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Sulfur Pentafluoride is a Preferred Reagent Cation for Negative Electron Transfer Dissociation

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Abstract

Negative mode proteome analysis offers access to unique portions of the proteome and several acidic post-translational modifications; however, traditional collision-based fragmentation methods fail to reliably provide sequence information for peptide anions. Negative electron transfer dissociation (NETD), on the other hand, can sequence precursor anions in a high-throughput manner. Similar to other ion-ion methods, NETD is most efficient with peptides of higher charge state due to the increased electrostatic interaction between reacting molecules. Here we demonstrate that NETD performance for lower charge state precursors can be improved by altering the reagent cation. Specifically, the recombination energy of the NETD reaction—largely dictated by the ionization energy (IE) of the reagent cation—can affect the extent of fragmentation. We compare the NETD reagent cations of $C_{16}H_{10}^{*+}$ (IE = 7.9 eV) and SF_5^{*+} (IE = 9.6 eV) on a set of standard peptides, concluding that SF_5^{*+} yields greater sequence ion generation. Subsequent proteome-scale nLC-MS/MS experiments comparing $C_{16}H_{10}^{*+}$ and SF_5^{*+} further supported this outcome: analyses using SF_5^{*+} yielded 4,637 peptide spectral matches (PSMs) and 2,900 unique peptides, whereas $C_{16}H_{10}^{*+}$ produced 3,563 PSMs and 2,231 peptides. The substantive gain in identification power with SF_5^{*+} was largely driven by improved identification of doubly deprotonated precursors, indicating that increased NETD recombination energy can increase product ion yield for low charge density precursors. This work demonstrates that SF_5^{*+} is a viable, if not favorable, reagent cation for NETD, and provides improved fragmentation over the commonly used fluoranthene reagent.

Graphical abstract

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INTRODUCTION

Modern proteome characterization relies on liquid chromatography coupled with tandem mass spectrometry (nLC-MS/MS) to detect and quantify proteins from complex biological samples [1–5]. Despite continual advances in proteomic depth achieved in such experiments, these technologies do not detect all the proteins present in a sample and, in fact, typically only monitor a portion of those proteins which are detected. More specifically, one or two peptides resulting from the enzymatic digestion of a protein can map uniquely to the parent protein and allow for its unambiguous detection and quantification. New approaches that can offer increased diversity of peptides measured are therefore of considerable significance as they can reveal new proteins and offer access to portions of proteins that were previously not detectable. One factor that may limit the scope of present technology is the unilateral use of positive electrospray ionization. Many proteins, and portions of most proteins, are acidic and thus are more easily ionized in the negative mode [6–8]. Negative electrospray ionization can generate multiply deprotonated peptide anions, but the commonly used collision-based dissociation methods are ineffective at producing sequence-informative fragmentation of negatively charged peptides [9, 10]. These limitations have driven the development of alternative dissociation methods for peptide anions that utilize electrons, photons, and metastable atoms [11–30].

Scott McLuckey, the recipient of the Award for Distinguished Contributions in Mass Spectrometry whom we honor in this issue of JASMS, has been a pioneer in this field,

especially in the development of gas-phase ion-ion chemistry[31, 32]. Indeed, this investigation stems from foundational work his group and others described. Most notably, in 1995 McLuckey and co-workers first introduced ion-ion reactions of positive reagent cations with anionic oligonucleotides and have continued this innovating in this space[33–35]. Inspired by McLuckey's experiment, we developed ion-ion reaction chemistries to abstract electrons from multiply deprotonated peptide anions using singly charged reagent cations, termed negative electron transfer dissociation (NETD) [11]. NETD has emerged as one of the most promising peptide anion dissociation methods and has been successfully utilized in nLC-MS/MS experiments to access the acidic proteome[11, 12, 16, 17, 30].

