.5% sulfolane, and the monomodal charge-state distribution indicates

that there is less perturbation of the folded form of the protein in the nESI droplet.

Protein-protein complexes, such as the 22 kDa heterodimer barnase-barstar (Figure 3,

Table 2), are also preserved better with 2.5% sulfolane than with 1.5% *m*-NBA. A nESI mass

spectrum of a 6:5 molar ratio mixture of barstar and barnase in sodiated ammonium bicarbonate

solution shows the 8+ through 10+ charge-state ions of the barnase-barstar complex and ions of

barstar, which is the excess reagent (Figure 3a). Because the barnase-barstar complex has a very

low dissociation constant (25 ± 5 pM in aqueous ammonium bicarbonate solution, pH 7.2, 1%

glycerol[57]), nearly all of the barnase, the limiting reagent, is present in the complex, and ions of free barnase are not observed in the mass spectrum. The most abundant charge state of the complex is 9+, and there is an average of 4.0 sodium ions adducted. In a mass spectrum of the same solution containing 1.5% *m*-NBA (Figure 3b), the charge-state distribution is shifted to higher charge, and the appearance of barnase ions indicates that some solution-phase unfolding and partial dissociation of the complex occur in the nESI droplet. The most abundant charge state of the complex shifts to 11+ (an average of 0.4 sodium ions adducted), and only 38% of the complex remains intact. In comparison, the charge of the most abundant ion of the complex increases to only 10+, and 84% of the barnase-barstar complex remains intact in the mass spectrum of the same solution with sodium and 2.5% sulfolane (Figure 3c). The average number of sodium ions adducted to the most abundant charge state with sulfolane is 0.6, similar to that obtained from the *m*-NBA solution. The examples of myoglobin and barnase-barstar illustrate that using sulfolane as a desalting agent can be particularly useful in native nESI experiments where measurement of ions from the intact complex is desired, such as when complex stoichiometry or the presence of ligand binding is being determined, although caution in interpreting these data is necessary owing to destabilization and possible dissociation of the complexes in the nESI droplet.

Improving mass accuracy for large proteins and protein complexes. The charge-state distributions of large proteins and protein complexes in native ESI shift to higher *m/z* with increasing molecular weight.[58] The inability to resolve individual sodium ion adducts at high *m/z* can reduce mass measuring accuracy and obscure the presence of different populations of covalently and non-covalently modified forms of the protein. Robinson and coworkers showed that retention of solvent and buffer molecules by large proteins and protein complexes in ESI

results in measured masses much higher than calculated masses. For example, the measured

mass of the 685 kDa 20S proteasome lacking an α-subunit is ~7 kDa higher than the calculated

mass.[23]

A native nESI mass spectrum of human holo-transferrin (79.7 kDa) in 10 mM ammonium

bicarbonate with no sodium salt added is shown in Figure 4a. Four peaks corresponding to four

different glycoforms of the protein are evident in the 18-20+ charge states. These glycoforms

were determined from the masses measured from a denaturing solution of the protein (Figure S-

1) to correspond well with the two oligosaccharide (one diantennary and one triantennary)

glycosylations to this protein that were identified by van Halbeek and coworkers[59] both with and

without a fucosylation site on the oligosaccharide. The glycoforms are labelled as **A**) two

diantennary oligosaccharides; **B**) **A** with one fucosylation; **C**) one di- with one triantennary

oligosaccharide; and **D**) **C** with one fucosylation. The average masses measured for each of

these four glycoforms formed by nESI from native solution (Figure 4a, Table 3) are on average

~6 Da higher than the measured masses of these ions formed from denaturing solution (the

calculated m/z is indicated by the dashed lines in Figure 4), indicating that on average, less than

one sodium ion is adducted to the protein. With 1 mM NaCl in the same solution (Figure 4b),

the centroids of each peak shift to higher m/z so that the masses of ions **A** and **C** increase by

~168 Da and ~189 Da, respectively. This additional mass corresponds to an average of 8.3 and

