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Pulsed Nanoelectrospray Ionization Boosts Ion Signal in Whole Protein Mass Spectrometry

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Abstract: Electrospray ionisation (ESI) is renowned for its ability to ionise intact proteins for sensitive detection by mass spectrometry (MS). However, the use of a conventional direct current ESI voltage can result in the formation of relatively large initial droplet sizes, which can limit efficient ion desolvation and sensitivity. Here, pulsed nanoESI (nESI) MS using nanoscale emitters with inner diameters of ~250 nm is reported. In this approach, the nESI voltage is rapidly pulsed from 0 to ~1.5 kV with sub-nanosecond rise times, duty cycles from 10 to 90%, and repetition rates of 10 to 350 kHz. Using pulsed nESI, the performance of MS for the detection of intact proteins can be improved in terms of increased ion abundances and decreased noise. The absolute ion abundances and signal-to-noise levels of protonated ubiquitin, cytochrome C, myoglobin, and carbonic anhydrase II formed from standard denaturing solutions can be increased by up to 82% and 154% using an optimal repetition rate of ~200 kHz compared to conventional nESI-MS. Applying pulsed nESI-MS to a mixture of four proteins resulted in the signal for each protein increasing by up to 184% compared to the more conventional nESI-MS. For smaller ions ($\leq 1032\ m/z$), the signal can also be increased by the use of high repetition rates (200–250 kHz), which is consistent with the enhanced performance depending more on general factors associated with the ESI process (e.g., smaller initial droplet sizes and reduced Coulombic repulsion in the spray plume) rather than analyte-specific effects (e.g., electrophoretic mobility). The enhanced sensitivity of pulsed nESI is anticipated to be beneficial for many different types of tandem mass spectrometry measurements.

Keywords: electrospray ionisation; nanoelectrospray; proteins; alternating current; top-down

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1. Introduction

Electrospray ionisation (ESI) is well known for its ability to form intact protein ions for sensitive detection by mass spectrometry [1]. For large biomolecules, a key characteristic of ESI is the formation of a distribution of highly charged ions [2,3]. This multiple charging effect has many advantages. High charging extends the effective mass range of instruments with upper m/z limits, such that proteins can be detected on essentially any type of ESI-equipped mass spectrometer [4]. For charge-sensitive mass analysers, the instrument response increases linearly with the charge state of the ion and thus, more highly charged ions can be detected with higher sensitivity and lower detection limits [5]. Moreover, protein ions that are formed with higher ion abundances and more extensive charging typically yield richer product ion spectra, corresponding to increased information regarding the sequence of the protein and any post-translational modifications. For example, in electron capture dissociation (ECD) [6–8], electron transfer dissociation (ETD) [9,10], and some types of ultraviolet photodissociation (UVPD) [11,12], the extent of the ion dissociation and

sequence coverage can increase significantly with both the charge state and the abundance of the precursor ion.

In 'top-down' MS, intact protein ions are often formed from denaturing solutions that are acidified and contain an organic modifier. Such solutions facilitate the elongation of the protein ions' conformations, which have higher surface areas and more exposed basic sites and can thus accommodate higher charge states than protein ions formed from more 'native-like' solutions [13]. However, a challenge with top-down MS is that, in ESI, charge state distributions tend to be broad, which effectively distributes the protein signal across multiple detection channels [8]. Moreover, the use of ECD, ETD, and/or UVPD can result in the formation of hundreds of product ions, further partitioning the ion signal and reducing signal-to-noise levels [14]. Thus, methods that can be used to increase the abundances of whole proteins formed by ESI are desirable.

In ESI-MS, the extent of ion charging, sensitivity, and detection limits depends on many factors including solution composition, emitter size and geometry, and instrumental factors. For example, the use of chemical additives in ESI solutions have been demonstrated to increase the charge states of proteins and peptides, which can improve the efficiency of MS-based proteomic workflows, in an approach termed 'supercharging' [2]. A number of different supercharging additives have been reported, including *m*-nitrobenzyl alcohol [15], dimethyl sulfoxide (DMSO) [16], sulfolane [17], and cyclic alkyl carbonates [7,9,14,18–20] such as 1,2-butylene carbonate (C2). Our group has demonstrated that the latter class of additives can be used to form positively charged proteins in higher charge states than by use of other additives [4,6], and such highly charged protein ions are sufficiently reactive that they can protonate Ar (g) and N₂ (g) in thermal, ambient temperature ion–molecule reactions [18]. Kuster and co-workers reported that DMSO can be doped into the eluent in LC-MS/MS to significantly increase the number of proteins and peptides identified in whole-cell digests by 10–25%, resulting in an improvement of the signal for peptide ions in bottom-up proteomics by up to ten fold [19].

The use of ESI at low flow rates (mid-to-low nL/min) and with narrow ion emitter capillaries (i.e., nanoelectrospray ionisation, nESI) in MS can be highly beneficial in the analysis of biomolecules [20–22]. The use of lower solution flow rates and narrow emitters in the range of nL/min can be used to form initial droplets that are an order of magnitude smaller than those in more conventional ESI [23,24]. The use of narrower tips lowers the voltage required to initiate ESI by more effectively concentrating the electric field at the emitter tip, and it reduces sample consumption resulting in initial ESI droplets with very high surface-to-volume ratios [25,26]. Such droplets can more readily desolvate and be transferred through narrow conductance apertures to under the vacuum of an atmospheric pressure interface to a mass spectrometer, thereby improving its sensitivity [27,28]. Moreover, the use of nanoscale ion emitters with inner diameters of less than 1 µm can significantly reduce the extent of the adduction of non-volatile salts and non-volatile molecules to protein ions [29,30], including those of protein–ligand complexes formed from native-like solutions, which can facilitate the accurate measurement of ligand–protein binding constants [31–33]. In addition, a number of instrument modifications have been developed to increase the efficiency of the transfer of ions from atmospheric pressure to the low vacuum required for MS detection, which include different types of capillaries [34–36], skimmers [37,38], electrodynamic ion guides [39,40], and ion funnels [41–44]. Although these strategies can be highly effective, new approaches that can be used to enhance ion signal further are desired.