One challenge of using NETD in large scale proteomic experiments is its limited product ion yield for low charge density precursors. In NETD experiments, peptide anions are oxidized by positively charged reagent cations to initiate dissociation and production of *a*[•]- and *x*-type product ions. Sometimes electron transfer from the anion to the cation occurs without concomitant dissociation (i.e., non-dissociative negative electron transfer, or NETnoD)[36–39]. NETnoD frequently occurs and is one of the primary causes for reduced product ion yields in NETD, especially in the case of low charge density precursors [39]. In these cases, once electron transfer has occurred, peptide backbone cleavage may be achieved; however, the resultant product ions can be held together by non-covalent bonding and detected as a charge-reduced product. To maximize the production of NETD product ions, a reduction of NETnoD species can be accomplished by supplying the charge-reduced product with more energy, either concurrent with, or post, electron transfer. The additional energy can disrupt the non-covalent interactions holding these ions together, yielding sequence informative products[40, 41]. One such approach, termed activated-ion NETD (AI-NETD), has been implemented by concurrently irradiating the ions with infrared photons as they are interacting; however, this approach requires the addition of an IR laser to the system[16, 30].

An alternate approach to increase the energy of the system during electron transfer events is to alter the reaction exothermicity, which is determined by the difference between the ionization energy of the reagent cation and the electron affinity of the peptide anion. For example, the ionization energy of fluoranthene, C₁₆H₁₀^{•+}, is 7.9 eV and the electron affinity of the carboxylate ion of a peptide is 3.4 eV, yielding a reaction enthalpy of 4.5 eV, known as the recombination energy. This energy surplus is redistributed into the peptide anion and drives fragmentation. Use of NETD reagent cations with higher ionization energies result in increased recombination energies and, potentially, an increase in NETD fragmentation efficiency [42]. The recombination energy of the reagent cation and its effect on NETD has been previously explored by Polfer and co-workers for use in determining phosphorylation sites on standard peptides[43], as well as by McLuckey et al. to investigate transition metal complexes and their interaction with peptide anions[44]. Polfer and colleagues compared two NETD reagents (fluoranthene and xenon) and determined that the increase in ionization energy of xenon led to considerable phosphate and side-chain neutral loss and therefore fluoranthene, having a lower ionization energy than xenon, should be used for sequencing phosphopeptides. Alternatively, McLuckey and co-workers showed that transition metal complexes yield both electron transfer as well as metal insertion reactions, allowing further control of the cation-anion interaction. To date, only xenon, fluoranthene, and phenanthroline complexes of Fe, Cu, and Co have been investigated as NETD reagent

cations for peptide analysis, and fluoranthene remains by far the most commonly used NETD reagent.

The primary motivation of this work is to investigate the hypothesis that increasing the ionization energy of the NETD reagent cation will increase the NETD conversion to product ions thereby yielding greater peptide identification rates and protein sequence coverage in large-scale shotgun proteome analyses. To test this hypothesis, we used a set of synthetic peptides to compare the NETD fragmentation efficiency of sulfur pentafluoride cations (SF_5^{*+} , IE = 9.6 eV) and fluoranthene cations ($\text{C}_{16}\text{H}_{10}^{*+}$, IE = 7.9 eV) over a range of available precursor charge states ($z = -2$ to -6). Concluding that SF_5^{*+} cations provided increased sequence ion production for low charge state precursors, we then performed nanoflow liquid chromatography-tandem mass spectrometry (nLC-MS/MS) experiments employing high pH separations and optimized NETD reaction kinetics to compare $\text{C}_{16}\text{H}_{10}^{*+}$ to SF_5^{*+} NETD reagent ions for analysis of a complex mixture of yeast peptides. From these data we revealed that up to 40% more peptide spectral matches (PSMs) could be made when using reagent ions from sulfur pentafluoride compared to fluoranthene. The overall peptide spectral match and unique peptide identification numbers improved 30% when using SF_5^{*+} as the NETD reagent instead of fluoranthene. From the data, we conclude that the use of SF_5^{*+} offers a direct route to boosting the performance of NETD dissociation.