9.3 sodium ions adducted to these two glycoforms, respectively, and the peak widths in the

spectrum are broad due to sodium adduction such that the fucosylated ions **B** and **D** are no longer

evident in the mass spectrum. Sodium adducts also lead to a significant increase in the baseline

at higher m/z. With 1.5% m-NBA (Figure 4c), the charge-state distribution is shifted to higher

charge, the most abundant charge state being 22+, and all of the peaks in the ion distribution are

still broad as a result of sodium adduction. The fucosylated ions **B** and **D** are not resolved, and

even ion **C** is evident only as a shoulder on the main **A** peak. Ions **A** and **C** have masses that are

~230 Da and ~300 Da higher than those formed from nESI from aqueous solutions without

sodium, and ~62 Da and ~111 Da higher than those from aqueous solutions with sodium. The

higher mass of these ions compared to those formed from solutions containing sodium without

supercharging reagents may be due to *m*-NBA adduction to the protein, which has been observed

before by Loo and coworkers for smaller proteins.[46] With 2.5% sulfolane (Figure 4d), the most

abundant charge state is 20+, and *all* glycosylated ions are evident in the mass spectrum, with

ions A, C, and D resolved, and ion B distinguishable as a shoulder on the peak of ion A. The

increase in mass for each glycosylated form of the protein is $\leq$ ~69 Da, or ~3 sodium ions. Thus,

addition of sulfolane not only enables a more accurate mass measurement from a solution

containing sodium, but it also reveals two additional fucosylated species present in solution that

were unresolvable in the mass spectrum from this same solution without sulfolane present

(Figure 4b).

A mass spectrum of the 146 kDa homotetramer of rabbit lactate dehydrogenase (LDH)

formed by nESI from 10 mM ammonium bicarbonate is shown in Figure 5a (dashed lines are the

calculated *m/z* values). The measured mass of the tetramer is 146,004 ± 18 Da, which is close to

the calculated mass of 145,996 Da based on the monomer mass determined from a spectrum of

LDH obtained under denaturing conditions (Figure S-2). The 8 Da mass difference corresponds

to an average of ~0.4 sodium ions adducted. There is also a distribution of LDH tetramer with a

mass of 146,619 ± 14 Da corresponding to bound NADH, a cofactor of LDH that has a mass of

664 Da.[60] With NaCl added at 5 mM concentration to this solution (Figure 5b), the LDH

tetramer peaks are broader due to sodium ion adduction, and the NADH adduct is unresolved.

The measured mass of the tetramer is $146,521 \pm 23$ Da, and corresponds to a mass increase of

~ 525 Da, or an average of ~ 23.9 sodium ions adducted. The peaks in a mass spectrum of rabbit

LDH in the same solution containing NaCl but with 2.5% sulfolane (Figure 5c) are significantly

narrower compared to spectra without the supercharging reagent (Figure 5b). The non-

covalently bound NADH is clearly resolved on all of the tetramer peaks, except for the lowest

charge state, and no dissociation of the NADH from the complex or increase in the average

charge of the protein complex is evident, indicating that sulfolane does not significantly disrupt

the structure of this protein complex. The addition of sulfolane also reduces the error in mass

measurement associated with sodium adduction. The measured masses for the tetramer with and

without NADH bound are $146,025 \pm 26$ Da and $146,763 \pm 13$ Da, respectively, with sulfolane,

~ 29 Da and ~ 103 Da higher than the calculated mass, corresponding to an average of only ~ 1.3

and ~ 4.7 sodium ions adducted, respectively. There is no data shown for LDH with 1.5% *m*-

NBA because the protein precipitates out of solution upon addition of the supercharging reagent

as evidenced by solution clouding, suggesting that the stabilities of some proteins can be affected

by even low concentrations of supercharging reagents.

  Effects on S/N and detection limits. Loo and coworkers reported binding of *m*-NBA to

ammonium and sodium cations at low *m/z*, but no protonated *m*-NBA was observed.[46] It is

possible that supercharging reagents reduce sodium ion adduction to proteins by binding to

sodium ions in solution. Mass spectra of 5 μM barstar formed by nESI from aqueous solutions

containing 10 mM ammonium bicarbonate and 1 mM NaCl with and without supercharging

reagents are shown in Figure 6. In the expanded region below *m/z* = 700, many clusters

containing sodium, chloride, bicarbonate, and ammonium are observed without supercharging

reagents (Figure 6a), resulting in significant chemical noise at high *m/z* where protein ion signal

is often observed (Table S-3). This can lower the protein ion S/N (Table S-3). With *m*-NBA