In ESI, typically a direct current (DC) high voltage potential is applied to the ESI solution relative to a capillary entrance to the mass spectrometer to initiate and maintain the stable formation of a plume of highly charged droplets. ESI droplets formed from DC ESI can have a natural pulse frequency (generally around 1–2 kHz) owing to the physics of the droplet formation process, which depends on the sample flow rate, applied DC voltage, and properties of the solution [45]. However, externally pulsed ESI-methods have the advantage that extremely small droplets (~30 fL) can be formed from relatively

large capillaries (e.g., ~11 μm) compared to those formed using DC ESI [46], which can potentially enhance the sensitivity via a more efficient ion desolvation. Externally pulsed ESI can result from either pulsing a high voltage continuously through a sample solution that is flowing through a capillary emitter [47], or by applying a constant DC voltage to sequentially dispense discrete droplets of a sample solution [48,49]. Traditionally, high frequency pulsed ESI refers to frequencies of ca. 1 kHz, whereas low frequency pulsed ESI corresponds to frequencies of 1–100 Hz [45]. In addition to externally pulsed ESI, the use of very high frequency alternating current (AC) ESI (up to 400 kHz) has been reported [50–52]. In AC ESI [50–52], and likely externally pulsed ESI, the Taylor cone can be significantly ‘sharper’ and longer than in DC ESI, which is an effect that has been attributed to the entrainment of low mobility species in the capillary meniscus that are rapidly charged and discharged owing to the high electrophoretic mobility of protons. After multiple cycles, the low mobility species (e.g., protein ions) are enriched within the Taylor cone, which substantially elongates at a half angle (~12°) that is significantly lower than that formed by DC ESI (~47°) [53,54]. Although the optimal conditions for DC ESI generally resulted in higher ion abundances than those of AC ESI—owing primarily to the far lower electrical breakdown limit of AC vs. DC for the same maximum applied voltage [50]—the formation of a sharper Taylor cone should result in the production of smaller initial ESI droplets than those formed by DC ESI and less radial dispersion of the resulting aerosol plume. These two effects should in principle improve the efficiency of ESI-MS particularly for narrower bore nESI emitters in which the electrical breakdown limit (<1 kV) is substantially lower than that of larger bore ion emitters.

Here, 10 to 350 kHz externally pulsed nanoelectrospray ionisation (pulsed nESI) with nanoscale ion emitters is demonstrated for use in whole protein MS. During the course of this project, Ninomiya and Hiraoka reported the use of a high frequency pulsed nESI source with microscale ion emitters (4 μm i.d.), in which a DC voltage of up to ~1500 V was superimposed onto a pulsed waveform of up to ~4000 V to initiate and maintain nESI [55]. However, a direct comparison between the analytical performance of such a source to conventional direct current nESI was not reported. Here, we report the use of high frequency pulsed nESI with nanoscale ion emitters can be used to efficiently ionise molecules by rapidly increasing the voltage from 0 to ~1.0 kV with pulse widths that range from 2.85 to 100 μs (duty cycles ranging from 10 to 90%) and frequencies from 10 to 350 kHz. As a proof of concept, four prototypical test proteins were selected as test analytes of relevance to top-down MS (ubiquitin, Ubq; cytochrome C, Cyt C; myoglobin, Myo; and carbonic anhydrase II, CAII). By the use of pulsed, high frequency nESI with nanoscale ion emitters, the performance of MS for the detection of protein ions can be improved in terms of an enhanced sensitivity and decreased background chemical noise.

2. Materials and Methods

2.1. Materials and Sample Preparation

Angiotensin II (Ang $\geq 95\%$), ubiquitin from bovine erythrocytes (Ubq $\geq 98\%$), myoglobin from equine heart (Myo $\geq 90\%$), and carbonic anhydrase isozyme II from bovine erythrocytes (CAII ≥ 3000 W-A units/mg protein) were purchased from Sigma Aldrich (St. Louis, MO, USA). Cytochrome C from equine heart (Cyt C $\geq 90\%$) was obtained from Alfa Aesar (Ward Hill, MA, USA). Methanol ($\geq 99.9\%$) was obtained from Honeywell Inc. (Charlotte, NC, USA). Acetic acid and chloroform were purchased from Merck Pty Ltd. Deionized water (18 M Ω) was obtained using a water purification system (MilliQ, Merck, Darmstadt, Germany). Stock solutions of Ang, Ubq, Cyt C, Myo, and CAII were prepared in 100% deionized water at a concentration of 200 to 500 μM . The stock solutions of Ang, Ubq, Cyt C, Myo, and CAII were diluted into 47.5:47.5:5 methanol:water:acetic acid to prepare solutions for ESI-MS at a concentration of 1 to 5 μM . A solution mixture containing 20 μM of each of the four proteins in 47.5:47.5:5 methanol:water:acetic acid was also prepared.