EXPERIMENTAL PROCEDURES

NETD on standard peptides

Synthetic peptides have the sequences SVFAVNWISYLASK, EEAQALEDLTGFK, and ELVNDDEDIDWVQTEK were obtained from New England Peptides (Gardner, MA) and were individually suspended in 3:1 methanol/water with 5 mM piperidine to a concentration of 10 ppm. The peptides were infused into a LTQ Velos mass spectrometer (Thermo Fisher Scientific, San Jose, CA) modified to perform NETD. For each peptide precursor, a 0 nce CAD MS/MS scan was performed, followed by a series of NETD MS/MS scans with increasing reaction time, encompassing the 2τ time point (see Supplementary Figure 1 for further experimental design diagram). This series was repeated at least 15 times. Once complete, the next precursor charge state was reacted until all accessible charge states were reacted. The precursor AGC target was set to 10,000 and the reagent AGC target was set to 1,000,000 for all MS/MS acquisitions. The q-value for the NETD reaction was kept at 0.4 for all experiments. This procedure was repeated using both SF_5^{*+} and $\text{C}_{16}\text{H}_{10}^{*+}$ reagent cations. The solid phase NETD reagent fluoranthene was introduced to the system using the standard glass vials contained in the ETD module's reagent vial heater. The gaseous sulfur hexafluoride reagent was introduced to the system by connecting the high purity SF_6 gas cylinder (Concorde Specialty Gases, Eatontown, NJ) to a precision regulator (Porter Instruments, Hatfield, PA) with 6 feet of 1/8 inch outer diameter and 0.065 inch inner diameter copper tubing. Then a 100 μm inner diameter capillary tube was attached to the out port of the precision regulator and fed directly into the vacuum manifold of the ETD module and the pressure reading of the precision regulator was adjusted to generate appropriate reagent cation signal (~ 2 psi). Reagent cations were generated via the EI/CI source in the presence of nitrogen gas, and reagent signal was optimized by varying lens voltages using an

automated calibration routine. The filament emission current was set to 70 μ A for both reagents. The nitrogen gas pressure was also optimized for maximum signal for each reagent cation. For fluoranthene, the reagent vial temperature was set to 108° C. For both reagents, the ion source, transfer line, and restrictor temperature were held at 160° C. The fluoranthene reagent produced ion radicals $C_{16}H_{10}^{*+}$, while the predominant cation for sulfur hexafluoride was the fluorine-loss species SF_5^{*+} , shown in Supplementary Figure 2.

Yeast sample preparation

Tryptic yeast (*Saccharomyces cerevisiae*) peptides were prepared as previously described[16]. Briefly, cultured yeast cells were lysed by glass bead milling (Retsch GmnH, Germany), and proteins were reduced and alkylated using 5 mM dithiothreitol and 15 mM iodoacetamide, respectively. Trypsin digestion was performed during an overnight incubation at room temperature with a 1:50 (w/w) enzyme to protein ratio. A second trypsin addition was done the following morning at 1:100 (w/w) enzyme to protein ratio for 1 hour, followed by desalting over a C18 SepPak (Waters Corporation, Milford, MA).

High pH nLC-MS/MS

An ETD-enabled hybrid dual cell-quadrupole ion trap-Orbitrap mass spectrometer (Orbitrap Elite, Thermo Fisher Scientific) coupled to a nanoACQUITY UltraPerformance liquid chromatograph (Waters) was used for the nLC-MS/MS analyses. The mass spectrometer was modified to perform NETD as described previously[41, 45–47]. Briefly, the higher-energy collisional dissociation cell (HCD) was replaced with a multi-purpose dissociation cell (MDC) that can conduct ion-ion reactions, allowing for NETD to be performed within. Fluoranthene and sulfur hexafluoride reagents were introduced as is described above, with the exception of the SF_6 pressure being adjusted to 10 psi. The solvent composition for liquid chromatography were mobile phase A (5 mM piperidine in water) and mobile phase B (5 mM piperidine in 85% ACN and 15% water). The reverse phase columns were prepared in house using 75 μ m ID, 360 OD bare fused silica capillary tubing packed to a 30 cm length with 3.5 μ m, 130 Å pore size, Ethylene Bridged Hybrid C18 particles (Waters). For each analysis, 1 μ g of yeast digest was loaded onto the column equilibrated with 95% A at 400 nL/minute. The gradient elution was performed at 400 nL/min increasing from 5% mobile phase B to 30% B over 70 minutes, followed by an increase to 70% B at 76 minutes and a wash at 70% B for 4 more minutes. Peptides were ionized in the negative mode using electrospray ionization with a spray voltage of –1.5 kV. The inlet capillary temperature was set to 300° C. Survey MS scans were analyzed in the Orbitrap mass analyzer with a resolving power of 60,000 at 400 m/z and an AGC precursor ion target value of 1,000,000 over a mass range of 300–1250 m/z. Data dependent MS/MS events were triggered off of the 10 most intense peaks in the survey scan. Each MS/MS scan used a precursor AGC target of 100,000 ions and was analyzed in the Orbitrap with a resolving power of 15,000 at 400 m/z. Precursors were isolated at ± 0.9 Th and an exclusion window of ± 10 ppm was created around the monoisotopic peak of the precursor for 45 seconds. All nLC-MS/MS experiments were performed in duplicate.