(Figure 6b), the dominant low *m/z* ions are ammonium and sodium ions complexed with one or

two *m*-NBA molecules, but there are also some low abundance ions containing sodium, chloride,

bicarbonate, and ammonium, as well as sodium chloride and sodium bicarbonate cluster ions

bound to *m*-NBA. Thus, *m*-NBA appears to inhibit the formation of salt cluster ions. There are

no clusters above *m/z* ~700 (Figure 6b, inset), resulting in very low chemical noise in the higher

m/z region of the spectrum where barstar ions are observed (Table S-3). With sulfolane, sodium

and ammonium complexed to one, two, or three sulfolane molecules are most abundant (Figure

6c). Clusters extend to *m/z* ~1600, resulting in high chemical noise in the region where protein

ions are often observed that is similar to the chemical noise without supercharging reagents

(Figure 6a and c insets; Table S-3).

Dearden and coworkers[61] showed that alkali cations can be removed from peptides and

small protein ions in the gas phase by crown ether molecules, leading to a concomitant decrease

in the peptide or protein charge as the cations are removed. The average charge of protein ions

formed from solutions containing *m*-NBA and sulfolane with sodium chloride is typically the

same or higher than that obtained without these reagents. This indicates that protein desalting

with *m*-NBA and sulfolane is not solely due to these supercharging reagents stripping sodium

ions from proteins in the gas phase. Supercharging reagents must be influencing the amount of

sodium ion adduction to proteins in solution, most likely in the nESI droplet, where enrichment

of both sodium ions and supercharging reagent occurs. It is likely that sequestration of sodium

ions by supercharging reagents is the cause of the reduced sodium ion adduction to protein ions

observed with *m*-NBA and sulfolane. Other groups have reported adduction of supercharging

reagents to protein ions,[46, 54, 62] although no obvious adduction was observed in our study. It is

plausible that the supercharging reagents may also block sites on the protein where sodium ions

bind.

Addition of *m*-NBA or sulfolane to aqueous solutions containing sodium substantially

increases the S/N of some protein ions owing to reduced chemical noise and fewer sodiated

species over which protein signal is spread (Table S-3, Figure S-3). For example, the S/N of the

most abundant ion of barstar formed from a 5 µM solution is 7-fold higher with *m*-NBA than

without this supercharging reagent, and there is a 2-fold enhancement with sulfolane (Table S-3).

Supercharging reagents can also decrease the limit of detection (LOD) for these protein ions

formed from solutions containing sodium. The LOD for barstar from sodiated solutions,

determined from protein concentration dependent measurements, is almost an order of magnitude

lower with *m*-NBA than without (0.02 µM versus 0.12 µM) (Table S-3). However, the effect of

supercharging reagents on the S/N and detection limit is protein dependent, and an improvement

was not observed for all proteins (Table S-3). This may be due to differences in the chemical

and salt impurities in the protein samples.

Conclusions

Supercharging reagents can increase the charge of protein ions formed by nESI from both

native and denaturing solutions, but they also have the added benefit of effectively reducing

sodium ion adduction to protein and protein complex ions formed from aqueous solutions.

Addition of <3% of *m*-NBA or sulfolane to aqueous solutions containing millimolar

concentrations of sodium can reduce sodium adduction to protein ions by an average of ~58%

and ~80%, respectively. The presence of *m*-NBA in protein solutions containing sodium reduces

chemical noise caused by clusters, resulting in up to a factor of 7 enhancement in S/N for some

15

protein ions, whereas the presence of sulfolane has little effect on protein ion S/N due to salt

cluster formation that extends to high m/z. Sodium adduction to both m-NBA and sulfolane

occurs, and this may sequester a sufficient number of sodium ions such that sodium adduction to

proteins is significantly reduced. In the future, it would be interesting to investigate whether the

use of supercharging reagents for desalting protein ions formed by nESI is effective for other

ions that commonly bind nonspecifically to proteins, such as potassium and calcium, or for

anions, such as sulfate and phosphate, which are often added as buffers to native protein

solutions.

Acknowledgements

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Table 1. Average charge and average number of sodium ions adducted to the most abundant protein charge state formed by nESI from aqueous solutions containing 5 µM protein, 10 mM ammonium bicarbonate, and 1 mM NaCl with no supercharging reagent, with 1.5% *m*-NBA, or with 2.5% sulfolane.