2.2. Mass Spectrometry

Most of the mass spectrometry experiments were performed using a linear quadrupole ion trap mass spectrometer (LTQ XL, Thermo Fisher Scientific, San Jose, CA, USA), which is modified with an electrodynamic ion funnel (Heartland Mobility, Wichita, KS, USA). The stainless-steel capillary entrance was heated to 120 °C. The ion funnel conditions were optimized for a 'maximum' ion signal for DC nESI prior to pulsed nESI. Specifically, the RF frequency and 'drive' were tuned between 700–900 kHz and 10–24 a.u. corresponding to a sinusoidal RF waveform of 100–400 V_{p-p}. Voltages applied to the MS inlet and the ion funnel electrode were set between 100–250 V. A second LTQ XL with an unmodified ion source (i.e., with the stock capillary-skimmer source) was used for nESI-MS experiments with the protein mixture. Nanoelectrospray ionization emitters were pulled from glass capillaries (1.0 mm o.d.; 0.78 i.d., Harvard Apparatus, UK) to an inner diameter of ~250 nm using a Flaming/Brown micropipette puller (Model P-97, Sutter Instrument, Novato, CA, USA). The inner diameters of emitters were confirmed by use of scanning electron microscopy as described elsewhere [34]. The nanoelectrospray emitter was positioned about 2–5 mm from the capillary inlet to the MS. A platinum wire with a diameter of 0.005" (SDR Scientific, Chatswood, NSW, Australia) was inserted into an uncoated glass capillary filled with ~15 µL of sample solution. A DC voltage of ~1.5 kV was applied to the platinum wire relative to the capillary entrance to the MS to initiate and maintain electrospray for conventional nESI-MS experiments. For pulsed nESI, the experiment was performed using the same conditions, except that a pulsed voltage of 0.8 to 1.5 kV was applied to the platinum wire.

2.3. Pulsed Nanoelectrospray Ionization

The pulsed nanoelectrospray ion source setup consisted of an external high voltage DC power supply (TSA4000-1.2/240SP; Magna-Power Electronics, Flemington, NJ, USA), a fast high voltage square wave pulser (Model FSWP 51-02, Behlke, Germany), an oscilloscope (200 MHz, Wavesurfer 3024, Teledyne Lecroy, Ramapo, NY, USA), a waveform generator (20 MHz; DG1022, Rigol, Beaverton, OR, USA), a stabilised power supply (model 272A, BWD Electronics, Melbourne, Australia), and a control panel and a picoammeter (Keithley 6485 Picoammeter, Beaverton, OR, USA). In Figure S1, the electrical circuit that was used to generate high voltage pulses is shown. A DC high voltage potential was applied to the internal circuit of the high voltage pulser, which included a logic control circuit, an isolated DC/DC converter for gate driver, and a bridge leg. A positive 5 V was connected to the input of the isolated DC/DC converter and the logic control circuit. The isolated power supply generated two isolated voltages for the dual channel isolated gate driver that drove the switching devices, S1 and S2, of the bridge leg on and off. S1 and S2 were operated in the complementary mode, with only one switch turned on at any time. When S1 was on and S2 was off, the output of the generator was connected to the positive rail of the HV DC power supply supplying a high voltage to the source. The time that S1 was on corresponded to the pulse width (T_P) (Figure 1). In contrast, when S1 was off and S2 was on, the output of the generator would connect to the ground, resulting in zero voltage applied to the source, which corresponded to the space width (T_S); i.e., the delay between high voltage pulses. The sum of T_P and T_S corresponded to a full cycle; i.e., a single pulse period (T) (Figure 1). The high voltage pulses were generated by applying a frequency and duty cycle of 10 to 350 kHz and 10 to 90%, respectively.

2.4. Data Analysis

Mass spectra were collected using Xcalibur™ (Thermo Fisher Scientific, Waltham, MA, USA) and exported to Excel for calculating the average background chemical noise (N_C)—which refers to the total chemical background signal not assigned to the signal arising from the analytes—and signal-to-chemical background noise (S/N_C) in terms of the measured ion signal of the analyte of interest divided by the chemical background [56,57]. The signal and S/N_C values were obtained from the average of three replicate measurements with

three different emitter tips. p -values were obtained using the standard T -test for two independent means (two tailed) at a 95% confidence interval. UniDec (Version 4.4.1) was used to calculate the average charge states of protein ions [58].

Figure 1. Diagram of pulsed nanoelectrospray ionization mass spectrometry. (a) A borosilicate capillary emitter pulled to an inner diameter of ~ 250 nm. The emitter was filled with a sample solution (blue); a thin platinum wire was inserted to apply the spray voltage into the solution. (b) A pulsed, positive polarity high voltage potential was applied to the emitter relative to the capillary entrance to the mass spectrometer (MS), which had sub-nanosecond rise times, pulse widths (T_P) from 2.85 to 100 μ s, and a delay between pulses (T_S) of 0.25 to 90 μ s.

3. Results

3.1. Pulsed Nanoelectrospray Ionization Increases Signal-to-Background Chemical Noise

Nanoelectrospray ionization mass spectra were obtained for solutions containing Ubq, Cyt C, Myo, and CAII at concentrations of 3 to 5 μ M. ‘Optimal’ pulsed nESI mass spectra in terms of their ion abundance were obtained for each test analyte using frequencies and duty cycle values ranging from 10 to 350 kHz and 10 to 90%, respectively. Lower duty cycles were not investigated because no ion signal was readily detected below a duty cycle of 10%. In general, pulsed nESI resulted in the same type of ions being formed as that for direct current nESI as expected; i.e., protonated ions were readily formed in both pulsed and direct current nESI-MS in comparable charge states (Figure 2). However, at the optimal frequencies and duty cycles, pulsed nESI-MS resulted in an increase in ion signal and a decrease in background chemical noise (N_C) levels. For example, the average ion signal for Ubq, Cyt C, Myo, and CAII increased by 12, 62, 82, and 37% respectively using pulsed nESI-MS compared to when direct current nESI-MS was used (Figure 2). The N_C also decreased for all samples by up to 46%. Such an increase in the absolute ion abundance and decrease in N_C by pulsed nESI resulted a significant increase in S/N_C across all samples by up to 154% compared to when direct current nESI-MS was used (Figure 2; Table 1).

