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Accelerated Articles

Peptide Sequencing and Characterization of Post-Translational Modifications by Enhanced Ion-Charging and Liquid Chromatography Electron-Transfer Dissociation Tandem Mass Spectrometry

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We have tested the effect of *m*-nitrobenzyl alcohol (*m*-NBA) as a method to increase the average charge state of protonated gas-phase molecular ions generated by ESI from tryptic peptides and phosphopeptides. Various concentrations of *m*-NBA were added to the mobile phases of a liquid chromatography system coupled to an ESI tandem mass spectrometer. Addition of just 0.1% *m*-NBA changed the average charge state for the identified tryptic BSA peptides from 2.2+ to 2.6+. As a result, the predominant charge states for BSA peptides were changed from 2+ to ≥3+. To evaluate the benefits of peptide charge enhancement, the ETD fragmentation efficiency and Mascot peptide score were compared for BSA peptides in charge states 2+ and 3+. In all cases but one, triply charged peptides fragmented more efficiently than the analogues 2+ peptide ions. On average, triply charged peptides received a 68% higher Mascot score (24 units) than doubly charged peptides. *m*-NBA also increased the average charge state of phosphopeptides by up to 0.5 charge unit. The ease of implementation and the analytical benefits of charge enhancement of tryptic peptides by addition of *m*-NBA to the LC solvents suggest the general application of this reagent in proteomic studies that employ ETD-MS/MS and related techniques.

Tandem mass spectrometry (MS/MS)[1] is an indispensable method for large-scale identification of proteins in proteomics studies.[2] This is largely due to the enhanced sensitivity, accuracy, and speed of modern mass spectrometry for mass determination and sequencing of peptides. Proteins extracted from cells or tissues from humans, animals, plants, and microbes are typically fractionated by liquid chromatography (LC) or gel electrophoresis[3] and then subjected to enzymatic digestion. Subsequently, complex peptide mixtures are separated and sequenced by LC interfaced to electrospray ionization (ESI)[4] MS/MS analysis.

The choice of protease is determined by the desired cleavage specificity, cleavage efficiency, turnover number, and cost. Trypsin is by far the preferred proteolytic enzyme in proteomics as it is efficient, low cost, and it cleaves specifically C-terminally to arginine and lysine residues[5] producing on average 10−12-residue-long peptides.

The purpose of MS/MS is to fragment peptides into a collection of sequence-informative product ions from which the peptide sequence can be deduced either by manual interrogation or by database searching. The confidence of the protein assignment by database searching relies heavily on the extent and the quality of the obtained sequence information generated by the MS/MS event. The most common MS/MS technique is collision-activated dissociation (CAD), where gaseous peptide cations are internally heated by multiple collisions with an inert neutral gas.

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This leads eventually to peptide backbone fragmentation of the C−N bond resulting in series of b- and y-fragment ions. Although backbone fragmentation can be abundant in CAD mass spectra, it is competing with other peptide/molecular dissociation channels producing internal fragment ions and neutral losses of H_2O, NH_3, CO_2, and labile groups (e.g., phosphoric acid, sulfuric acid, and glycan groups). Unfortunately, in many cases, the fragmentation data gained by CAD alone are not sufficient to identify the peptide.

In addition, the nature of CAD as a slow-heating technique can result in losses of many labile groups such as common post-translational modifications (PTM) on peptides.[6−9] This complicates the localization of the site at which the modification was originally attached and often limits the range of informative backbone cleavages. The preferential cleavage of the weakest bond can result in overall lower sequence coverage, especially for larger proteins where only a fraction of the sequence information can be obtained with slow-heating dissociation techniques.[10,11]

A complement technique to CAD for fragmentation of multiply protonated peptides is electron capture dissociation (ECD).[12] The principle of ECD is to react multiply protonated polypeptide cations with low-energy electrons. Capturing of a near-thermal free electron (EI = 0) releases 4−7 eV of energy (depending on the charge state of the precursor ions) that is available for peptide fragmentation. This induces fragmentation in the charge-reduced radical peptide ions of the peptide backbone at the amide (N−C_α) bond to produce c-type and z-type fragment ions. Due to different cleavage sites of the two fragmentation techniques, the product ions of ECD (c′, $z^\bullet$) are complementary to those of CAD (b, y′). The analytical application of using the information of the complementary fragment ions is to increase the specificity of peptide identification.[13,14] Importantly, ECD cleaves the peptide backbone bonds, even in the presence of potentially labile PTMs, such as phosphorylation and glycosylation, leaving them attached to fragment ions.[7−9,15] A comprehensive review on ECD and its utility in proteome research was recently published by Zubarev.[16]

A few years ago, an analogous technique, electron-transfer dissociation (ETD), was developed.[17] In ETD, the electron transfer from the radical anions with low electron affinity (EA) to multiple protonated peptide cations also initiates peptide backbone fragmentation of the N−C_α bond to produce predominantly c′ and $z^\bullet$ ion series similar to fragmentation with ECD.[17−21] ETD is so far realized both in Paul traps and in linear ion traps.

From a thermodynamical point of view, ETD is fundamentally different from ECD. Electrons in ETD are delivered by radical anions (typically fluoranthene) unlike being free electrons as in ECD. In ETD, the peptide cations have first to overcome the barrier of abstracting the electrons from the radical anions for the cleavage reaction to proceed. Depending on the structure and chemical property of the anions, the energy barrier for this reaction is close to the EA of the anions, which is on the order of 0.5−1.5 eV of energy.[18] Therefore, one can speculate that less energy is available for peptide fragmentation in ETD than in ECD. This may explain why several studies report on reduced fragmentation efficiency and limited sequence coverage for doubly charged peptides in ETD.[22−24] Another indication is that so far the majority of the previous ETD studies were conducted on peptide ions predominately >2+ in the charge states.[17−19,25−27] The problem with reduced ETD fragmentation of doubly charged ions is even more pronounced in the light of the overwhelming production of doubly charged peptides from tryptically digested proteins. As a consequence of the basic nature of Arg and Lys, and the typical length of tryptic peptides, most (>70%)[14] multiple charged peptides adopt two positive charges as gaseous ions upon ESI. Obviously, low ETD fragmentation efficiency of most peptides in standard proteomics strategies calls for a solution. McLuckey and co-workers have shown that elevated bath gas temperature can increase the overall sequence coverage for tryptic peptides larger than seven residues. Although the ETD efficiency did not increase as a result of the higher bath gas temperature, it was suggested that the observed greater peptide sequence coverage resulted from a broader distribution of charges within the peptide giving rise to more fragmentation channels.[22] Another approach to increase peptide ion charge states is to use proteases such as Lys-C or Asp-N, which produces longer (20−25 residues) peptides[17] that tend to hold more charges (3−6) than tryptic peptides. An associated advantage of this strategy is that the false positive rate of protein identification decreases with the increasing size of peptides.[28] However, Asp-N and Lys-C are much more expensive than trypsin and larger highly charged peptides are usually not