Protein	Average charge	Average # Na adducts to (most abundant charge state)	Protein size	Protein pI	# Disulfide bonds
Ubiquitin	5.33 ± 0.01	3.5 ± 0.1 (5+)	8.6 kDa	6.6	0
m-NBA	7.1 ± 0.1	1.2 ± 0.2 (7+)			
sulfolane	6.3 ± 0.1	0.44 ± 0.03 (6+)			
Barstar	5.5 ± 0.1	3.5 ± 0.4 (5+)	10.2 kDa	4.7	0
m-NBA	7.1 ± 0.1	1.4 ± 0.2 (7+)			
sulfolane	5.7 ± 0.1	1.0 ± 0.4 (6+)			
Cytochrome *c*	6.5 ± 0.1	2.5 ± 0.2 (7+)	12.3 kDa	9.6	0
m-NBA	9.42 ± 0.04	0.93 ± 0.01 (9+)			
sulfolane	8.6 ± 0.2	0.17 ± 0.01 (9+)			
Ribonuclease A	6.9 ± 0.1	4.5 ± 0.2 (7+)	13.7 kDa	9.6	4
m-NBA	9.16 ± 0.03	1.2 ± 0.2 (9+)			
sulfolane	7.7 ± 0.1	0.5 ± 0.1 (8)			
α-Lactalbumin	6.8 ± 0.1	3.3 ± 0.5 (7+)	14.2 kDa	4.8	4
m-NBA	8.9 ± 0.1	1.7 ± 0.1 (9+)			
sulfolane	7.2 ± 0.2	1.0 ± 0.3 (8+)			
Lysozyme	7.5 ± 0.1	1.7 ± 0.1 (8+)	14.3 kDa	11.4	4
m-NBA	9.9 ± 0.1	0.60 ± 0.03 (10+)			
sulfolane	8.8 ± 0.3	0.4 ± 0.1 (9+)			
holo-Myoglobin	8.2 ± 0.1	3.9 ± 0.4 (8+)	17.6 kDa	7.4	0
m-NBA	11.2 ± 0.2	0.79 ± 0.02 (11+)			
sulfolane	9.8 ± 0.4	0.3 ± 0.1 (10+)			
apo-Myoglobin	--	--	17.0 kDa	7.4	0
m-NBA	12.4 ± 0.6	0.71 ± 0.04 (11+)			
sulfolane	9.2 ± 0.3	0.28 ± 0.01 (9+)			
β-lactoglobulin	8.13 ± 0.04	4.0 ± 0.2 (8+)	18.3 kDa	4.8	3
m-NBA	10.41 ± 0.03	2.88 ± 0.03 (10+)			
sulfolane	8.5 ± 0.2	1.9 ± 0.2 (9+)			
Carbonic Anhydrase	9.8 ± 0.1	4.5 ± 0.6 (10+)	29.0 kDa	6.4	0
	13.5 ± 0.3	3.0 ± 0.5 (13+)			
m-NBA	11.7 ± 0.1	0.7 ± 0.2 (12+)			
sulfolane					

Table 2. Average charge state, average number of sodium adducts to the most abundant charge state, and fractional population of barnase-barstar and free barnase formed by nESI from aqueous solutions containing 6 µM barstar, 5 µM barnase, 10 mM ammonium bicarbonate, and 1 mM NaCl with no supercharging reagent, with 1.5% *m*-NBA, or with 2.5% sulfolane.

Protein	Average charge	Average # Na adducts to most abundant charge state	% Total protein ion population
Barnase-Barstar	8.8+	4.0	100%
+ *m*-NBA	11.0+	0.4	38%
+ sulfolane	9.6+	0.6	84%
Free Barnase	--	--	0%
+ *m*-NBA	6.4+	0.7	62%
+ sulfolane	5.7+	0.3	16%

Table 3. Measured masses and average sodium ion adduction for four different glycoforms of holo-transferrin formed by nESI from aqueous solutions containing 10 mM ammonium bicarbonate (ABC) and 1 mM NaCl with no supercharging reagent, with 1.5% *m*-NBA, or with 2.5% sulfolane.