3.2. Effects of Frequency and Pulse

In Figure 3, mass spectra of Myo that were obtained by conventional direct current nESI and pulsed nESI at a frequency of 50, 100, 200, and 300 kHz are shown. To investigate the effects of the waveforms used in pulsed nESI, mass spectra were first obtained at a frequency ranging from 10 to 350 kHz using a fixed duty cycle of 50% to determine the ‘optimal’ frequency (Figure 4a). With increasing frequency from 10 kHz, the absolute ion abundance of Myo generally and steadily increased to a maximum at 200 kHz and then decreased as the frequency increased further to 350 kHz. Mass spectra were then obtained using the optimal frequency, but with different duty cycles. The signal intensity increased as the duty cycle increased, resulting in a peak at a duty cycle of 60% followed by a gradual decrease with higher duty cycles (i.e., $\geq 70\%$ to up to 90%; Figure 4c). The optimal frequency of 200 kHz and duty cycle of 60% were used to collect all other proteins’

spectra and compared to those collected from the conventional direct current nESI method (Figure 2).

Figure 2. Mass spectra of ubiquitin (3 μ M) (a,e), cytochrome C (5 μ M) (b,f), myoglobin (5 μ M) (c,g), and carbonic anhydrase (5 μ M) (d,h) obtained from pulsed (a–d) and conventional direct current nESI (e–h), respectively. The pulsed nESI spectra were collected at an ‘optimum’ frequency of 200 kHz. † The heme group of myoglobin; * peaks corresponding to the chemical noise that are present in the solution blank.

Table 1. Background chemical noise (N_C), signal-to-background chemical noise (S/N_C), and average charge state for angiotensin II, ubiquitin, cytochrome C, myoglobin, and carbonic anhydrase II calculated from pulsed and conventional direct current nESI mass spectrometry.

Analytes	N_C ^a			S/N_C ^a			Average Charge State ^a	
	Pulsed nESI	nESI	% Decrease ^b	Pulsed nESI	nESI	% Increase ^b	Pulsed nESI	nESI
Ubq	105 $\pm$ 3.99	155 $\pm$ 10.5	32.5%	2334 $\pm$ 71.4	1406 $\pm$ 117	66.0%	12.1 $\pm$ 0.54	11.9 $\pm$ 0.33
Cyt C	206 $\pm$ 52.2	273 $\pm$ 35.2	24.4%	1313 $\pm$ 143	615 $\pm$ 66.2	114%	15.6 $\pm$ 0.42	15.9 $\pm$ 0.40
Myo	158 $\pm$ 9.63	185 $\pm$ 11.3	14.6%	709 $\pm$ 23.9	461 $\pm$ 17.9	53.8%	22.0 $\pm$ 0.34	20.6 $\pm$ 0.36
CAII	272 $\pm$ 48.0	504 $\pm$ 102	46.1%	518 $\pm$ 128	204 $\pm$ 66.9	154%	37.5 $\pm$ 0.44	36.5 $\pm$ 0.47
Heme	148 $\pm$ 7.63	181 $\pm$ 9.31	18.2%	124 $\pm$ 8.32	89.1 $\pm$ 5.91	39.2%	N/A ^c	N/A ^c
Ang	92.1 $\pm$ 7.31	165 $\pm$ 8.37	44.2%	3943 $\pm$ 103	2082 $\pm$ 24.7	89.3%	2.08 $\pm$ 0.09	1.97 $\pm$ 0.10

^a Values correspond to the mean $\pm$ standard deviation of at least three replicate measurements; ^b p -value < 0.05; ^c Not applicable.

The use of pulsed nESI resulted in an increase in the average charge state distributions of the two larger protein ions (Figures 2 and S2, Table 1). For example, the average charge state distribution of Myo increased from 19.5 ± 0.07 to 20.6 ± 0.09 as the frequency increased from 10 to 200 kHz and then decreased slightly as the frequency decreased further (Figure S2). Likewise, the most abundant charge state of Myo shifted from the 20+ for DC nESI to the 23+ for pulsed nESI at 200 kHz (Figure 2c,g). For CAII, the average charge state increased slightly from 36.5 ± 0.47 for DC nESI to 37.5 ± 0.44 for pulsed nESI at 200 kHz (Table 1). Such a shifting to a higher charge state distribution at a higher frequency is consistent with results reported previously for protein ions formed from denaturing solutions using AC and pulsed ESI [52,55].

Figure 3. Mass spectra of myoglobin (5 μM) (a–e) and angiotensin II (1 μM) (f–j) obtained from pulsed and conventional direct current nESI methods. (a,f) Conventional direct current nESI mass spectra of myoglobin and angiotensin II. (b–e) Pulsed nESI mass spectra of myoglobin obtained using a frequency of 50, 100, 200 and 300 kHz, respectively. (g–j) Pulsed nESI mass spectra of angiotensin II obtained using a frequency of 50, 100, 200, and 250 kHz, respectively. All pulsed nESI experiments were conducted using a duty cycle of 50%. $\dagger$ The heme group of myoglobin.

Figure 4. Absolute ion abundance of myoglobin (5 μM) (a,c) and angiotensin II (1 μM) (b,d) as a function of frequency (a,b) and duty cycle (c,d) in pulsed nESI-MS. The spectra were collected at a frequency ranging from 10 to 350 kHz (duty cycle of 50%) and a duty cycle ranging from 10 to 90% (frequency of 250 and 200 kHz for angiotensin II and myoglobin, respectively).

3.3. Pulsed Nano Electrospray Ionization of Protein Mixture

Pulsed and DC nESI mass spectra of a solution mixture containing all four proteins are shown in Figure 5. These data were collected using an LTQ XL with an unmodified ion source; i.e., a skimmer–capillary atmospheric pressure ion source rather than a capillary–ion funnel interface. The use of pulsed nESI-MS resulted in a respective increase in the ion counts for the most abundant charge states of Ubq, Cyt C, Myo, and CAII of 158%, 74%, 47%, and 184% compared to when DC nESI-MS was used (Table 2). The extent of protein ion charging was comparable to that obtained using the ion funnel interface (Figure 2). Overall, these results indicate that the enhanced signal obtained by use of pulsed nESI-MS compared to DC nESI-MS under these conditions is likely to apply more generally to additional types of atmospheric pressure ion sources and more complex mixtures of proteins.

