Pulsed nanoelectrospray ionisation mass spectrometry

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Pulsed nanoelectrospray ionisation mass spectrometry

by

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A thesis in fulfilment of the requirements

for the degree of

Master of Philosophy

School of Chemistry

Faculty of Science

May 2021

THE UNIVERSITY OF NEW SOUTH WALES
Thesis/Dissertation Sheet

Surname or Family name **Liu**

First name: **Qinwen**   Other name/s: **n/a**

Abbreviation for degree as given in the University calendar: **MPhil**

School: **Chemistry**   Faculty: **Science**

Title: **Pulsed nanoelectrospray ionisation mass spectrometry**

Abstract 350 words maximum: (PLEASE TYPE)

Pulsed nanoelectrospray ionisation (pulsed nESI) is an emerging technique for ionising volatile biomolecules for sensitive detection by mass spectrometry (MS). In conventional nESI, direct current high voltage (DC HV) is applied to the analyte solution resulting in the formation of ions with high sensitivity, low sample consumption and low limits of detection. Alternatively, in pulsed nESI, molecules are ionized by using a pulsed high voltage square waveform. During the course of this research project, another group reported that pulsed nESI can be used to measure peptide and protein ions by applying short, repetitive pulses of high voltage to the nESI emitter. However, the performance of such an ion source was not compared to conventional nESI. Thus, the extent that pulsed nESI can be used to improve performance of MS was unclear. Here, pulsed nESI is reported in which the voltage is rapidly pulsed from 0 to up to ~3 kV with a rise time of low to sub-nanoseconds and with duty cycles ranging from 1-50% corresponding to pulse durations of 9 to 450 µs. By use of pulsed nESI, the performance of MS for the detection of many different classes of molecules can be improved in terms of decreasing background chemical noise and enhancing the sensitivity for analytes ranging from small molecules to whole proteins. Specifically, pulses that are less than ~450 µs can be used to decrease the background chemical noise and increase signal-to-background chemical noise ratio in pulsed nESI MS by up to 93% and 691% for six test analytes compared to conventional nESI. Pulsed nESI can also be used for native mass spectrometry to improve signal to background chemical noise ratios. Overall, pulsed nESI can be used to significantly improve the performance of MS.

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Acknowledgements

A heartfelt thanks to my supervisor A/Prof. Alex Donald for all his support and encouragement throughout the two years of my MPhil degree. Your constant optimism and patience has pushed me to work hard and help me grow. I appreciate everything and will remember your support all the time.

A huge thanks to Dr. Ezaz Ahmed for your support during my project, training me how to use the pulsing power supply and helping me to solve some of the problems that we encountered. To the rest of the Donald group, you truly felt more like family than colleagues. Without your help, this thesis would not be possible. I hope we can keep in touch in the future as I truly cherish our friendship. I am grateful to Dr. Morphy Dumlao for your support during my 2 years Master studying, thank you for the guidance, friendship, and encouragement.

To my parents, thank you for supporting me mentally and financially to finish my undergraduate and master studies. Thank you for your encouragement during the many times I said I wanted to give up.

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C2	1,2 butylene carbonate
PC	34:2-L-α-phosphatidylcholine
ACOH	Acetic acid
AC	Alternative current
Angio	Angiotensin
Ar	Argon
N_C	Background chemical noise
CAII	Carbonic anhydrase
CytC	Cytochrome c
Da	Dalton
ºC	Degree celsuis
DI	Desorption ionisation
DMMP	Dimethyl methylphosphonate
DMSO	Dimethyl sulfoxide
DC HV	Direct current high voltage
ESI	Electrospray ionisation
EI	Electron ionisation
et al.,	Et alia - "and other"
e.g.,	Exempli gratia - "for example"
	Frequency
GC-MS	Gas chromatography mass spectrometry
kHz	Kilohertz
kV	Kilovolt
LC	Liquid chromatography
MALDI	Matrix-assisted laser desorption ionisation
MS	Mass spectrometry
m/z	Mass and charge ratio
m-NBA	Meta-nitrobenzyl alcohol