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fragmented efficiently with CAD fragmentation.[29] Thus, Lys-C or Asp-N peptides are less compatible for complementary fragmentation experiments where both ETD/ECD and CAD are applied. Finally, longer peptides will generate many highly charged fragment ions, which are difficult to resolve when using low-resolution mass spectrometers (e.g., ion traps). Even with subsequent charge reduction by proton-transfer reactions of the multiple charged product ions,[19] most of the product ions will have m/z values beyond the mass range of the instrument capability. Recently, Coon et al. showed that supplemental collisional activation of the nondissociation population of the electron-transfer ions (charge-reduced intact species, $[M + nH]^{(n-1)+\bullet}$) can increase the ETD efficiency up to 77% for doubly charged ions.[24] With this strategy, more backbone cleavages were also observed for phosphorylated peptides and neutral losses of phosphoric acid were observed.

We present here an ETD fragmentation strategy based on charge-state manipulation of tryptic peptide ions. Shifting the tryptic peptide charge-state distributions to greater values will allow more ≥3+ peptides to be fragmented by ETD. In such case, ≥3+ peptides will benefit from higher ETD fragmentation efficiency. Importantly, this strategy is compatible with the use of trypsin as the protease for protein digestion. Several fundamental studies by Williams et al. have shown that enhanced charging is possible for a variety of analytes by the addition of *m*-nitrobenzyl alcohol (*m*-NBA) to the electrospray solution.[30−32] For instance, addition of 1% *m*-NBA to the ESI solution of cytochrome *c* changed the maximum charge state from 21+ to 24+ and increased the average charge state from 17.3+ to 20.8+.[31] Common for the above-mentioned studies is that *m*-NBA was mainly tested on larger molecular systems like intact proteins or polymers. However, one of the smallest molecular systems tested was the highly basic tetrapeptide Lys_4 that changed its predominant charge state from 2+ to 3+ upon addition of 6.7% *m*-NBA to the ESI solution.[30] The charge-enhancing ability of *m*-NBA in these and other studies was explained by the combination of low vapor pressure and high surface tension of *m*-NBA. As a result, ESI droplets can tolerate more surface-exposed charges before they reach the Rayleigh limit[33] of fission. Hence, the higher charge density on the surface of the droplet translates into higher charge states of the analytes. Despite the above-mentioned results, it still remains unclear if it is possible to enhance the overall charge-state distribution of tryptic peptides by the addition of *m*-NBA. Contrary to previously studied molecular systems, tryptic peptides are much smaller than proteins and less basic than Lys_4. Moreover, for efficient identification of complex peptide mixtures, it is necessary to test the compatibility of *m*-NBA with conventional solvent solutions used in a LC−MS/MS setup. We therefore test the charge-enhancing phenomenon of *m*-NBA on a representative set of tryptic peptides and phosphopeptides using liquid chromatography coupled with ETD peptide fragmentation (LC-ETD-MS/MS). The results of this study have relevance to the broad field of proteome research and for the basic understanding of the ESI charging process.

EXPERIMENTAL SECTION

Samples and Solutions. Bovine serum albumin (BSA) was reduced, alkylated, and digested with trypsin (Promega, Madison, WI) using standard protocol. *m*-NBA (99.5% puriss) was purchased from Fluka. Phosphopeptides from a trypsin-digested protein mixture consisting of 12 proteins (carbonic anhydrase, BSA, α-casein (S1 and S2), β-casein, ovalbumin, β-lactoglobulin, RNAse B, alcohol dehydrogenase, myoglobin, transferrin, lysozyme, α-amylase) were enriched using TiO_2 microcolumns as described.[34] In all experiments, an amount of 100 fmol of material was analyzed by LC-ETD-MS/MS.

Liquid Chromatography Solutions. Mobile phase A consisted of 0.1% formic acid (FA) in water. Mobile phase B consisted of 90% acetonitrile (ACN) and 0.1% FA in water. Various amount of *m*-NBA were added to mobile phases A and B. *m*-NBA was dissolved in mobile phase B directly and in mobile phase A using ultrasound. Several different mobile-phase concentrations of *m*-NBA were tested to optimize both the enhanced charging and the chromatography. The following mobile-phase solutions were tested and listed with concentrations of *m*-NBA (w/v of *m*-NBA) in mobile phases A and B, respectively: 0%:0%, 0.1%:0.1%, 0.25%:0.25%, and 0.5%:0.5%.

LC-MS/MS. In all experiments was used an 1100 Agilent nanoflow LC system connected via a HPLC-Chip Cube ESI interface to an MSD Trap XCT Ultra (Agilent Technology, Santa Clara, CA). Peptides were separated on an 43 × 75 μm analytical HPLC-Chip packed with 5-μm Zorbax 300SB C18 reversed-phase material (Agilent Technology) containing a 40-nL enrichment precolumn. After a 2-min loading time (400 nL/min), the peptides were eluted from the column with linear gradients over a 12-min period (1−50% B in 10 min and 50−90% B in 2 min) at a flow rate of 300 nL/min. This LC gradient was used for all mobile-phase compositions.

MS/MS analysis was performed using unattended data-dependent acquisition mode. After a survey scan (enhanced mode, 300−2000 m/z) consisting of four scans, a maximum of five peptides were selected for ETD-MS/MS (ultrascan, 100−2200 m/z). The automated gain control was set to 500 000. The maximum accumulation time was set to 150 ms. For each peptide, one ETD mass spectrum was recorded. Since ETD fragmentation of doubly charged peptide ions is generally poor, we applied a small resonance excitation during the ETD event to promote the separation of fragments.