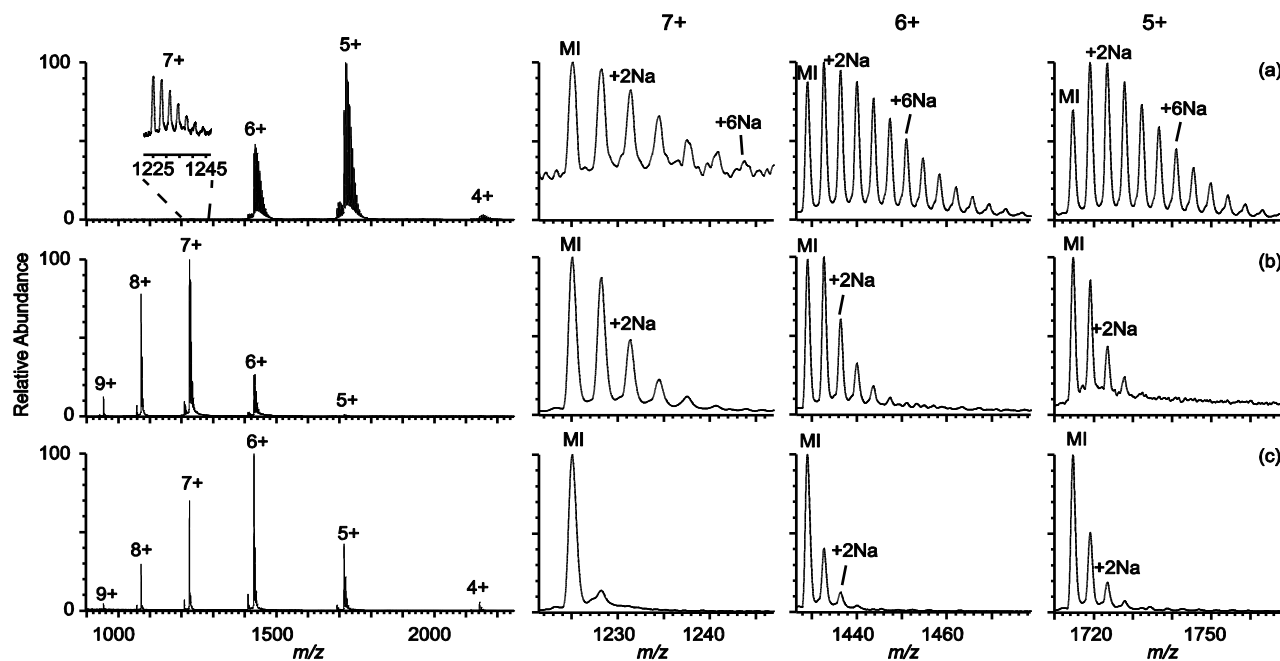|	Protein mass (Da)				
Protein glycoforms	10 mM ABC	10 mM ABC 1 mM NaCl	+1.5% *m*-NBA	+2.5% sulfolane	Calculated mass
A	79,714 ± 4	79,882 ± 21	79,944 ± 12	79,764 ± 5	79,700 Da
B	79,848 ± 10			79,910 ± 15	79,846 Da
C	80,372 ± 7	80,561	80,675 ± 21	80,425 ± 10	80,356 Da
D	80,505 ± 12			80,567 ± 20	80,502 Da
	Average # Na adducts				
	10 mM ABC	10 mM ABC 1 mM NaCl	+1.5% *m*-NBA	+2.5% sulfolane	
A	0.6 ± 0.2	8.3 ± 1.0	11.1 ± 0.6	2.9 ± 0.2	
B	0.1 ± 0.5			2.9 ± 0.7	
C	0.7 ± 0.3	9.3	14.5 ± 1.0	3.1 ± 0.5	
D	0.1 ± 0.6			3.0 ± 0.9	