Figure 5. Pulsed (a) and direct current nESI (b) mass spectra of a mixture of ubiquitin (●), cytochrome C (◀), myoglobin (▲), and carbonic anhydrase II (▶) formed from a solution containing 20 μ M of each protein. The frequency and duty cycle of the pulsed high voltage was 200 kHz and 60% respectively.

Table 2. Most abundant charge state, ion count of the most abundant charge state (arbitrary units), percentage increase in ion count, and average charge state of ubiquitin, cytochrome C, myoglobin, and carbonic anhydrase II obtained from pulsed and conventional direct current nESI mass spectra of a solution mixture containing all four proteins. Refer to Figure 5 for the corresponding mass spectra.

Analytes	Most Abundant Charge State		Ion Count ($\times 10^5$) for the Most Abundant Charge State ^a		% Increase in Signal ^b	Average Charge State ^a	
	Pulsed nESI	nESI	Pulsed nESI	nESI		Pulsed nESI	nESI
Ubq	9+	8+	2.83 ± 0.11	1.10 ± 0.10	158	9.19 ± 0.05	8.99 ± 0.13
Cyt C	13+	13+	1.75 ± 0.08	1.01 ± 0.10	74.0	13.2 ± 0.21	12.9 ± 0.07
Myo	18+	18+	3.04 ± 0.09	2.07 ± 0.09	47.1	17.4 ± 0.08	17.3 ± 0.04
CAII	31+	34+	2.02 ± 0.10	0.72 ± 0.06	184	31.8 ± 1.03	34.0 ± 0.79

^a Values correspond to the mean $\pm$ standard deviation of at least three replicate measurements; ^b p -value < 0.05.

3.4. Mechanistic Considerations

The effects of electrophoretic mobility on the abundances of ions formed in pulsed and direct current nESI was examined for ions with significantly different mobilities. Proteins have electrophoretic mobilities that are substantially lower than Ang. For example, the electrophoretic mobilities of Ang and Myo are $6 \times 10^{-5} \text{ cm}^2/\text{Vs}$ and $1.4 \times 10^{-5} \text{ cm}^2/\text{Vs}$ [59,60]. The highest ion signal and S/N_C values for Ang were obtained at a frequency and duty cycle of 250 kHz and 50%. The abundance of the singly positively charged Fe(III)-heme prosthetic (i.e., m/z 616) also had a maximum abundance and S/N_C at 200 kHz and 50%, which was similar to that of Myo (200 kHz and 60%) formed from the exact same solution and ion emitter tip (Figures 3, 4 and S3). Thus, electrophoretic mobility alone cannot fully account for the enhanced abundance of these ions. There is a lack of a significant electrophoretic entrainment effect in pulsed nESI using nanoscale emitters. In AC ESI with larger bore emitters, the RF amplitudes are higher, volume of the Taylor cone is larger, and polarity of the ESI waveform is fully reversed every period unlike in pulsed nESI, which may contribute to the reduced dependence on electrophoretic mobility.

The improved performance by use of pulsed high voltage nESI compared to DC nESI can be attributed to at least two factors. First, the use of shorter pulses may result in the formation of smaller sized ESI droplets owing to the lower nESI current. It is well established that larger initial droplets tend to be formed in ESI using higher currents [61]. The use of shorter pulses lowers the ESI current and in principle should result in the formation of smaller initial sized droplets. Such droplets should result in more efficient droplet desolvation and ionisation. Secondly, the use of shorter pulses should reduce charge–charge repulsion in the nESI aerosol plume, which may improve ion transmission through the narrow capillary entrance to the mass spectrometer. This may be beneficial to the detection of lower abundance ions and result in the signal not being linearly dependant on the duty cycle. It is established that in AC ESI using larger emitter tips with higher frequencies can result in a significant narrowing of the Taylor cone angle [50–52].

4. Conclusions

The use of high voltage and high frequency pulses in nESI-MS can result in significant performance gains in terms of the signal intensity, background chemical noise (N_C), and signal-to-background chemical noise (S/N_C) in the detection of intact proteins compared to conventional DC nESI-MS. For example, the use of pulsed high voltages from 0 to ~1.5 kV with sub-nanosecond rise times, a frequency of 200 kHz, and a duty cycle of 50–60% can increase signal and S/N_C values by up to 82% and 154%, respectively, while the N_C can be decreased by up to 46% compared to DC nESI. The use of pulsed high voltage waveforms in nESI-MS can also be used to significantly increase the abundances of protein ions formed from mixtures of proteins by up to 184% compared to DC nESI-MS. Given that the abundances of both small molecules (protonated angiotensin II and Fe(II)-heme) and protein ions with substantially different electrophoretic mobilities peaked at very high frequencies (200–250 kHz), these data indicate that factors other than electrophoretic mobility contribute to the enhanced performance of pulsed nESI. Alternatively, the use of pulsed nESI may result in the formation of smaller ESI droplets and less Coulombic repulsion in the ESI plume, which should result in improved ion desolvation and a more efficient transfer of ions from atmospheric pressure to under vacuum through the narrow capillary entrance of the mass spectrometer, thereby increasing the signal. Enhancing the signal for intact protein ions formed using pulsed nESI should be beneficial in many different types of tandem mass spectrometry experiments for the quantitative and qualitative analysis of complex chemical mixtures including the contents of single cells.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/app112210883/s1>, Figure S1: Electrical circuit to generate high voltage pulses for pulsed nESI-MS, Figure S2: Effects of frequency and duty cycle on average charge states and signal-to-noise

ratios, Figure S3: Effects of frequency and duty cycle on average charge states and signal-to-noise ratios, Figure S4: Mass spectra for angiotensin.