Data Analysis. Tandem mass spectra were retrieved and stored in a combined mgf file using the 6300 series ion trap LC/MS software 6.1. MS/MS data were searched using the Mascot search engine. Since the sample ID was known and in order to save search time a premade small protein database consisting of BSA, α-casein (S1, S2), β-casein, and 15 other common proteins was used. With the following search parameters, peptide mass 1.8 Da, MS/MS 0.8 Da, and missed cleavages 2, the Mascot determined threshold score was 9 for non-phosphopeptides and 16 for phosphopeptides). This threshold ensures >95% statistical

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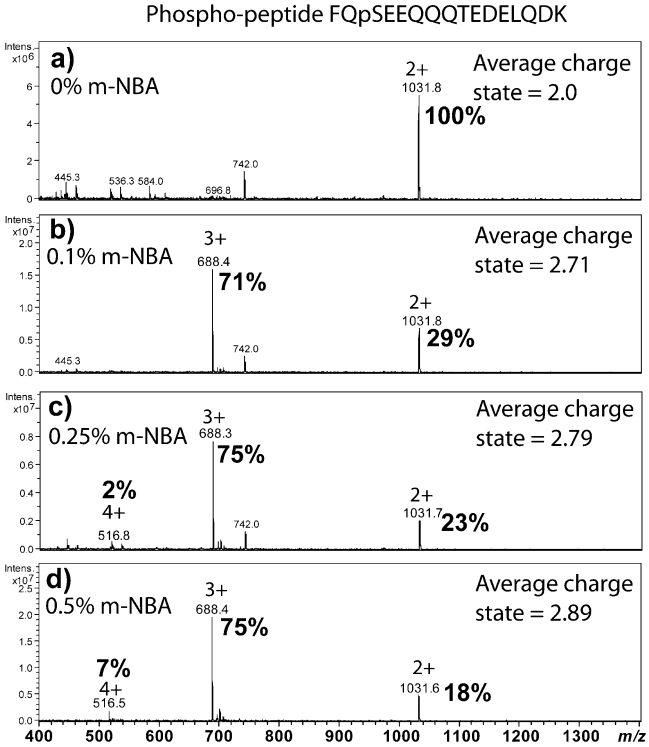

Figure 1. Electrospray ionization mass spectra of the phospho-peptide FQpSEEQQQTEDELQDK eluting with liquid chromatography mobile phases containing (a) 0, (b) 0.1, (c) 0.25, and and (d) 0.5% *m*-NBA. The mobile phase is (A) 0.1% FA in H_2O and (B) 0.1% FA and 90% ACN.

confidence in the peptide identification.[35] Peptide hydrophobicity and pI values were calculated using a recently described in-house software.[36]

Weighted Average Charge State. Throughout the text and figures, the term average charge state ($q_{average}$) is used as one of the parameters to describe the effect of *m*-NBA on the peptide charge-state distribution (eq 1). In eq 1, N is the number of analyte

$$q_{average} = \frac{\sum_i^N q_i W_i}{\sum_i^N W_i} \quad (1)$$

charge states in the spectrum, q_i is the net charge of the ith charge state, and W_i is the signal abundance of the ith charge state.

RESULTS AND DISCUSSION

Peptide Charge-State Increment with *m*-NBA. The ESI mass spectra shown in Figure 1 exhibits the charge-state distribution of the phosphopeptide (FQpSQQQTEDELQDK) eluting from the LC column when different concentrations of *m*-NBA were added to the solutions of the two mobile phases. Four different concentrations were tested: 0, 0.1, 0.25, and 0.5% w/v *m*-NBA. Without addition of *m*-NBA, only the doubly charged species of the phosphopeptide was observed, giving an average charge state of 2.0+. Notably, with only 0.1% of m-NBA the charge-state distribution shifts dramatically towards higher charge states (2+ (29%); 3+ (71%)) and result in an average charge state of 2.71+. Increasing the concentration of m-NBA to 0.25% and 0.5% shifts the charge-state distributions even further (average charge states of 2.79+ and 2.89+, respectively), although the effect is not increasing linearly. With 0.25% and 0.5% m-NBA the maximum achieved charge state for the phosphopeptide was 4+ being only 2.5 times less intense than the 2+ ions Figure 1d). No adverse effect was observed in the signal intensity for the phospho-peptide upon addition of m-NBA up to 0.5%. In fact, addition of m-NBA to the mobile phase slightly improved the total signal intensity (all charge states summed) from an average (triplicate experiments) of $5*10^6$ with 0% m-NBA to $1.6*10^7$, $0.9*10^7$ and $2*10^7$ with 0.1%, 0.25% and 0.5% m-NBA, respectively. However, extending the concentration of m-NBA to 1% was tested, but resulted in significant chemical noise and lowering of the signal intensity (data not shown). Furthermore, column backpressure increased approximately 85% with 1% m-NBA and thereby surpassed the upper pressure limit for the column. The prompt effect on the charge-state distribution for the phospho-peptide by addition of only 0.1% m-NBA was very encouraging and turned out to be a general observable effect for all tested peptides. Since the steepest increase in the charge-state distribution was observed at 0.1% m-NBA and in order to keep the actual chemical changes of the mobile phase solutions to a minimum, we decided to continue with this condition in the following studies.

Benefits of Charge Enhancement. The application of charge-state manipulation toward higher charge states was tested in relation to ETD fragmentation efficiency and peptide identification confidence. Peptide fragmentation by ETD of the phosphopeptide (FQpSEEQQQTEDELQDK) as doubly, triply, and quadruply charged precursor ions is shown in Figure 2. ETD of doubly charged precursor ions m/z 1031.6 (Figure 1a) result in nine inter-residue cleavages and a Mascot peptide score of 30. The ETD efficiency was determined as the ratio of the summed abundances of all ETD products, except for the reduced undissociated precursor ions $[M + nH]^{(n-1)+\bullet}$, divided by the total ion abundance (eq 2):

$$\text{ETD frag eff} = \frac{\sum \text{ETD products (c, z and neutral losses)}}{\sum \text{total ion abundance}} \quad (2)$$

For the doubly charged precursor ions, it was measured to 29%. Unlike CAD of doubly charged peptides, which often gives rise to the appearance of complementary b and y′ ion pairs, ETD of 2+ ions can only produce one fragment ion per cleavage due to charge neutralization of the counter fragment ion. The preferential charge neutralization of the least basic site prevents complementary ion formation for doubly charged peptides. For instance, in a study of ~3000 high-quality ECD spectra of doubly charged precursor ions, only 14% of the ECD data contained complementary fragment ions.[37] Given the similarity of ETD and ECD, it is likely that preferential charge neutralization of the least basic site in peptide ions also prevails in ETD. For peptide identification, the appearance of two complementary fragments from a cleavage