Figure 1. Mass spectra of ubiquitin (5 µM) formed by nESI from aqueous solutions containing 10 mM ammonium bicarbonate and 1 mM NaCl (a) with no supercharging reagent, (b) with 1.5% *m*-NBA, and (c) with 2.5% sulfolane. Expansions showing adduction of sodium ions to the 5-7+ molecular ions are shown on the right; MI indicates the protonated molecular ion, $(M+nH)^{n+}$.
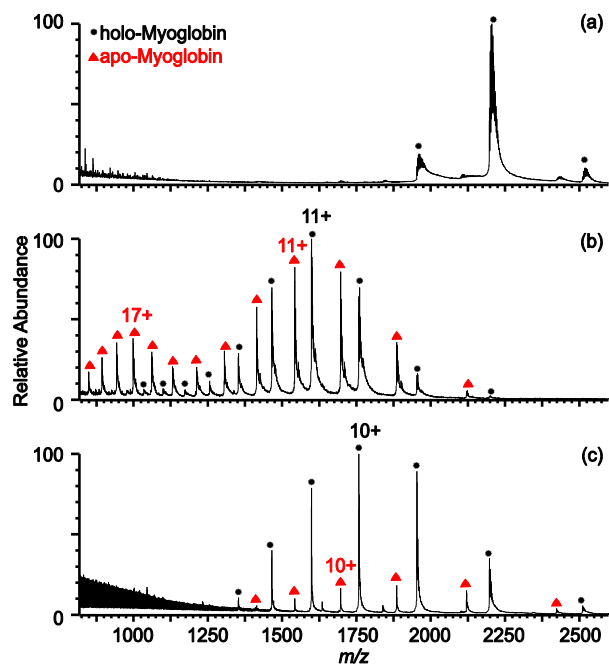
Figure 2. Mass spectra of myoglobin (5 µM) formed by nESI from aqueous solutions containing 10 mM aqueous ammonium bicarbonate and 1 mM NaCl (a) with no supercharging reagent, (b) with 1.5% *m*-NBA, and (c) with 2.5% sulfolane.
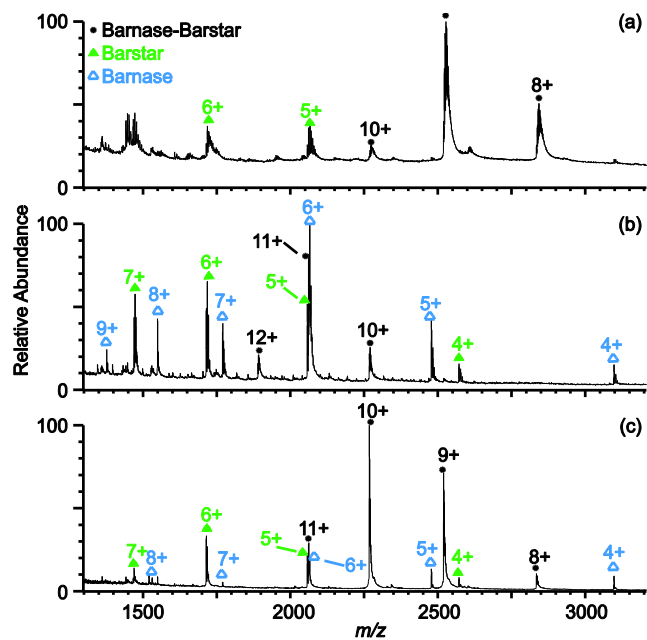
Figure 3. Mass spectra of barnase-barstar (5 μM) with excess barstar formed by nESI from aqueous solutions containing 10 mM ammonium bicarbonate and 1 mM NaCl (a) with no supercharging reagent, (b) with 1.5% *m*-NBA, and (c) with 2.5% sulfolane.