Data analysis

Peptide standard infusion data was searched using an in-house C# script, which extracted ion current intensities directly from raw data files. These values were normalized relative to the ion current of the precursor from a 0 nce CAD scan collected previous to the NETD reacted spectra (see Supplementary Figure 1 for further experimental design diagram). The nLC-MS/MS raw data files were searched using the Open Mass Spectrometry Search Algorithm (OMSSA), modified to allow for anionic peptide $a^{\bullet-}$ and x -type fragment ions to be searched. Search parameters included carbamidomethylation of cysteine as a fixed modification and oxidation of methionine as a variable modification[17, 48]. A multi-isotope search was employed using three isotopes with a mass tolerance of ± 125 ppm for the precursors and a monoisotopic mass tolerance of ± 0.02 Da for product ions. Three missed cleavages were allowed for the trypsin digestion. The data processing was done through the COMPASS software suite designed for OMSSA searching. A UniProt database for *Saccharomyces cerevisiae* (downloaded September 29, 2014) was concatenated with reversed sequences and used to determine peptide spectral matches (PSMs). Scored spectra were filtered using a false discovery rate of 1% at the unique peptide level. False discovery rates for spectra from each set of duplicate nLC-MS/MS experiments were calculated for the combined set of spectra as opposed to separate calculations for each nLC-MS/MS run. Additionally, prior to OMSSA searching, each spectrum was preprocessed to remove the unreacted precursor ion (± 3 Da) and neutral loss ions of the oxidized precursor by removing ions within a window of 55 to 5 Da below the oxidized precursor ion. The nLC-MS/MS experiments were also searched using an inhouse C# script to extract ion intensities for expected sequencing ions as well as ions resulting from neutral losses from both oxidized precursor ions and $a^{\bullet-}$ and x -type product ions. Ion intensities from spectra in nLC-MS/MS experiments were normalized relative to the total ion current (TIC) of each individual MS/MS scan.

RESULTS AND DISCUSSION

NETD of standard peptides with alternative reagent cations

The primary metric in determining an effective reagent cation is the production of sequence informative fragment ions relative to all other product ions produced. In these experiments, reagent ions $C_{16}H_{10}^{++}$ and $SF_5^{\bullet+}$ were tested for their effectiveness as NETD reagent cations using an ETD-enabled dual-pressure linear ion trap mass spectrometer. These species were reacted with three standard peptides (sequences given in top left of Figure 1), each with a C-terminal lysine to mimic those yielded from protein digestion with trypsin, and all were synthesized without additional post-translational modifications. The peptides had lengths of 13, 14, and 16 amino acid residues, isoelectric points of 3.62, 4.00, and 8.31, and generated peptide anions having charge states ranging from $z = -2$ to -6 .