MeOH	Methanol
μL	Microliter
μM	Micromolar
μm	Microminute
μs	Microsecond
Myo	Myoglobin
nESI	Nanoelectrospray ionisation
N_2	Nitrogen
O_2	Oxygen
Pulsed nESI	Pulsed nanoelectrospray ionisation
R	Radius of droplet
T	Pulse width
RF	Radio frequency
S	Signal
S/N_C	Signal-to-background chemical noise
NaCl	Sodium chloride
γ	The solvent's surface tension
Q_R	The amount of charge on the droplet
TOF	Time-of-flight
ε_0	The vacuum permittivity
V	Voltage
Ubq	Ubiquitin
Pd	Palladium
Pt	Platinum

Abstract

Pulsed nanoelectrospray ionisation (pulsed nESI) is an emerging technique for ionising volatile biomolecules for sensitive detection by mass spectrometry (MS). In conventional nESI, direct current high voltage (DC HV) is applied to the analyte solution resulting in the formation of ions with high sensitivity, low sample consumption and low limits of detection. Alternatively, in pulsed nESI, molecules are ionized by using a pulsed high voltage square waveform. During the course of this research project, another group reported that pulsed nESI can be used to measure peptide and protein ions by applying short, repetitive pulses of high voltage to the nESI emitter. However, the performance of such an ion source was not compared to conventional nESI. Thus, the extent that pulsed nESI can be used to improve performance of MS was unclear. Here, pulsed nESI is reported in which the voltage is rapidly pulsed from 0 to up to ~3 kV with a rise time of low to sub-nanoseconds and with duty cycles ranging from 1-50% corresponding to pulse durations of 9 to 450 μs. By use of pulsed nESI, the performance of MS for the detection of many different classes of molecules can be improved in terms of decreasing background chemical noise and enhancing the sensitivity for analytes ranging from small molecules to whole proteins. Specifically, pulses that are less than ~450 μs can be used to decrease the background chemical noise and increase signal-to-background chemical noise ratio by up to 93% and 691% in pulsed nESI MS for six test analytes compared to conventional nESI. Pulsed nESI can also be used for native mass spectrometry to improve signal-to-background chemical noise ratios. Overall, pulsed nESI can be used to significantly improve the performance of MS.

Contents

Chapter 1: Introduction

1.1. General background overview

Mass spectrometry (MS) is a powerful and robust instrumental analytical method that can accurately and efficiently be used to analyse many different molecules nearly simultaneously and rapidly with high sensitivity. Over the last century, continuous research and development in MS and ionisation sources has significantly improved the mass accuracy, mass range, data acquisition speeds, and type and size of analytes that can be measured. Such research and development efforts have established MS as one of the leading approaches for performing quantitative and qualitative analysis.

There are four major classes of biomolecules that are considered building blocks of life: nucleic acids (including DNA and RNA), proteins, lipids and glycans. By 'constructing', modifying and interacting with these components, cells can develop and function, which is critical to cell survival. DNA is synthesised from four nucleic acids, the sequence of which defines our genes stored in the cell nucleus. A type of RNA called messenger RNA, carries the information to be translated into polypeptide chains that, ultimately, will form proteins. These proteins are essential for all living organisms as they regulate most cellular functions that occur in the body.[1] Therefore, it is important to develop robust and precise chemical analysis techniques to characterise such biomolecules.

The analysis of biomolecules is often performed in biology and chemistry and allied fields. The ability to efficiently separate and characterise biomolecules is crucial in understanding health and disease.[2-3] In cells, proteins have major roles in many different critical biochemical processes including energy storage and use, regulating bodily functions, and metabolism.[4-5] MS-based proteomics is a powerful method to identify and quantify many different proteins rapidly in a complex mixture in a limited number of measurements. Typically

in protein MS, the individual protein or its complexes are digested into peptides by an enzyme (often trypsin), and those peptides are separated by one or more stages of chromatography with 'online' detection by MS.[6] By using tandem MS, peptide ions can be isolated based on their m/z ratios, induced to fragment, and a mass spectrum of the fragment ions can be obtained. From the fragment spectra, peptides can be identified, and by detecting multiple peptides from a single protein, the presence of the protein can be inferred. There has been significant interest in the development of so-called top-down strategies in which whole proteins are analyzed directly without enzymatic digestion, which can enable more information to be obtained regarding protein post-translational modifications.[7] Currently, electrospray ionisation (ESI) is a Nobel prize winning method of choice to ionize biomolecules such as proteins and peptides using MS. However, ESI is typically considered incompatible with non-volatile buffers, salts, and molecules which are often present at high concentrations in biological samples. Therefore, signal suppression is a major challenge for ESI MS measurements, which usually require significant amounts of sample preparation and chromatographic separation prior to MS analysis of complex biological samples.[8]