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References

- Donnelly, D.P.; Rawlins, C.M.; DeHart, C.J.; Fornelli, L.; Schachner, L.F.; Lin, Z.; Lippens, J.L.; Aluri, K.C.; Sarin, R.; Chen, B. Best practices and benchmarks for intact protein analysis for top-down mass spectrometry. *Nat. Methods* **2019**, *16*, 587–594. [\[CrossRef\]](#)
- Iavarone, A.T.; Jurchen, J.C.; Williams, E.R. Supercharged protein and peptide ions formed by electrospray ionization. *Anal. Chem.* **2001**, *73*, 1455–1460. [\[CrossRef\]](#) [\[PubMed\]](#)
- Konermann, L.; Rodriguez, A.D.; Liu, J. On the formation of highly charged gaseous ions from unfolded proteins by electrospray ionization. *Anal. Chem.* **2012**, *84*, 6798–6804. [\[CrossRef\]](#)
- Teo, C.A.; Donald, W.A. Solution additives for supercharging proteins beyond the theoretical maximum proton-transfer limit in electrospray ionization mass spectrometry. *Anal. Chem.* **2014**, *86*, 4455–4462. [\[CrossRef\]](#)
- Stahl-Zeng, J.; Lange, V.; Ossola, R.; Eckhardt, K.; Krek, W.; Aebersold, R.; Domon, B. High sensitivity detection of plasma proteins by multiple reaction monitoring of N-glycosites. *Mol. Cell. Proteom.* **2007**, *6*, 1809–1817. [\[CrossRef\]](#)
- Zenaidee, M.A.; Donald, W.A. Electron capture dissociation of extremely supercharged protein ions formed by electrospray ionisation. *Anal. Methods* **2015**, *7*, 7132–7139. [\[CrossRef\]](#)
- Skinner, O.S.; Breuker, K.; McLafferty, F.W. Charge site mass spectra: Conformation-sensitive components of the electron capture dissociation spectrum of a protein. *J. Am. Soc. Mass Spectrom.* **2013**, *24*, 807–810. [\[CrossRef\]](#) [\[PubMed\]](#)
- Zenaidee, M.A.; Donald, W.A. Extremely supercharged proteins in mass spectrometry: Profiling the pH of electrospray generated droplets, narrowing charge state distributions, and increasing ion fragmentation. *Analyst* **2015**, *140*, 1894–1905. [\[CrossRef\]](#) [\[PubMed\]](#)
- Syka, J.E.; Coon, J.J.; Schroeder, M.J.; Shabanowitz, J.; Hunt, D.F. Peptide and protein sequence analysis by electron transfer dissociation mass spectrometry. *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 9528–9533. [\[CrossRef\]](#)
- Gunawardena, H.P.; He, M.; Chrisman, P.A.; Pitteri, S.J.; Hogan, J.M.; Hodges, B.D.; McLuckey, S.A. Electron transfer versus proton transfer in gas-phase ion/ion reactions of polyprotonated peptides. *J. Am. Chem. Soc.* **2005**, *127*, 12627–12639. [\[CrossRef\]](#) [\[PubMed\]](#)
- Becher, S.; Wang, H.; Leeming, M.G.; Donald, W.A.; Heiles, S. Influence of protein ion charge state on 213 nm top-down UVPD. *Analyst* **2021**, *146*, 3977–3987. [\[CrossRef\]](#)
- Girod, M.; Sanader, Z.; Vojkovic, M.; Antoine, R.; MacAleese, L.; Lemoine, J.; Bonacic-Koutecky, V.; Dugourd, P. UV photodissociation of proline-containing peptide ions: Insights from molecular dynamics. *J. Am. Soc. Mass Spectrom.* **2014**, *26*, 432–443. [\[CrossRef\]](#)
- Wang, H.; Yong, G.; Brown, S.L.; Lee, H.E.; Zenaidee, M.A.; Supuran, C.T.; Donald, W.A. Supercharging protein ions in native mass spectrometry using theta capillary nanoelectrospray ionization mass spectrometry and cyclic alkylcarbonates. *Anal. Chim. Acta* **2018**, *1003*, 1–9. [\[CrossRef\]](#) [\[PubMed\]](#)
- Lermyte, F.; Valkenborg, D.; Loo, J.A.; Sobott, F. Radical solutions: Principles and application of electron-based dissociation in mass spectrometry-based analysis of protein structure. *Mass Spectrom. Rev.* **2018**, *37*, 750–771. [\[CrossRef\]](#)
- Going, C.C.; Williams, E.R. Supercharging with m-nitrobenzyl alcohol and propylene carbonate: Forming highly charged ions with extended, near-linear conformations. *Anal. Chem.* **2015**, *87*, 3973–3980. [\[CrossRef\]](#) [\[PubMed\]](#)
- Meyer, J.G.; Komives, E.A. Charge state coalescence during electrospray ionization improves peptide identification by tandem mass spectrometry. *J. Am. Soc. Mass Spectrom.* **2012**, *23*, 1390–1399. [\[CrossRef\]](#)
- Going, C.C.; Xia, Z.; Williams, E.R. New supercharging reagents produce highly charged protein ions in native mass spectrometry. *Analyst* **2015**, *140*, 7184–7194. [\[CrossRef\]](#) [\[PubMed\]](#)
- Zenaidee, M.A.; Leeming, M.G.; Zhang, F.; Funston, T.T.; Donald, W.A. Highly charged protein ions: The strongest organic acids to date. *Angew. Chem.* **2017**, *129*, 8642–8646. [\[CrossRef\]](#)
- Hahne, H.; Pachl, F.; Ruprecht, B.; Maier, S.K.; Klaeger, S.; Helm, D.; Médard, G.; Wilm, M.; Lemeer, S.; Kuster, B. DMSO enhances electrospray response, boosting sensitivity of proteomic experiments. *Nat. Methods* **2013**, *10*, 989–991. [\[CrossRef\]](#)
- Tang, S.; Cheng, H.; Yan, X. On-demand electrochemical epoxidation in nano-electrospray ionization mass spectrometry to locate carbon–carbon double bonds. *Angew. Chem. Int. Ed.* **2020**, *59*, 209–214. [\[CrossRef\]](#)