To provide a straightforward comparison of their product ion generation efficiencies the extent of reaction for the two reagent cations was carefully controlled. To do so, we standardized several conditions that dictate the number of electron transfer events that occur per reaction, isolating the chemistry of the reagent cations as the main variable of the experiment. The rate of ion-ion reactions, and thus the number of electron transfer events,

are governed by a number of parameters including ion population, reaction q-value, reaction cell architecture, and reaction time. Previous work has shown that to maximize peptide identifications, the ideal electron transfer extent occurs when the precursor ion population has been reduced by 86% [49]. Such reaction conditions minimize the amount of secondary electron transfer while still offering sufficient sequence informative fragment ion production. Using this as a model, we created a method, illustrated in Supplementary Figure 1, where a series of increasing reaction times were employed surrounding the optimal reaction extent (13.5% unreacted precursor remaining), keeping all other reaction parameters constant. Figure 1 summarizes the production of sequence informative *a*^{•+}- and *x*-type fragment ions produced when all accessible charge states of the three standard peptides were reacted with SF₅^{•+} or C₁₆H₁₀^{•+}. Use of SF₅^{•+} as the reagent cation more than quadrupled the *a*^{•+}-type ion signal relative to C₁₆H₁₀^{•+} for all doubly deprotonated precursors. An increase in *x*-type fragment ion production was observed for two out of three doubly deprotonated precursors.

As charge density increased, the difference in fragment ion production was reduced. In the case of triply deprotonated precursors, SF₅^{•+} generated spectra with more *a*^{•+}-type fragment ions while use of C₁₆H₁₀^{•+} cations produced more *x*-type fragment ions, but the magnitude of the difference is considerably less than for the lower charge state precursors. Generally, the C₁₆H₁₀^{•+} reagent cation produced spectra with marginally more *x*-type fragment ions for higher charge state species, but the product ion signal is largely comparable between the two reagent cations for *z* ≥ 3 precursors. This suggests that the higher IE of SF₅^{•+} can benefit fragmentation of low charge density precursors (i.e., *z* = -2) where the predominance of NETnoD can adversely affect dissociation product ion generation. Note, however, SF₅^{•+} reagent cations retain the good performance of C₁₆H₁₀^{•+} for more highly charged ions. Supplementary Figure 3 also considers the distribution of even and odd electron fragment ions (odd electron species containing a radical electron and an additional hydrogen atom), showing that *a*^{•+}-type and *x*-type fragment ions are the predominant species formed upon NETD, and that there is no significant difference in the even and odd electron ratios between the two reagent cations.

nLC-MS/MS of yeast tryptic digest using SF₅^{•+} and C₁₆H₁₀^{•+}

To expand the scope of our study, we compared the performance of C₁₆H₁₀^{•+} and SF₅^{•+} as reagent cations in nLC-MS/MS analyses of peptides derived following tryptic digestion of yeast proteins. Importantly, the majority of precursors sampled in negative mode nLC-MS/MS experiments are doubly deprotonated [16], suggesting the use of SF₅^{•+} reagent cations could improve the depth of analysis in whole-proteome shotgun sequencing. Indeed, in 90-minute nLC-MS/MS experiments, NETD with SF₅^{•+} generated 30% more PSM and unique peptide identifications than did NETD using C₁₆H₁₀^{•+} (Figure 2). Figure 2b displays the charge state distribution of peptide spectral matches (PSMs) following use of the two NETD reagents. We note a substantial increase in the identification of *z* = -2 peptides for analyses with SF₅^{•+} (1,039 more *z* = -2 PSMs than those with C₁₆H₁₀^{•+}), which comprised more than 70% of all PSMs identified in either dataset. This finding is consistent with the previous results using standard peptides, further showing that peptides with lower charge density benefit more from excess recombination energy, while higher charge density peptides are not as impacted.