In this work, an emerging technology entitled pulsed nanoelectrospray ionisation (pulsed nESI) was investigated for the characterization of different classes of biomolecules. During the course of this research project, Ninomiya and Hiraoka published a paper reporting the use of a pulsed nESI source in which a direct current of up to ~1500 V was superimposed on a pulsed waveform of up to ~4000 V to initiate and maintain nESI.[9] However, a direct comparison between the analytical performance of such a source to conventional nESI was not reported. It is reported in this thesis that pulsed nESI without a biasing voltage can be used to efficiently ionise molecules by rapidly by increasing the voltage from 0 to ~3 kV and with pulse widths ranging from 9 to 450 μs (duty cycles ranging from 1-50% with a frequency of 1.1 kHz).[9] The performance of the new pulsed nESI technique was directly compared to more

conventional nESI source using the same instrument and emitter tips. By use of pulsed nESI, the performance of MS for the detection of many different classes of molecules can be improved in terms of decreased background chemical noise and enhanced sensitivity. In this work, pulsed nESI was demonstrated for small molecules with masses as low as 124 Da to proteins with masses as high as 29 kDa. Comparisons of background noise, signal-to-background chemical noise ratios, signal-to-noise ratios and protein charge states were also performed and discussed. Pulsed nESI can potentially be used in many different types of ESI MS applications to increase sensitivity and potentially to prolong the use of batteries in portable MS instruments.

1.2. Proteins

Thousands of different proteins exist with many different functions that support the life cycles of prokaryotic and eukaryotic cells.[10] For example, proteins have important roles in maintaining cell shape, modulating biochemical reactions, receiving environmental signals and regulating movement.[11] Proteins normally have complex three dimensional and higher order structures.[12] The order of the amino acid residues in the linear sequence of protein corresponds to the DNA sequence resulting from transcription and translation.[13] The resulting polypeptides can form into secondary structures, tertiary structures and quaternary protein structures, which results in functionally diverse three-dimensional structures.[14] Rapid, sensitive and precise approaches for analysing proteins are highly desirable. In the field of pharmaceutical and clinical chemistry, the chemical analysis of proteins is important for discovering biomarkers for diseases, understanding biochemical mechanisms, and ensuring medicines are safe. For example, an agonist or antagonist drug matches the structure of signal proteins to stimulate or stop the biochemical reaction in the cells.[44] Therefore, protein structural analysis is crucial in drug discovery and development.[15]

1.3. Small molecules

Small organic molecules are chemicals with low molecular weight that can serve various biological roles, including as cell signalling molecules. The major classes of small organic compounds are amino acids, lipids, peptides, nucleic acids, phenolic compounds, alkaloids, sugars and fatty acids. In the body, small molecules have the major function of binding with the receptors on the cell membrane to stimulate or stop biochemical reactions in cells.[16] Peptides are comprised of multiple amino acid residues joined together by peptide backbone amide bonds. Peptides are much smaller than proteins, their sequences are simple and easily break down to amino acids, and can more easily penetrate the skin and intestine.[17-

[18] Within cells, peptides can perform biological functions, for example, some peptides can act as hormones that regulate the metabolism. Therefore, peptides are broadly used in health and cosmetic industries, such as anti-aging, anti-inflammatory and muscle building.[19-20] Lipids are hydrocarbon molecules that have a long fatty acid chain as hydrophobic tail. The functions of lipids include energy storage, signalling and acting as structural components of cell membranes. Lipids also have applications in the cosmetic, food and clinical industries. Xenobiotic small molecules are widely used and designed in the biomedical industry because their biological regulatory processes tend to be more predictable and bioavailable in the cells than for macromolecules (such as proteins).[21] Analysing and developing small molecules can increase the efficacy and accuracy of the target drug. Therefore, small molecule analysis is essential to predict the bind sites for the developing of drug discovery.[22]