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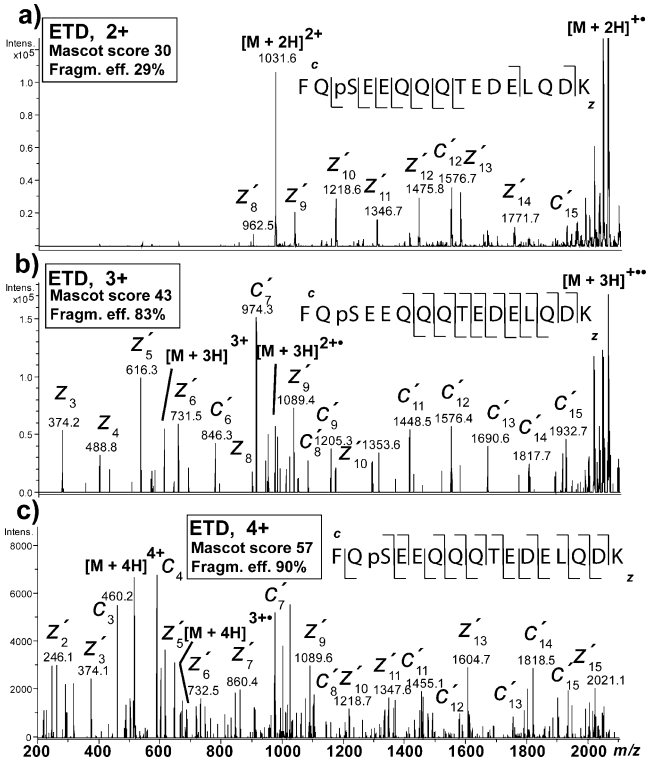

Figure 2. ETD mass spectra of the phosphopeptide FQpSEEQQQTEDELQDK in precursor ion charge states (a) 2+, (b) 3+, and (c) 4+. For each charge state, the Mascot score and ETD fragmentation efficiency is given.

of a peptide bond increases the certainty for a correct peptide match.

ETD fragmentation of triply charged precursor ions (m/z 974.3) is shown in Figure 2b. A total of 10 inter-residue bonds were cleaved of which 8 (c'_6–c'_{13}) had complementary ions. The more extensive ETD fragmentation of the 3+ ions is also reflected in the peptide Mascot score of 43 (13 units larger). Since the Mascot score is a 10-log function ($-10\log(p)$) of the probability (p) calculated for a peptide identification to be chance event,[35] it makes the identification of the triply charged phosphopeptide 20 times more certain than that of the doubly charged peptide. The ETD fragmentation efficiency was almost three times larger for 3+ precursor ions (83%) than for 2+ precursor ions (29%). Finally, ETD of 4+ ions (m/z 516.3) (Figure 2c) resulted in the most extensive fragmentation yield. A total of 13 inter-residues cleaved of which 7 (c_3, c'_6, c'_7, c'_9, c'_{11}, c'_{13}, c'_{14}) had complementary fragment ions. The fragmentation efficiency was measured to 90%, and the Mascot peptide score of 57 was assigned for that ETD mass spectrum. The higher ETD fragmentation efficiency for charge states higher than 2+ can be explained by an increase in the recombination energy (RE) for these species. The RE is inversely related to the gas-phase proton affinity (PA) of the peptide cation, and since the gas-phase PA decreases with increased charge,[38] it makes the electron-transfer reaction significantly more exothermic for triply than doubly charged peptides. Finally, ETD of ≥3+ precursor ions produces a significant amount of complementary ions that due to Coulombic repulsion between charged fragments separates easier than a charged–neutral complex found in ETD products of doubly charged precursor ions.

Charge Enhancement and ETD Analysis of Tryptic Peptides of BSA. In order to study the charge enhancement effect of *m*-NBA on a larger peptide population, we digested BSA with trypsin and analyzed the peptides using LC-ETD-MS/MS with 0% *m*-NBA and 0.1% *m*-NBA, respectively, in the mobile phases.

In Table 1 are listed all the BSA peptides that were sequenced and identified in the two experiments. Determined by the MS survey scan, the relative charge-state distribution and their corresponding average charge state are given for each peptide. The calculated average charge state for all peptides in each experiment was 2.20+ with 0% *m*-NBA and 2.59+ with 0.1% *m*-NBA. The average increase in the charge state for the 33 peptides that were identified in both experiments (Table 1, right column) was 0.25+ with a standard deviation of 0.15+. The overall increase in peptide average charge state confirms the charge-enhancing effect of *m*-NBA on tryptic peptides. The average increase of ~0.25+ may appear rather low; however, it has a considerable impact. Consider as an example the peptide AWSVAR that changed its average charge state by 0.31+ upon addition of *m*-NBA. This translates into a change in the charge-state distribution from 63% 2+ and 37% 3+ to 32% 2+ and 68% 3+. This change in predominant charge state becomes particular important in MS/MS experiments where data-dependent acquisition is used. Now the 3+ charge-state peptide ions are more likely to be picked for MS/MS than the 2+ ions.

Regarding the overall BSA sequence yield, 45 BSA peptides were identified in the two experiments. A total of 33 peptides were sequenced both in the control and with addition of 0.1% *m*-NBA. Eight peptides were unique to the control, and four peptides were unique to the experiments using 0.1% *m*-NBA. This resulted in an overall protein sequence coverage of 60% without *m*-NBA and 55% with *m*-NBA. It is very common that results obtained under different experimental conditions vary. Therefore, differences in protein sequence coverage observed under the two different experimental conditions are not surprising. In fact, addition of *m*-NBA changes the preferences for which peptides were successfully sequenced, although the overall sequence coverage was slightly smaller than in the control experiment. Larger studies including more complex samples will clarify whether this is a general trend.