Figure 3 presents representative spectra from the nLC-MS/MS experiments illustrating the improved fragmentation afforded by use of SF_5^{*+} reagent cations as compared to $\text{C}_{16}\text{H}_{10}^{*+}$. For each spectrum, the doubly deprotonated precursor of the peptide ETAESYLGAKE was reacted with either NETD reagent to a reaction extent of 37.9% and 38.4% for $\text{C}_{16}\text{H}_{10}^{*+}$ and SF_5^{*+} , respectively. Reaction extent is defined as the ion current of the unreacted precursor divided by the total ion current of the scan. While in the standard peptide infusion data a 0 nce CAD scan was used to measure the total ion abundance for each peptide and fragment ion currents could be normalized to it, allowing direct calculation of the precursor to product ion conversion ratio, in the discovery nLC-MS experiments no 0 nce CAD scans were performed and therefore this normalization approach was not possible, and reaction extent was calculated instead. Comparing spectra with similar reaction extents ensures that the main contributor to the difference is the ion chemistries of the reagents themselves. The spectra show many similarities but with considerably more product ions generated when SF_5^{*+} was used. Specifically, SF_5^{*+} produced two more *a*⁺-type and three more *x*-type fragment ions than the corresponding NETD spectrum produced when $\text{C}_{16}\text{H}_{10}^{*+}$ was used as the reagent, and it also generated three CO_2 neutral losses from *x*-type fragment ions compared to only one when $\text{C}_{16}\text{H}_{10}^{*+}$ cations were used. In all, use of SF_5^{*+} as the reagent cation produced a spectrum with 100% peptide sequence coverage for ETAESYLGAKE while the corresponding spectrum when $\text{C}_{16}\text{H}_{10}^{*+}$ was used as the reagent yields only 55.5% coverage. Note, we define peptide sequence coverage as the ratio of the number of inter-residue positions broken to the total possible positions (residue length – 1) for a given sequence, expressed here as a percentage.

Figure 4a expands on the change in peptide sequence coverage between reagent cations SF_5^{*+} and $\text{C}_{16}\text{H}_{10}^{*+}$ by showing the composite difference for all peptides identified in the NETD experiments. Only peptides found in both data sets were considered, and of the 1,427 peptides in common, 812 peptides yielded an increase in sequence coverage when SF_5^{*+} was used as the NETD reagent while only a fourth of that ($n = 202$) showed an increase when $\text{C}_{16}\text{H}_{10}^{*+}$ was used. On average, SF_5^{*+} accounted for a nearly 10% improvement in peptide sequence coverage for all overlapping peptides.

Differences in peptide sequence coverage are even more pronounced when delineating across charge states. Figure 4b displays peptides categorized by charge state, where charge states of $z = -2, -3, -4$, and -5 showed an average sequence coverage difference of 9.0%, 4.7%, 2.8%, and -4.7% respectively. Note, as indicated in the Sequence Coverage equation at the top of Figure 4, a positive value indicates higher sequence coverage with SF_5^{*+} . Interestingly, $\text{C}_{16}\text{H}_{10}^{*+}$ as the NETD reagent only performs better on $z = -5$ precursors, which account for 1.3% of the total precursors sampled in the experiments. In all other cases, peptide sequence coverage was improved by use of SF_5^{*+} , especially for lower precursor charge states. Supplementary Figure 4 considers the impact of reagent cation on total protein sequence coverage for all proteins in common between the two data sets, as the increased number of peptide identifications with SF_5^{*+} translates to high coverage of the proteins mapped in the experiments. Here protein sequence coverage is defined as the number of amino acid residues comprising each identified peptide divided by all amino acid residues in the protein sequence. Use of SF_5^{*+} improved coverage of 298 proteins, $\text{C}_{16}\text{H}_{10}^{*+}$

improved coverage of 125 proteins, and 84 showed no difference between the reagent cations, with an average improvement of 3.1% in favor of SF_5^{*+} .

Comparison of neutral losses

Utilizing the higher ionization energy SF_5^{*+} reagent cation yields greater fragmentation in regards to sequence informative fragment ions. As the Polfer group noted with xenon, however, high ionization energies can drive the production of neutral losses from fragment ions. Using the same pool of doubly deprotonated peptides in common between the $\text{C}_{16}\text{H}_{10}^{*+}$ and SF_5^{*+} data sets discussed above, Figure 5 examines how the oxidized (i.e., charge reduced) precursor ions and their associated neutral losses are affected by the use of the two reagent cations. Figure 5a displays the average percent ion current accounted for by the oxidized precursor as well as the signal from corresponding neutral losses of CO_2 and either NH_3 or OH from this charge-reduced species. Doubly charged peptide precursors reacted with $\text{C}_{16}\text{H}_{10}^{*+}$ show on average 8.5% more CO_2 neutral loss while the SF_5^{*+} data yielded greater NH_3 and OH neutral loss. Figure 5b compares the abundance of amino-acid specific side chain neutral losses from the oxidized precursor [12]. The predominant differences are found in methionine, serine, and threonine residues. Interestingly, methionine and serine show a substantial increase in neutral loss when $\text{C}_{16}\text{H}_{10}^{*+}$ is used, despite the lower recombination energy relative to SF_5^{*+} . However, in total, very little difference is found in amino-acid specific side chain neutral loss when comparing the two NETD reagent cations.