1.4. Mass spectrometry

MS is a robust and emerging technique that is commonly used for the analysis of all kinds of molecules from single atoms to large biomolecules and non-covalently bound assemblies including whole viruses.[7] The application of MS dates back to the early 1900s, when Sir J.J. Thomson and William Francis Aston developed the first MS that separated ions using different parabolic trajectories in an electromagnetic field. The first MS had the capability to separate ions but had poor resolution and sensitivity.[23] A year later, Aston designed and built a succession of 'mass spectrographs' with increasing resolution, incorporating both magnetic and electric fields. In this case, the MS could be used to measure the isotopic composition of chemical elements, which supported the early structure elucidation of the atom.[24] In 1918, electron 'impact' ionisation (EI) was first reported by J. Dempster, in which gases and vapours of volatile and semivolatile molecules were bombarded by electrons to generate a radical cation ($M^{+\bullet}$) by removing an electron.[25-26] This method of ionisation

ultimately became widely used for deducing the structures of small molecules including from natural extracts based on ion fragmentation mechanisms and rules. In the 1930s, Alfred O. C. Nier revolutionised mass spectrometry by constructing a sector field MS, including associated vacuum technology and an electric sector component. Such innovatively designed and constructed mass spectrometers of all shapes and sizes have played an important role in the tremendous growth of mass spectrometry until today.[7, 24] As a notable example, in the second world war, Nier and co-workers developed a high mass-resolution double-focusing instrument that could be used to separate uranium-235 from uranium-238, which was useful in the development of the first nuclear bomb.[23] In 1952, the invention of gas chromatography (GC) can be attributed to A. T. James and A. J. P. Martin, in which chemical compounds can be separated from mixtures in the gas phase.[27] In the 1950s to 60s, MS was widely used for the fragmentation of complex molecules (such as natural product molecules), and this led to the application of MS for structural determination of unknown molecules.[28] In this period, Fred McLafferty and Roland Gohlke coupled GC 'online' to MS allowing the structural analysis of analytes separated by GC, which substantially improved the analytical capability of GC.[29] By the 1980s, small organic molecules could be readily analysed by MS. However, analysing larger molecules (proteins, complex carbohydrates and other macromolecules, such as nucleic acids) by MS provided a significant challenge.[28] In 1984, John Fenn reported the coupling of electrospray ionisation to a modern mass spectrometer (building on the earlier work of Dole), which was demonstrated to be highly useful for transferring large molecules into the gas phase without extensive fragmentation and decomposition.[30] In 1985, the matrix-assisted laser desorption ionisation (MALDI) was first developed by Franz Hillenkamp, Michael Karas and their colleagues.[31] Over the past several decades and concurrent with the rapid development in ionisation methods, sector based instruments were gradually replaced with faster scanning and higher resolution mass analysers. After decades of accelerating development, MS has become

significantly more rapid, sensitive and specific for analysing wider classes of biomolecules. In particular, there has been a breathtaking explosion in the number of different types of ionisation methods that have been developed for MS, and specifically for detecting large biomolecules both with or without sample pre-treatment.

1.4.1. Modern mass spectrometry for protein analysis

Protein analysis by using modern MS can be highly beneficial for detecting and identifying proteins in complex mixtures, and obtaining structural information.[7] By using particular ionisation sources, intact proteins can be converted into the gas-phase and the molecular mass can be precisely measured.[32] For many applications, the separation of proteins is one of the major challenging steps compared to the separation of smaller molecules like peptides.[33]

There are two major methods used in protein analysis by mass spectrometry that are referred to as the top-down and bottom-up approaches. The bottom-up approach is the most widely used to identify proteins by first digesting the proteins into peptides prior to their separation and MS measurement, usually by liquid chromatography tandem mass spectrometry.[34] The fragmentation patterns of isolated peptides from tandem MS data can then be compared to predicted fragmentation patterns from sequence databases using bioinformatics methods to identify peptides and infer the proteins that gave rise to such peptides. A limited number of peptides can precisely identify a specific protein.[35] However, different protein isoforms can generate a set of identical peptides, which results in the information on isoform and stoichiometry of post-translational modifications (PTMs) on a particular peptide often being lost.[36]