Figure 3 (left) shows the distribution of predominant charge states for the BSA peptides observed under conventional ESI conditions (0% *m*-NBA) and with the addition of 0.1% *m*-NBA. With 0% *m*-NBA, 56% of all BSA peptide ions were predominantly doubly charged. This corresponds to 66% of all multiple charged peptides, which is similar to previous reports.[14] Only 29% of the peptides were triply or quadruply charged. Note that 15% of all peptides had a predominant ion charge of 1+. With the addition of 0.1% *m*-NBA, 48% of the peptides were now doubly charged and the combined fraction of triply and quadruply charged peptides increased to 52%. Thus, the significant increase in charge state of peptides upon addition of 0.1% *m*-NBA provides the foundation for more efficient ETD analysis. In addition, no peptides were found to be predominantly singly charged with 0.1% *m*-NBA. The conversion of predominantly singly to doubly charged peptide ions

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Table 1. Lists of Charge-State Distributions and Calculated Average Charge States for Identified BSA Peptides Found in LC-ESI-ETD-MS/MS Experiments Using 0 and 0.1% *m*-NBA in the Mobile Phases

	0% *m*-NBA					0.1% *m*-NBA						
	charge-state distrib					charge-state distrib						
peptide	1+	2+	3+	4+	av charge state	1+	2+	3+	4+	5+	av charge state	diff in av charge state
IETMR	50	50			1.50							
KFWGK		100			2.00							
AWSVAR	63	37			1.37	32	68				1.68	0.31
GACLLPK	60	40			1.40	31	69				1.69	0.29
LVTDLTK	54	46			1.46	22	78				1.78	0.32
ATEEQLK	51	49			1.49	21	79				1.79	0.30
LSQKFPK	85	15			1.15							
LCVLHEK		83	17		2.17		46	54			2.54	0.37
AEFVEVTK	13	87			1.87	3	94	3			2.00	0.13
YLYEIAR	37	63			1.63	11	86	3			1.92	0.29
DLGEEHFK		100			2.00		53	47			2.47	0.47
LVVSTQTALA	60	40			1.40	18	82				1.82	0.42
QTALVELLK	5	95			1.95		100				2.00	0.05
NECFLSHK		85	15		2.15							
SHCIAEVEK		93	7		2.07		81	19			2.19	0.12
EACFAVEGPK							100				2.00	
CCTESLVNR		100			2.00							
KQTALVELLK		100			2.00		81	19			2.19	0.19
LVNELTEFAK	5	95			1.95		100				2.00	0.05
FKDLGEEHFK		13	87		2.87		4	72	24		3.20	0.33
ECCDKPLLEK		67	33		2.33		33	67			2.67	0.34
HLVDEPQNLIK		100			2.00		89	11			2.11	0.11
TVMENFVAFVDK		100			2.00							
SLHTLFGDELCK		36	64		2.64		17	78	5		2.88	0.24
RHPEYAVSVLLR		23	77		2.77			100			3.00	0.23
YICDNQDTISSK		100			2.00		97	3			2.03	0.03
TCVADESHAGCEK		57	43		2.43		35	65			2.65	0.22
ETYGDMADCCEK		100			2.00		85	15			2.15	0.15
EYEATLEECCAK		100			2.00		92	8			2.08	0.08
LGEYGFQNALIVR							100				2.00	
VPQVSTPTLVEVSR		100			2.00							
LKECCDKPLLEK		18	66	16	2.98			50	50		3.50	0.52
DDPHACYSTVFDK		59	41		2.41		22	78			2.78	0.37
DAFLGSFLYEYSR		100			2.00							
AEFVEVTKLVTDLTK									100		4.00	
LKPDPNTLCDEFK		27	73		2.73		7	93			2.93	0.20
KVPQVSTPTLVEVSR		18	82		2.82		8	92			2.92	0.10
QEPERNECFLSHK			100		3.00			61	39		3.39	0.39
YNGVFQECCQAEDK		100			2.00		38	62			2.62	0.62
ECCHGDLLECADDR		18	82		2.83		13	87			2.87	0.04
RPCFSALTPDETYVPK		18	82		2.82			100			3.00	0.18
NECFLSHKDDSPDLPK			90	10	3.10			61	39		3.39	0.29
CCAADDKEACFAVEGPK		23	77		2.77			100			3.00	0.23
QEPERNECFLSHKDDSPDLPK				100	4.00				60	40	4.40	0.40
HPEYAVSVLLRLAKEYEATLEECCAK									88	12	4.12	
average					2.20						2.59	0.25
standard deviation												0.15

by the addition of *m*-NBA is also of great importance when fragmenting with ECD/ETD since these techniques are not applicable to singly charged ions. CAD fragmentation of singly charged peptide ions is also known to produce limited backbone cleavages.

The change in charge state of the BSA peptides by *m*-NBA was also reflected by peptide ion charge states that were selected for ETD-MS/MS and identified by Mascot (Figure 3, right). With 0.1% *m*-NBA, 51% of the identified peptides had a charge state of ≥3+, while this figure was only 33% without the addition of *m*-NBA. By adding the highest obtained Mascot score for all different peptides under the two different conditions, the average peptide score improved from 41.9 to 48.1.

A closer inspection of the differences between ETD of 2+ and 3+ peptide ions was made (Figure 4a and b). The ETD fragmentation efficiency varies with the peptide mass and the charge state (Figure 4a). The data shown represent those BSA peptides that were sequenced and identified both as doubly and as triply charged species. For each peptide and charge state, the measurement of two independent data points were used. In all cases but one, triply charged peptides fragmented more efficiently than the corresponding doubly charged peptide. Interestingly, determined

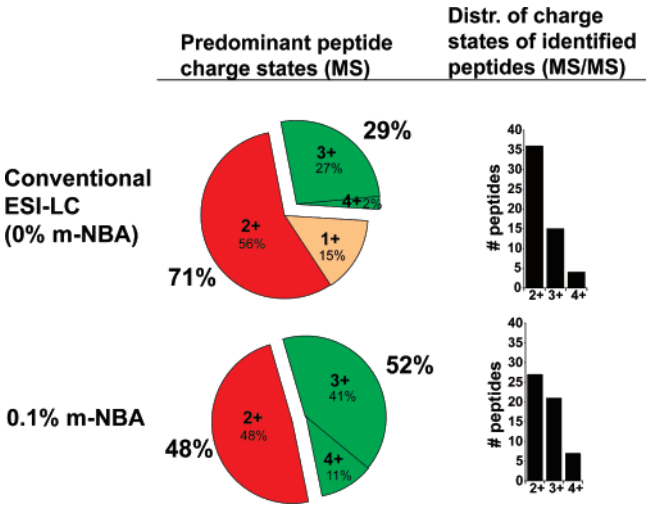

Figure 3. (Left) Pie chart showing the distribution of predominant charge states (MS mode) for identified BSA peptides and (right) distribution of charge states that were selected for ETD-MS/MS and successfully identified with Mascot, with and without addition of 0.1% *m*-NBA to the mobile phases.