Lastly, we investigated the $a^{\bullet}$ - and x -type product ions generated in these experiments, including how CO_2 neutral losses from product ions differed between the two reagent cations. Figure 6a provides a holistic look at the total number of $a^{\bullet}$ - and x -type fragment ions produced in the NETD datasets. As shown with our standard peptide data above, SF_5^{*+} yields more fragment ion species than $\text{C}_{16}\text{H}_{10}^{*+}$, particularly more $a^{\bullet}$ -type fragments, with an increase of 32%. Figure 6b shows the percentage of those sequence ions which also yield a CO_2 neutral loss peak. SF_5^{*+} cations generated 5.4% more x -type ion species with CO_2 neutral losses. To investigate how the occurrence of these neutral losses from sequencing ions impacts data analysis, we searched our yeast peptide database allowing for a variety of different fragment ion combinations. No combination of product ions and neutral losses yielded a greater number of PSMs when searching the SF_5^{*+} dataset than just $a^{\bullet}$ - and x -type fragment ions alone, which concurs with the same analysis performed on the fluoranthene dataset (not shown). We conclude that the amount of CO_2 neutral loss is not significant enough to impact or adversely affect automated spectral annotation.

CONCLUSION

NETD provides direct access to analysis of proteomes in the negative mode, but improvements are still needed to make NETD amenable to the large proportion of low charge density precursors generated in whole-proteome analyses. In this experiment, we sought to improve peptide anion fragmentation using SF_5^{*+} , a reagent cation with a higher IE of 9.6 eV, compared to $\text{C}_{16}\text{H}_{10}^{*+}$, the most common NETD reagent cation with an IE of 7.9 eV. Using peptide fragmentation efficiency and unique peptide identifications as our

primary metrics, we determined that SF_5^{*+} significantly improves shotgun proteomic analyses with NETD, especially considering the fragmentation of doubly deprotonated precursors. We contribute this gain in identification power to the increase in ionization energy for SF_5^{*+} compared to $\text{C}_{16}\text{H}_{10}^{*+}$, which impacts the exothermicity of the ion-ion reaction and provides greater fragment ion yield and less non-dissociative negative electron transfer. The predominant gain in identification for low charge density precursors may also be useful when considering protein digestion using proteases other than trypsin, such as Lys-C, which often yields longer, less charge-dense peptides. Another benefit of using a gaseous reagent for NETD is the simplification of the ion source as no reagent vial heaters or heated transfer lines are required to volatilize and transfer the solid fluoranthene reagent.

While SF_5^{*+} appears to be a favorable choice for large-scale negative mode proteomics analyses, its use in other types of NETD applications may benefit as well. While previous work has shown that lower recombination energy NETD reactions reduces the occurrence of labile PTM neutral loss [43], many other molecules might benefit from the more energetic reaction. In addition to peptides, NETD has been useful in the study of polynucleotides[35] and carbohydrates[50–53] and further improvements may be obtained through the utilization of SF_5^{*+} as the reagent cation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Comparison of product ion signal from NETD reactions using reagents $C_{16}H_{10}^{*+}$ and SF_5^{*+} for all precursor charge states of three synthetic peptides (sequences shown top left). The intensities of both *a*⁺- and *x*-type fragment ions were normalized to the total ion current of the unreacted precursor from a preceding scan as explained in the text. Following this normalization, the $\log_2$ fold change of the normalized fragment ion current intensity was calculated between SF_5^{*+} and $C_{16}H_{10}^{*+}$. All precursor ion populations were reacted to the 2τ time point (i.e., $13.5 \pm 3\%$ unreacted precursor ion current remaining in the MS/MS spectrum).