The top-down method is a beneficial approach for the analysis of intact proteins in which the entire protein is introduced into the mass spectrometer, where the entire protein and

its fragmented ion masses are measured.15 In a majority of top-down proteomics methods, intact protein ions are generated from acidified solutions that contain both aqueous and organic components (e.g., 50:50:1 (v/v) methanol:water:formic acid). In such solutions, proteins are largely unfolded meaning a higher number of basic sites are available for protonation (in addition to a higher surface accessible area) such that the resulting ions are highly charged, facilitating effective fragmentation upon ion activation in the gas-phase. Compared to the bottom-up approach, sample preparation for top-down MS does not need any chemical modification steps, such as reduction and alkylation, which can potentially reduce experimental artifacts.8 Therefore, the major advantages of the top-down ability to introduce the whole protein chain into the MS can include: (i) more detailed sequence information (including PTM information), and (ii) potentially increased sequence coverage and structural characterization of proteins.14 Therefore, the identification of information resulting from genetic variants, alternative splicing and post-translational modification is possible, unlike in typical bottom up proteomics measurements. However, compared to the bottom-up technique, the top-down method tends to be less sensitive, is more technically demanding, and results is far fewer protein identifications.[37]

During the past decade, protein identification methods have significantly improved in sensitivity and accuracy, leading to the analysis of large biological complexes. Innovations in protein analysis methods have led to an improved understanding of biochemical mechanisms of individual proteins, and drug compounds that can bind to specific locations and interact with such compounds.[38] High-precision and high-throughput detection methods have the capability to obtain information regarding protein sequence, and sequence coverage has been further expanded by high resolution MS.[15]

1.5. Ionisation

Ionisation refers to the process of charging a sample molecule to form ions, which in atmospheric pressure ion sources occurs prior to entering the inlet to the MS. The charged ions can be either negatively or positively charged.[23] There are several different ionisation methods developed for different applications. For example, ionisation can be achieved through electron ejection, electron capture, protonation, deprotonation, or cationisation.[23] Improving the performance of ionisation sources can increase the accuracy and sensitivity of sample analysis. Many kinds of ionisation sources have been created and developed over the years, which charge atoms and molecules based on their polarity, size and stability. Different ionisation sources have specific features. Some of the most common ionisation sources are described below.

1.5.1. Plasma ionisation

A plasma is a charged gas in which ample energy is provided to release electrons from atoms or molecules.[39] That is, plasma is a state of matter in which positively charged ions and negatively charged electrons are present in the gas phase. Complicated ion-neutral and neutral-neutral reactions in such plasmas can result in the formation of a wide range of positive and negative ions, and reactive neutral species (e.g., ozone in oxygen plasmas and electronically excited metastable atoms in helium plasmas). Plasmas can be generated in a number of different ways and used to ionize molecules for detection by mass spectrometry by charge transfer or Penning ionisation mechanisms. For example, in atmospheric pressure chemical ionisation, a high voltage can be applied to a needle electrode to cause a corona discharge, which can ionize ambient air and small organic molecules that are introduced in to the discharge.[40-41] One of the advantages of some plasma ionisation sources is that small molecules with low- and high-polarity can be detected simultaneously.[42] Both liquid and solid samples can be detected by plasma. Liquid samples are often introduced into plasma sources as a

nebulized gas; solid samples are introduced following laser ablation or electro/thermal vaporisation.[43] Plasma ionisation can be used for the rapid detection of a variety of industry-relevant chemical and biological samples, such as drug metabolites,[44-45] agrochemicals,[46-47] and environmental contaminants. Plasma ionisation methods depend strongly on the experimental conditions and solvents that may reduce the sensitivity of specific analytes, resulting in the generation of high background chemical noise.[48]

1.5.2. Desorption ionisation

Desorption ionisation (DI) refers to an atom or molecule being released from a solid surface into the gas phase, which can be ionized by acid-base or redox reactions. Desorption and ionisation of molecules from solid and liquid surfaces can be achieved by the use of high energy beams of photons (e.g., from a laser source), ions, atoms or molecules that are directed at the surface. Energy is deposited that can result in the release of the analyte from the interface. Ionisation typically occurs during the desorption process. For example, matrix-assisted laser desorption and ionisation (MALDI), a "soft" ionisation method where a sample is mixed with a matrix and the mixture is transferred onto a metal plate, is one example of DI. The mixture can then be irradiated by pulsed laser, which results in sample desorption and ionisation.[23]

The development of DI methods have solved the inherent restrictions in EI that only volatile or semi-volatile analytes can be analysed. The applications of DI method in non-volatile sample analysis includes the *in situ* screening of cells, biological fluids and tissues.[49] DI can also be used to measure bacterial colonies, dry blood spots, and many other samples. The other major advantage of some DI methods is the ability to obtain real-time information of molecules on nearly any surface and in some situations without sample preparation.[50]

1.5.3. Spray ionisation

Spray ionisation refers to the application of a high voltage to an analyte solution to form an aerosol of charged droplets which ultimately results in the ionisation of the analytes that are then transferred to the detector of the MS as ions. The major type of spray ionisation source is electrospray ionisation and many variants have been developed including paper spray ionisation[51] and desorption electrospray ionisation.[52] In electrospray ionisation, a high voltage is applied to an ESI capillary containing a sample solution. Spray ionisation sources tend to be highly sensitive and enable a very wide mass range of analytes to be detected.[19] ESI is described in more detail in the next section.