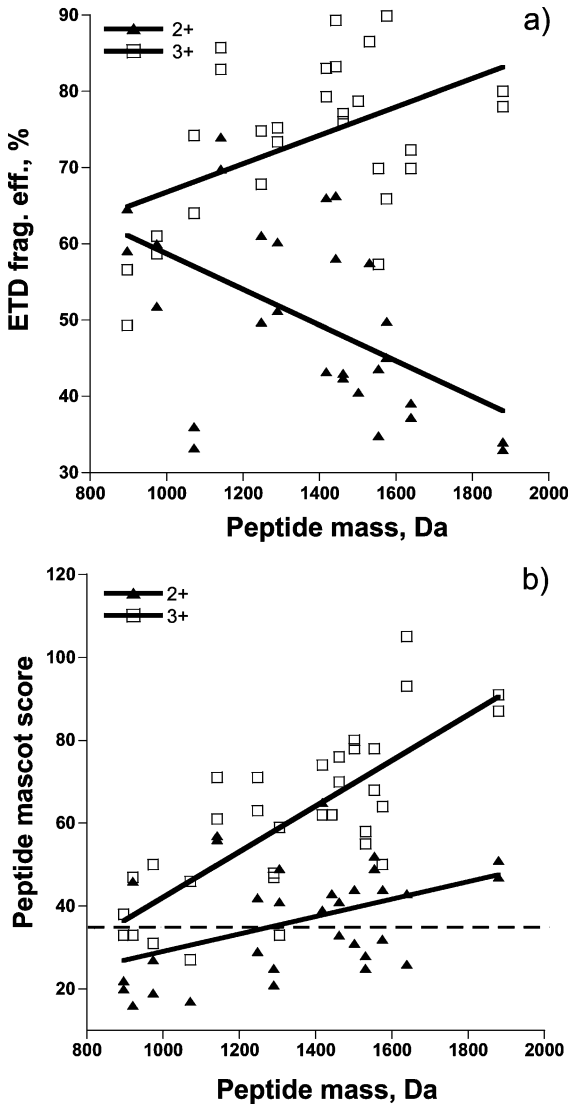

Figure 4. Display of the relationship between (a) the ETD fragmentation efficiency and the peptide mass and (b) the peptide Mascot score and the peptide mass. Results from parent 2+ and 3+ peptide ions are separated.

by the charge state of the peptides, two different trends are observed. The doubly charged peptides have a descending trend with increasing peptide mass, while the same peptides carrying three charges have an ascending trend with higher peptide mass. Lower ETD fragmentation efficiency for higher mass doubly charged peptides is consistent with previous reports.[22,24] The general higher fragmentation efficiency of triply charged peptides over doubly charged species is also expected. However, the ascending trend for the fragmentation efficiency of larger 3+ peptide ions is unexpected. This is because larger peptide ions in principle can form more noncovalent bonds, which should hamper the fragment dissociation. Although a direct comparison is difficult, our observation is also contrary to the descending trend in the sequence coverage for larger 3+ ions described in a recent study of ~1000 tryptic peptides.[29] This discrepancy is difficult to explain, and it is uncertain at this point whether this is due in part to poorer statistics (34 data points) of our study or to differences in the experimental design.

Figure 4b shows how the peptide Mascot score varies with the peptide mass and charge state. As expected, larger peptides are more specific probes than lower mass peptides for protein identification. This is because larger precursor ions have fewer potential peptide candidate matches in the protein sequence database.[28] Overall, product ions from triply charged precursor peptide ions result in much higher peptide scores than doubly charged precursor peptides. Again, the formation of complementary fragment ions as well as higher fragmentation efficiency adds to the higher confidence of protein identification. On average, the BSA peptides received a 68% (24.6 units) higher Mascot score for triply charged peptides than for the same doubly charged peptides. This makes ETD products from triply charged peptides more than 280 times more confident on average than their corresponding doubly charged ions. The steeper slope of the fitted line for 3+ peptide ions over 2+ peptide ions reflects the higher fragmentation efficiency of triply charged ions.

In practical terms, poor ETD fragmentation efficiency and subsequent lower Mascot peptide scores can have severe consequences in typical protein identification studies. For example, assume that the protein sample is unknown and the current data set is evaluated only by a Mascot-determined identification threshold of 35 (determined using the SwissProt (Other mammalian) protein sequence database). This would revoke 15 of 34 (44%) peptide identifications of 2+ peptide ions, whereas only 5 out of 34 (15%) peptide identifications based on 3+ peptide ions would be rejected.

Chromatography and ESI. The addition of *m*-NBA to the eluting mobile-phase solutions influences the retention time of peptides in reversed-phase chromatography. Figure 5 (left) shows the elution profiles (base peak chromatogram) of BSA peptides with 0 , 0.1 , and 0.5% *m*-NBA, respectively. *m*-NBA makes peptides elute earlier as a linear function of the added *m*-NBA concentration. In Figure 5 (right) is shown the retention time plotted against the *m*-NBA concentration for 19 different BSA peptides. For all peptides, a linear relationship was observed, which was confirmed by correlations ranging from $R = 0.983$ to $R = 0.999$. The effect of *m*-NBA to reduce the retention time was general for all peptides

Table 2. Chromatographic Peak Width (fwhm) and Peak Height for Three BSA Peptides Measured Using Mobile Phases Added 0, 0.1, and 0.5% *m*-NBA

	% *m*-NBA					
	0		0.1		0.5	
peptide	fwhm	height	fwhm	height	fwhm	height
YICDNQDTISSK	5.7	1.8×10^8	7.0	10^8	8.9	0.5×10^8
EYEATLEECCAK	5.0	1.1×10^8	6.3	0.9×10^8	8.2	3.2×10^7
HLVDEPQNLIK	6.0	10^8	6.1	0.4×10^8	6.3	2.8×10^7