Figure 2. Summary of peptide spectral matches (PSM) and unique peptide identifications from nLC-MS/MS experiments using a yeast tryptic digest (a). Experiments were conducted using either SF_5^+ or $\text{C}_{16}\text{H}_{10}^+$ as reagent cations. The charge state distributions of successfully sequenced PSMs from these experiments are also shown (b), revealing a significant increase in $z = -2$ peptides identified with SF_5^+ .

Figure 3.

Single-scan spectra for the NETD fragmentation of the peptide ETAESYLGA K , $z = -2$. The unreacted precursor signal accounts for 37.9% and 38.4% of the total ion current in each MS/MS scan for $\text{C}_{16}\text{H}_{10}^{*+}$ and SF_5^{*+} , respectively, indicating both precursors were reacted to similar extents. Use of SF_5^{*+} as the NETD reagent provided greater sequence coverage than $\text{C}_{16}\text{H}_{10}^{*+}$, yielding 11 sequence informative fragment ions compared to 5 for $\text{C}_{16}\text{H}_{10}^{*+}$. The NETD reagent SF_5^{*+} produced three CO_2 product ion neutral loss species while the fluoranthene spectrum only contains a single CO_2 product ion neutral loss fragment. Both spectra were acquired in the nLC-MS/MS experiments and represent a single scan (*i.e.*, un-averaged).

Figure 4.

(a) Percent peptide sequence coverage was calculated for each of the 1427 peptides in common between the $\text{SF}_5^{\bullet+}$ and $\text{C}_{16}\text{H}_{10}^{\bullet+}$ analyses. The difference between peptide sequence coverage with $\text{SF}_5^{\bullet+}$ and $\text{C}_{16}\text{H}_{10}^{\bullet+}$ ($\Delta \text{Peptide Sequence Coverage}$) was calculated for each peptide, and the distribution of the $\Delta \text{Peptide Sequence Coverage}$ values are shown. The orange distribution shows peptides with greater sequence coverage with $\text{SF}_5^{\bullet+}$ ($n = 812$), and the blue shows peptides with better sequence coverage with $\text{C}_{16}\text{H}_{10}^{\bullet+}$ ($n = 202$). Panel (b) shows the average $\Delta \text{Peptide Sequence Coverage}$ for all peptides in common between the two analyses as a function of peptide precursor charge state. The number of precursors averaged is shown in black text above the bar for each charge state. Concordant with previously shown data, lower charged precursors benefit most from the use of $\text{SF}_5^{\bullet+}$.

Figure 5.

(a) The average percent of total ion current accounted for by the oxidized precursor ion and neutral losses of CO₂ and either NH₃ or OH from the oxidized precursor ion are shown for $z = -2$ PSMs from C₁₆H₁₀^{•+} (blue) and SF₅^{•+} (orange) nLC-MS/MS analyses. (b) The average percent of total ion current is shown for side chain neutral losses from the oxidized precursor ion for $z = -2$ PSMs. In order for a spectrum to be searched for a given side chain loss, the PSM sequence had to contain that residue. Note, amino acids are organized by their side chain properties: non-polar (yellow), polar (green), acidic (red), and basic (blue).

Figure 6.

(a) The number of total *a*[•]- and *x*-type fragment ions generated from the peptides common to both C₁₆H₁₀^{•+} (blue) and SF₅^{•+} (orange) analyses are shown. For both fragment ion types SF₅^{•+} produces the greater number of sequencing ions and the percent gains over the number of ions from C₁₆H₁₀^{•+} analyses are shown in bold. Panel (b) compares the percentage of *a*[•]- and *x*-type product ions from Panel (a) that have a corresponding neutral loss of CO₂. (c) Despite the small increase in CO₂ neutral losses observed, incorporating CO₂ neutral losses from *a*[•]- and *x*-type product ions as fragment ion types to query in a database search does not improve peptide identifications over using standard *a*[•]- and *x*-type product ions only for the SF₅^{•+} data. The combination of product ion types used in the database searches are shown in black at the top, and the number of identified peptide spectral matches are shown in orange at the bottom. Similar results were obtained with the C₁₆H₁₀^{•+} analyses (data not shown).