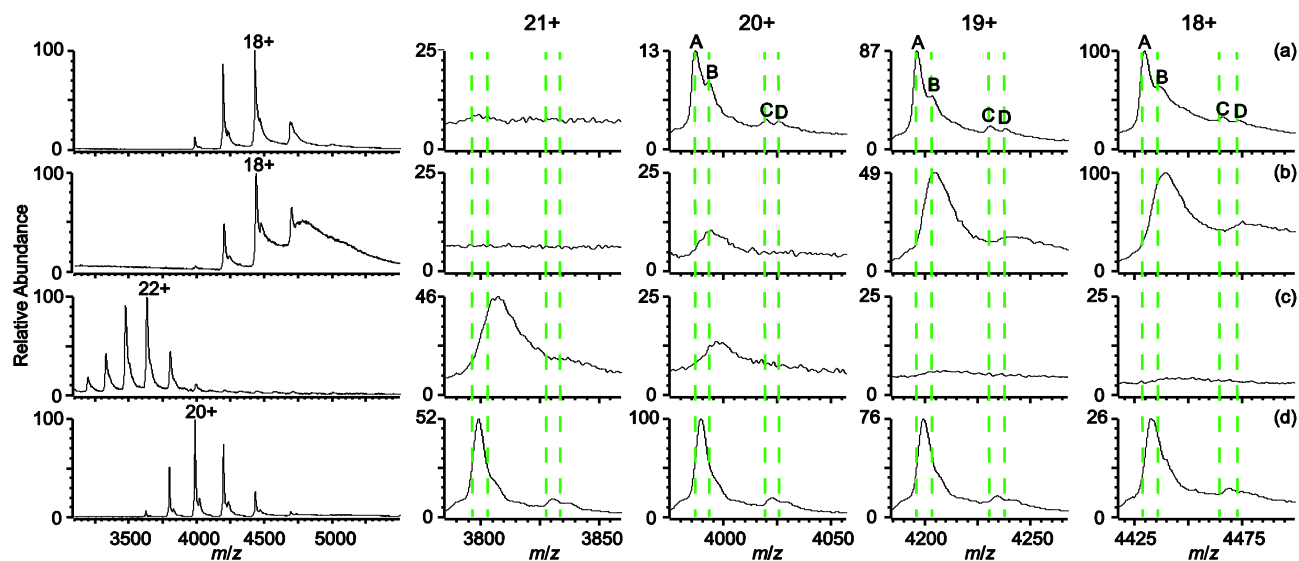
Figure 4. Mass spectra of human holo-transferrin (79.7 kDa) formed by nESI from aqueous solutions containing 10 mM ammonium bicarbonate with (a) no additional salt added, (b) 1 mM NaCl, (c) 1 mM NaCl with 1.5% *m*-NBA, and (d) 1 mM NaCl with 2.5% sulfolane. Expansions of the 18-21+ charge states are shown to the right. Dashed lines indicate the calculated average *m/z* for each glycoform without adducts.
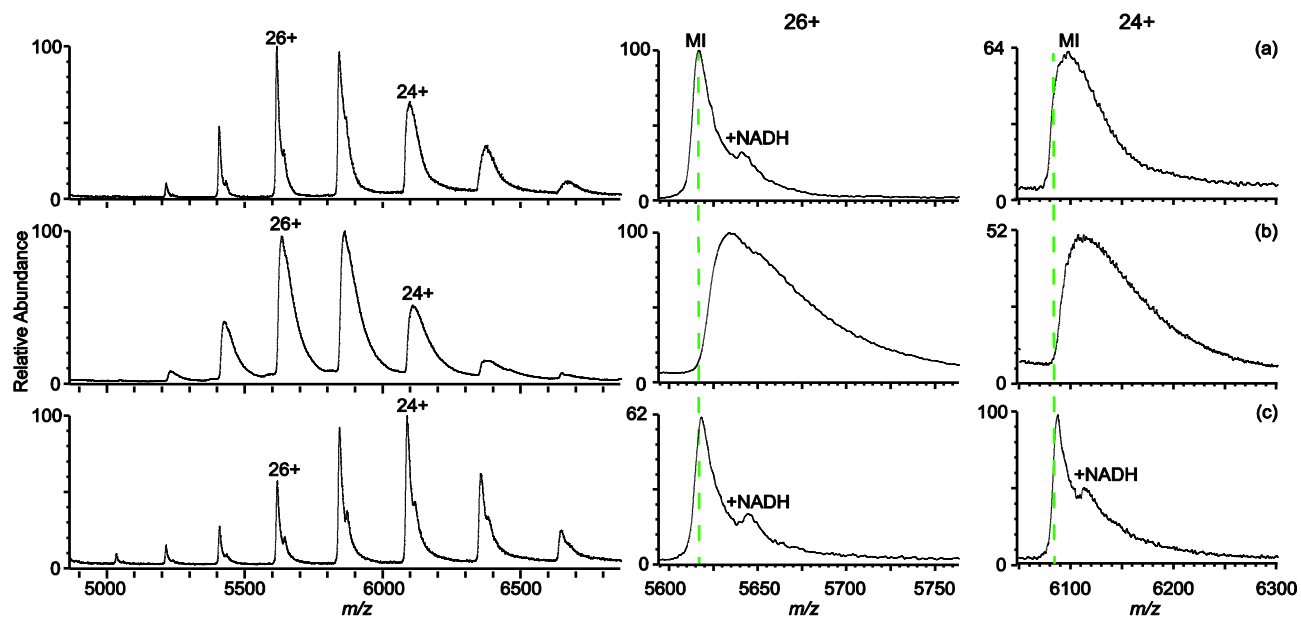
Figure 5. Mass spectra of rabbit LDH tetramer (146 kDa) formed by nESI from aqueous solutions containing 10 mM ammonium bicarbonate with (a) no additional salt added, (b) 5 mM NaCl, and (c) 5 mM NaCl with 2.5% sulfolane. Expansions