21. Mandal, M.K.; Chen, L.C.; Hashimoto, Y.; Yu, Z.; Hiraoka, K. Detection of biomolecules from solutions with high concentration of salts using probe electrospray and nano-electrospray ionization mass spectrometry. *Anal. Methods*. **2010**, *2*, 1905–1912. [\[CrossRef\]](#)
22. El-Faramawy, A.; Siu, K.M.; Thomson, B.A. Efficiency of nano-electrospray ionization. *J. Am. Soc. Mass Spectrom.* **2005**, *16*, 1702–1707. [\[CrossRef\]](#) [\[PubMed\]](#)
23. Marginean, I.; Tang, K.; Smith, R.D.; Kelly, R.T. Picoelectrospray ionization mass spectrometry using narrow-bore chemically etched emitters. *J. Am. Soc. Mass Spectrom.* **2013**, *25*, 30–36. [\[CrossRef\]](#) [\[PubMed\]](#)
24. Marginean, I.; Kelly, R.T.; Prior, D.C.; LaMarche, B.L.; Tang, K.; Smith, R.D. Analytical characterization of the electrospray ion source in the nanoflow regime. *Anal. Chem.* **2008**, *80*, 6573–6579. [\[CrossRef\]](#)
25. Schmidt, A.; Karas, M.; Dülcks, T. Effect of different solution flow rates on analyte ion signals in nano-ESI MS, or: When does ESI turn into nano-ESI? *J. Am. Soc. Mass Spectrom.* **2003**, *14*, 492–500. [\[CrossRef\]](#)
26. Susa, A.C.; Xia, Z.; Williams, E.R. Small emitter tips for native mass spectrometry of proteins and protein complexes from nonvolatile buffers that mimic the intracellular environment. *Anal. Chem.* **2017**, *89*, 3116–3122. [\[CrossRef\]](#)
27. Page, J.S.; Tang, K.; Kelly, R.T.; Smith, R.D. Subambient pressure ionization with nanoelectrospray source and interface for improved sensitivity in mass spectrometry. *Anal. Chem.* **2008**, *80*, 1800–1805. [\[CrossRef\]](#)
28. Shen, Y.; Zhao, R.; Berger, S.J.; Anderson, G.A.; Rodriguez, N.; Smith, R.D. High-efficiency nanoscale liquid chromatography coupled on-line with mass spectrometry using nanoelectrospray ionization for proteomics. *Anal. Chem.* **2002**, *74*, 4235–4249. [\[CrossRef\]](#)
29. Susa, A.C.; Xia, Z.; Williams, E.R. Native mass spectrometry from common buffers with salts that mimic the extracellular environment. *Angew. Chem. Int. Ed.* **2017**, *56*, 7912–7915. [\[CrossRef\]](#)
30. Wilm, M.; Mann, M. Analytical properties of the nanoelectrospray ion source. *Anal. Chem.* **1996**, *68*, 1–8. [\[CrossRef\]](#)
31. Nguyen, G.T.; Tran, T.N.; Podgorski, M.N.; Bell, S.G.; Supuran, C.T.; Donald, W.A. Nanoscale ion emitters in native mass spectrometry for measuring ligand–protein binding affinities. *ACS Cent. Sci.* **2019**, *5*, 308–318. [\[CrossRef\]](#)
32. Bennett, J.L.; Nguyen, G.T.; Donald, W.A. Protein–Small Molecule Interactions in Native Mass Spectrometry. *Chem. Rev.* **2021**. [\[CrossRef\]](#)
33. Nguyen, G.; Bennett, J.; Liu, S.; Hancock, S.; Winter, D.; Glover, D.; Donald, W. Multiplexed screening of thousands of natural products for protein–ligand binding in native mass spectrometry. *chemRxiv* **2021**. [\[CrossRef\]](#)
34. Chowdhury, S.K.; Katta, V.; Chait, B.T. An electrospray-ionization mass spectrometer with new features. *Rapid Commun. Mass Spectrom.* **1990**, *4*, 81–87. [\[CrossRef\]](#)
35. Lin, B.; Sunner, J. Ion transport by viscous gas flow through capillaries. *J. Am. Soc. Mass Spectrom.* **1994**, *5*, 873–885. [\[CrossRef\]](#)
36. Fenn, J.B.; Mann, M.; Meng, C.K.; Wong, S.F.; Whitehouse, C.M. Electrospray ionization for mass spectrometry of large biomolecules. *Science* **1989**, *246*, 64–71. [\[CrossRef\]](#)
37. Yamashita, M.; Fenn, J.B. Electrospray ion source. Another variation on the free-jet theme. *J. Phys. Chem.* **1984**, *88*, 4451–4459. [\[CrossRef\]](#)
38. Kantrowitz, A.; Grey, J. A high intensity source for the molecular beam. Part I. Theoretical. *Rev. Sci. Instrum.* **1951**, *22*, 328–332. [\[CrossRef\]](#)
39. Douglas, D.; French, J.B. Collisional focusing effects in radio frequency quadrupoles. *J. Am. Soc. Mass Spectrom.* **1992**, *3*, 398–408. [\[CrossRef\]](#)
40. Tolmachev, A.; Chernushevich, I.; Dodonov, A.; Standing, K. A collisional focusing ion guide for coupling an atmospheric pressure ion source to a mass spectrometer. *Nucl. Instrum. Methods. B* **1997**, *124*, 112–119. [\[CrossRef\]](#)
41. Kelly, R.T.; Tolmachev, A.V.; Page, J.S.; Tang, K.; Smith, R.D. The ion funnel: Theory, implementations, and applications. *Mass Spectrom. Rev.* **2010**, *29*, 294–312. [\[CrossRef\]](#) [\[PubMed\]](#)
42. Shaffer, S.A.; Tolmachev, A.; Prior, D.C.; Anderson, G.A.; Udseth, H.R.; Smith, R.D. Characterization of an improved electrodynamic ion funnel interface for electrospray ionization mass spectrometry. *Anal. Chem.* **1999**, *71*, 2957–2964. [\[CrossRef\]](#)
43. Kim, T.; Udseth, H.R.; Smith, R.D. Improved ion transmission from atmospheric pressure to high vacuum using a multicapillary inlet and electrodynamic ion funnel interface. *Anal. Chem.* **2000**, *72*, 5014–5019. [\[CrossRef\]](#) [\[PubMed\]](#)
44. Ahmed, E.; Xiao, D.; Kabir, K.M.; Fletcher, J.; Donald, W.A. Ambient Pressure Ion Funnel: Concepts, Simulations, and Analytical Performance. *Anal. Chem.* **2020**, *92*, 15811–15817. [\[CrossRef\]](#)
45. Wei, J.; Shui, W.; Zhou, F.; Lu, Y.; Chen, K.; Xu, G.; Yang, P. Naturally and externally pulsed electrospray. *Mass Spectrom. Rev.* **2002**, *21*, 148–162. [\[CrossRef\]](#) [\[PubMed\]](#)
46. Yogi, O.; Kawakami, T.; Yamauchi, M.; Ye, J.Y.; Ishikawa, M. On-demand droplet spotter for preparing pico-to femtoliter droplets on surfaces. *Anal. Chem.* **2001**, *73*, 1896–1902. [\[CrossRef\]](#) [\[PubMed\]](#)
47. Lu, Y.; Zhou, F.; Shui, W.; Bian, L.; Guo, Y.; Yang, P. Pulsed electrospray for mass spectrometry. *Anal. Chem.* **2001**, *73*, 4748–4753. [\[CrossRef\]](#) [\[PubMed\]](#)
48. Berggren, W.T.; Westphall, M.S.; Smith, L.M. Single-pulse nanoelectrospray ionization. *Anal. Chem.* **2002**, *74*, 3443–3448. [\[CrossRef\]](#)
49. Tracey, P.J.; Vaughn, B.S.; Roberts, B.J.; Poad, B.L.; Trevitt, A.J. Rapid profiling of laser-induced photochemistry in single microdroplets using mass spectrometry. *Anal. Chem.* **2014**, *86*, 2895–2899. [\[CrossRef\]](#)
50. Sarver, S.A.; Chetwani, N.; Dovichi, N.J.; Go, D.B.; Gartner, C.A. A Comparison of Alternating Current and Direct Current Electrospray Ionization for Mass Spectrometry. *J. Am. Soc. Mass Spectrom.* **2014**, *25*, 524–529. [\[CrossRef\]](#)