studied, although not to the same extent. Since *m*-NBA by itself is hydrophobic, it could be speculated that the addition of *m*-NBA would make hydrophobic peptides elute earlier than hydrophilic peptides. However, no correlation ($R = 0.02$) could be established between the peptide hydrophobicity and the change in retention time upon *m*-NBA addition. The same conclusion was drawn for the relation between change in retention times and the pI values of peptides ($R = 0.01$). For instance, the peptide represented by (▲) GACLLPK (pI 9.0; hydrophobicity 0.86) and the peptide represented by (X) ATEEQLK (pI 4.1; hydrophobicity −1.35) show almost similar response to the *m*-NBA addition. This is despite the fact that the peptide ATEEQLK is acidic and hydrophilic, while the peptide GACLLPK is basic and hydrophobic. The only direct correlation that could be established was that peptides eluting early in the chromatogram were more affected by the addition of *m*-NBA than peptides eluting later. This was confirmed by the correlation analysis ($R = 0.86$) made on the peptide retention times without addition of *m*-NBA and the degree of response to *m*-NBA (measure as the slope in Figure 5 (right)). This observation suggests that *m*-NBA is competing with peptides for binding sites in the column material. As the gradient progresses, the mobile phase becomes more organic, which makes *m*-NBA bind less efficiently. As a consequence, the effect on peptide retention times is greatest in the beginning of the gradient where the mobile phase is more polar. The addition of *m*-NBA also influences other chromatographic parameters. In Table 2 is shown as an example the peak width (full width at half-maximum, fwhm) and the peak height for three BSA peptides. The values are representing the average of two consecutive injections using 0, 0.1, and 0.5% *m*-NBA. From Table 2, it is apparent that addition of *m*-NBA to some extent increases the peak width of the analytes consequently reducing the peak heights. Smaller peak intensities will reduce the sensitivity of the analysis. While this effect appears to be marginal with 0.1% *m*-NBA, it highlights the importance of using an *m*-NBA concentration that maximizes the charge-enhancing effect as well as keeping the chromatographic performance as close to the optimum as possible. Notably, the effect of *m*-NBA to increase the peak width is not observed to the same extent for the three studied peptides. This discrepancy is similar to the observation of reduced retention times of peptides by *m*-NBA.

The addition of *m*-NBA was found to increase the charge state for all peptides. However, the rather large spread 0.25 ± 0.15 (one standard deviation) (Table 1) reveals that different peptides respond differently to the *m*-NBA addition in terms of adopting higher charge states. To explain this phenomenon, we first tested the assumption that larger peptides can increase their relative charge state more than smaller peptides due to a more extended ion conformation and potentially greater charge stabilization. In that respect, the correlation between the peptide length and the increment in average charge states was only 0.07, showing no relationship. Furthermore, according to our study, the chemical properties of hydrophobicity and pI of the studied peptide ions had little or no effect on the charge-enhancing effect of *m*-NBA. This conclusion is based on the absence of correlation between the hydrophobicity ($R = 0.06$) and the pI value ($R = 0.04$) of the peptides and the relative change in charge state. Therefore, from these data it still remains unclear exactly what determines these differences in peptide charge-state increments. However, the missing correlation between the basicity and hydrophobicity of peptides and the different degree of charge increment is an interesting finding as it shows that the action of charge enhancement by *m*-NBA appears to be independent of two major chemical properties of tryptic peptides.

Improvement for Phosphopeptides. Phosphopeptides are generally more acidic species than their nonphosphorylated analogues. Because of that, it becomes even less likely that tryptic phosphopeptides retain more than two charges under conventional LC-ESI conditions. This can result in low fragmentation efficiency with ETD and in worst case ambiguous localization of the phosphorylation site(s). The ability to enhance the overall charge-state distribution of phosphopeptides will therefore have significant impact in functional phospho-proteomics. Therefore, we tested the effect of *m*-NBA on a number of tryptic phosphopeptides from α-casein (S1, S2) and β-casein. A summary of the results are presented in Table 3. The phosphopeptide sample was analyzed both with *m*-NBA (0.1%) and without the addition of *m*-NBA. As for nonphosphorylated peptides, no adverse effect was observed in the signal intensity for phosphopeptides by the addition of 0.1% *m*-NBA. The comparison shows that when no *m*-NBA was used only one out of nine phosphopeptides (YKVPQLEIVPNpSAEER) had some of the ion population in a charge state higher than 2+ (56% 3+). The equivalent number was six out of eight when 0.1% *m*-NBA was used in the mobile-phase solutions. The shift in the charge-state distribution ranged from moderate (0.10+) to significant (0.50+). For instance, the phosphopeptide VPQLEIVPNpSAEER shifted its ion charge-state distribution from 100% doubly charged to achieve 10% triply charged ions. And TVDMEpSTEVFTK went from 100% doubly charged ions with no *m*-NBA to 50% triply charged ion with 0.1% *m*-NBA. As expected, the shift to more triply charged phosphopeptide ions was beneficial in terms of the resulting ETD fragmentation efficiency. Overall, triply charged phosphopeptides had a 36−60% larger ETD fragmentation efficiency than doubly charged phosphopeptides. This resulted in overall improved peptide sequence coverage and greater peptide Mascot score. On average, the peptide Mascot score for phosphopeptides identified under the condition of no addition of *m*-NBA was 47, while the same number was 59 with the addition of 0.1% *m*-NBA. Assuming this trend holds for even larger data sets, phosphopeptides can be identified on average with 16 times higher confidence using this strategy of charge enhancement with ETD fragmentation.

CONCLUSIONS

ETD is a novel and gentle fragmentation technique with a great potential in modern proteome research. However, since the ETD