of the 26+ and 24+ charge states are shown to the right. Dashed lines indicate the calculated average *m/z* of the tetramer without adducts.
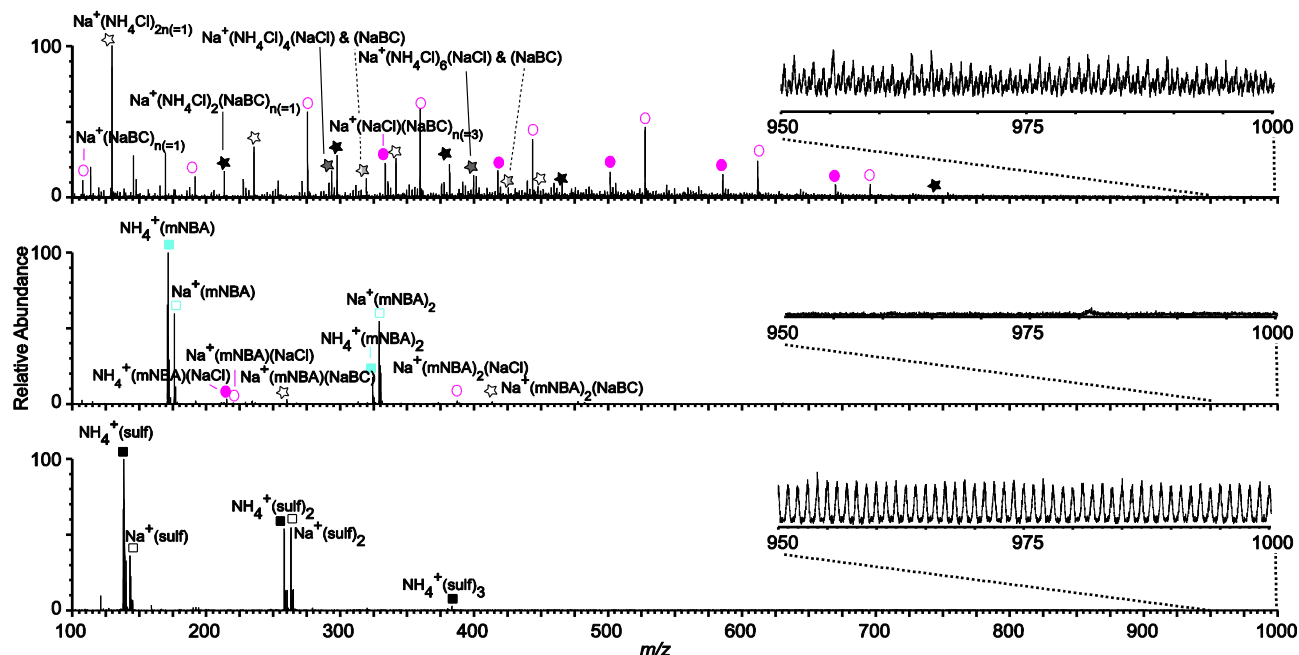
Figure 6. Mass spectra of barstar (5 µM) formed by nESI from aqueous solutions containing 10 mM ammonium bicarbonate and 1 mM NaCl (a) with no supercharging reagent, (b) with 1.5% *m*-NBA, and (c) with 2.5% sulfolane. The low *m/z* region of the spectra are expanded. Spectra are normalized to the most abundant ion, but insets are on the same absolute scale for comparison.

Table of Contents Entry

Low concentrations of supercharging reagents *m*-NBA (1.5%) and sulfolane (2.5%) effectively

desalt protein ions produced by electrospray ionization from aqueous solutions, improving mass

measuring accuracy for large proteins.