51. Chetwani, N.; Cassou, C.A.; Go, D.B.; Chang, H.-C. High-frequency AC electrospray ionization source for mass spectrometry of biomolecules. *J. Am. Soc. Mass Spectrom.* **2010**, *21*, 1852–1856. [[CrossRef](#)]
52. Chetwani, N.; Cassou, C.A.; Go, D.B.; Chang, H.-C. Frequency dependence of alternating current electrospray ionization mass spectrometry. *Anal. Chem.* **2011**, *83*, 3017–3023. [[CrossRef](#)]
53. Maheshwari, S.; Chang, H.-C. Effects of bulk charge and momentum relaxation time scales on ac electrospraying. *J. Appl. Phys.* **2007**, *102*, 034902. [[CrossRef](#)]
54. Chetwani, N.; Maheshwari, S.; Chang, H.-C. Universal cone angle of ac electrosprays due to net charge entrainment. *Phys. Rev. Lett.* **2008**, *101*, 204501. [[CrossRef](#)]
55. Ninomiya, S.; Hiraoka, K. Pulsed nano-electrospray ionization with a high voltage (4000 V) pulse applied to solutions in the range of 200 ns to 1 ms. *J. Am. Soc. Mass Spectrom.* **2020**, *31*, 693–699. [[CrossRef](#)]
56. Hassell, K.M.; LeBlanc, Y.C.; McLuckey, S.A. Chemical noise reduction via mass spectrometry and ion/ion charge inversion: Amino acids. *Anal. Chem.* **2011**, *83*, 3252–3255. [[CrossRef](#)] [[PubMed](#)]
57. Ahmed, E.; Xiao, D.; Dumlao, M.C.; Steel, C.C.; Schmidtke, L.M.; Fletcher, J.; Donald, W.A. Nanosecond Pulsed Dielectric Barrier Discharge Ionization Mass Spectrometry. *Anal. Chem.* **2020**, *92*, 4468–4474. [[CrossRef](#)]
58. Cleary, S.P.; Li, H.; Bagal, D.; Loo, J.A.; Campuzano, I.D.; Prell, J.S. Extracting charge and mass information from highly congested mass spectra using Fourier-domain harmonics. *J. Am. Soc. Mass Spectrom.* **2018**, *29*, 2067–2080. [[CrossRef](#)] [[PubMed](#)]
59. Xin, Y.; Mitchell, H.; Cameron, H.; Allison, S.A. Modeling the electrophoretic mobility and diffusion of weakly charged peptides. *J. Phys. Chem. B* **2006**, *110*, 1038–1045. [[CrossRef](#)] [[PubMed](#)]
60. Lewis, U.; Schweigert, B. Biochemistry of myoglobin: III. Homogeneity studies with crystalline beef myoglobin. *J. Biol. Chem.* **1955**, *214*, 647–655. [[CrossRef](#)]
61. Tang, K.; Page, J.S.; Kelly, R.T.; Marginean, I. Electrospray Ionization in Mass Spectrometry. In *Encyclopedia of Spectroscopy and Spectrometry*, 2nd ed.; Lindon, J.C., Ed.; Academic Press: Oxford, UK, 2010; pp. 467–474.