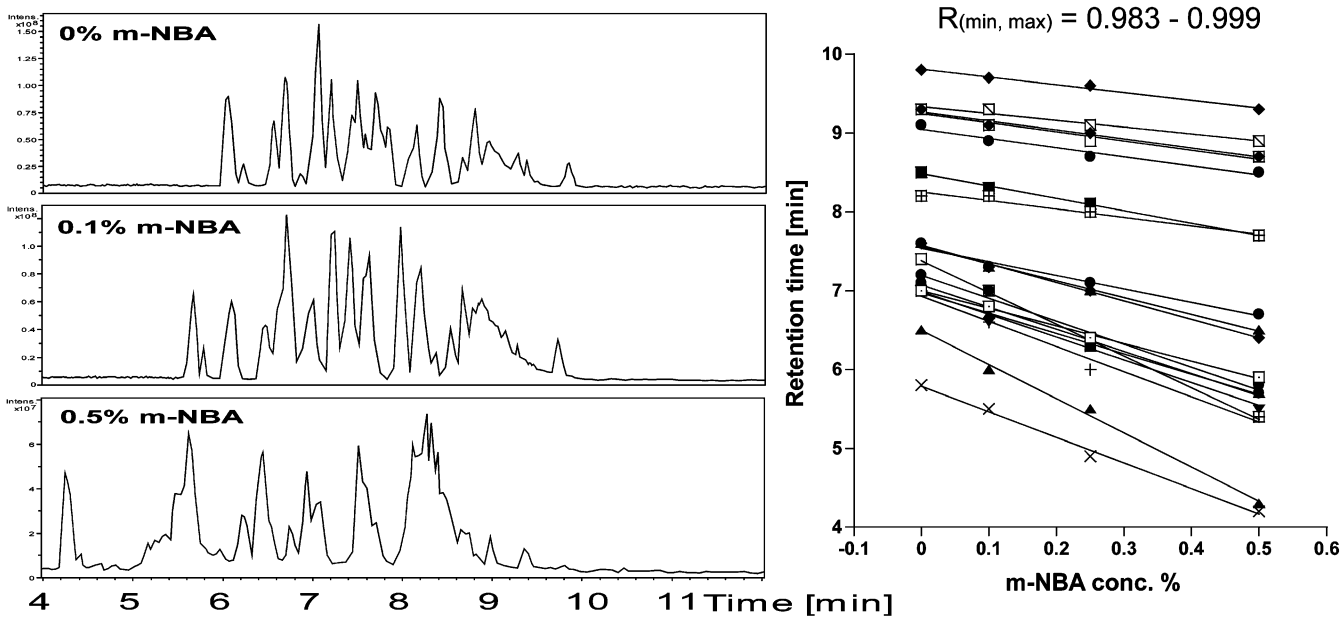

Figure 5. Left panel: Three chromatograms showing the base peak elution profile with addition of 0, 0.1, and 0.5% *m*-NBA, respectively. Right panel: the retention times of 19 BSA peptides plotted against the *m*-NBA concentration.

Table 3. List of Identified Phosphopeptides Using 0 and 0.1% *m*-NBA

	0% *m*-NBA						0.1% *m*-NBA					
peptide sequence	charge-state distrib	frag charge state	Frag Eff	Seq Cov	peptide Mascot score	total analyte abundance (arb units)	charge-state distrib	frag charge state	Frag Eff	Seq Cov	peptide Mascot score	total analyte abundance (arb units)
					α-Casein (S1)							
EKVNEL**pS**K	2+ (100%)	2+	42%	100%	26	1.5×10^5	not detect.	-	-	-	-	-
VPQLEIVPN**pS**AEER	2+ (100%)	2+	11%	46%	36	3.1×10^6	2+ (90%); 3+ (10%)	3+	71%	100%	83	5.0×10^6
DIGSE**pS**TEDQAMEDIK	2+ (100%)	2+	58%	53%	39	1.5×10^6	2+ (100%)	2+	48%	80%	33	4.8×10^5
DIG**pS**E**pS**TEDQAMEDIK	2+ (100%)	2+	35%	73%	73	1.5×10^6	2+ (75%); 3+ (25%)	3+	81%	100%	94	4.2×10^6
YKVPQLEIVPN**pS**AEER	2+ (44%); 3+ (56%)	3+	42%	73%	57	5.8×10^5	2+ (10%); 3+ (90%)	3+	68%	93%	63	8.5×10^6
					α-Casein (S2)							
EQLST**pS**EENSK	2+ (100%)	2+	43%	100%	58	3.8×10^5	2+ (83%); 3+ (17%)	2+	46%	72%	42	3.9×10^5
EQL**pS**T**pS**EENSK	2+ (100%)	2+	33%	90%	51	8.4×10^5	2+ (100%)	2+	46%	90%	60	8.0×10^5
TVDME**pS**TEVFTK	2+ (100%)	2+	29%	81%	57	1.8×10^6	2+ (50%); 3+ (50%)	3+	65%	100%	54	4.3×10^6
					β-Casein							
FQ**pS**EEQQQTEDELQDK	2+ (100%)	2+	29%	60%	30	5.7×10^6	2+ (32%); 3+ (68%)	3+	87%	67%	43	2.3×10^7
av peptide mascot score					47						59	

fragmentation efficiency is greatest for ≥3+ peptide ions, it presents a problem in proteomics strategies where protein mixtures are enzymatically digested with trypsin prior to MS analysis (bottom-up approach). This is because doubly charged peptide ions are the most abundant ion species from trypsin-digested proteins. Achieving higher (≥3+) charge-state peptide ions for efficient ETD fragmentation was the goal of the current study. We demonstrated the effect of *m*-NBA to shift the overall charge-state distribution of a variety of different tryptic peptides. We found that addition of just 0.1% *m*-NBA to the two mobile-phase solutions was optimal in terms of achieving high charge-state shifts without jeopardizing the chromatographic performance or increasing the chemical noise level. Fragmentation of ≥3+ peptides ions resulted in greater ETD efficiency and greater peptide identification confidence for both phosphorylated and nonphosphorylated peptides. Today, most mass spectrometers are using data-dependent acquisition where the most intense peptides ion species are picked for MS/MS. The overall charge increment by the addition of *m*-NBA increases the probability for ≥3+ peptide ions to be preferentially picked for peptide fragmentation. Furthermore, the use of charge-state enhancement will have even greater application with the coming implementation of ETD in orbitrap mass spectrometers. Besides increased ETD fragmentation efficiency, the accuracy of mass determination increases with lower m/z values. This is particularly important with larger-sized peptide or protein ions typically analyzed in the middle-down[39] or

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top-down[40] approach. Since the ion signal intensity is proportional to the ionic charge state in Fourier transforms mass spectrometry[41,42] (orbitrap and FT-ICR-MS), the generation of higher charge-state peptide/protein ions will increase their overall signal abundance.

The underlying mechanism of peptide charging is complex. The observed shifts in charge-state distributions of tryptic peptides confirm the charge-enhancing ability of *m*-NBA. However, most charge-enhancing studies so far have been focused on testing different additives on a subset of analytes, whereas this study has tested *m*-NBA on a great variety of tryptic peptides. Clearly, the effect of *m*-NBA to increase the relative average charge state varies for different peptides. However, this appears to be independent of the peptide length, hydrophobicity, and pI values of the peptides. In order to further illuminate and understand the mechanistic action of *m*-NBA, more detailed studies of the physical or chemical parameters of peptides that govern the relative charge enhancement are needed.

In conclusion, charge-state manipulation by using *m*-NBA will have significant impact on both single peptide/protein analysis as well as the outcome of large-scale proteomics studies where ETD is used for polypeptide fragmentation.

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