1.6. Electrospray ionisation (ESI)

1.6.1. Introduction

ESI is one of the most widely used ionisation sources. It is an increasingly important method in the clinical laboratory for structure identification (when combined with tandem MS) and quantitative measurements, including those involving large and complex biomolecules.[53] Furthermore, ESI can be used in top-down proteomic methods where an intact protein is introduced into the MS and both its intact form and fragment ions can be detected. Intact protein analysis can be used to improve the information obtained by MS regarding protein sequences.[54] ESI can also generate a distribution of multiply charged protein ions for larger molecules, i.e., $[M, zH+]^{z+}$, which can be beneficial for detecting large molecules on mass spectrometers with upper m/z limits and for increasing the extent of ion fragmentation in tandem mass spectrometry experiments.

1.6.2. Fundamentals

ESI is a soft ionisation technique that can ionise macromolecules by formation of small charged droplets.[51] In ESI, a diluted sample solution (< 20 µM) is typically injected by a syringe pump through a capillary emitter at a relatively low flow rate (<100 µL/min for infusion/protein measurements; Figure 1).[55] A high voltage is applied to create a fine spray of highly charged ionic droplets which are 'suctioned' into the capillary entrance to the MS (Figure 1a). Due to the application of a high voltage to the emitter, an intense electrostatic field is generated resulting in the formation of a Taylor cone.[56] Then, a fine spray of highly charged droplets undergo jet fission as a result of the electrostatic forces and the internal repulsion between charges.[57] The initially formed droplets reduce in radius during the solvent evaporation process which results in an increase in the electrostatic repulsion in the charged droplet. As a result, Coulombic fission occurs forming smaller charged droplets that increase the surface charge density. The critical radius at which droplet fission occurs is known as the Rayleigh limit (Equation 1)[58] in which Q_R corresponds to the amount of charge on the droplet. γ represents the solvent's surface tension, ε_0 refers to the vacuum permittivity and R is the droplet's radius.

$$Q_R = 8\pi(\gamma\varepsilon_0 R^3)^{1/2} \qquad\qquad \text{(Equation 1)}$$

The formation of charged ions is one of the main advantages of ESI which includes singly charged small molecular ions and/or multiply charged ions of large complex biomolecules. ESI is particularly useful for generating protein ions with higher charge states, which is important for methods, such as electron capture dissociation where high charge states allow for greater fragment ions and higher sequence coverage.[53]

1.6.3. Nanoelectrospray ionisation (nESI)

In nanoelectrospray ionisation (nESI), a modified ESI source that requires lower sample flow rates (< 10 nL/min) is used.[59] The aperture size of the spray emitter determines the solution

flow rate and size of charged droplets. In comparison to conventional ESI which uses relatively large emitter tips (> 1-70μm in internal diameter), nESI uses emitter tips that are 1 to ~70 μm in internal diameter, although more recent advances have resulted in the use of capillary nESI tips sub-micron emitter diameters. Such emitters can be used to form initial ESI droplets that are an order of magnitude smaller than in more conventional ESI and uses 100-1000 times less sample volume.[60] Smaller aperture spray needles also have the advantage of reducing background chemical noise partly owing to more efficient desolvation during the ionisation process.[23]

As lower amount of analyte solution is required for nESI, and the position of the nESI emitter is much closer to the entrance of the mass spectrometer than ESI, the transmission of charged ion droplets is far more effective than conventional ESI. Furthermore, effusing solutions at lower flow rates increases the sensitivity (Figure 1b). In addition, since low concentrations are typically used in nESI, sample solution waste is considerably reduced by up to 100 times compared to normal ESI.[23] nESI is considered more sensitive than ESI. However, many biomarkers are very low in abundance and in a typical proteomics experiment using nESI, only a fraction of total proteins in a given sample can be measured. Thus, it is important to develop methods that can be used to increase ion signal for biomolecules in nESI further.
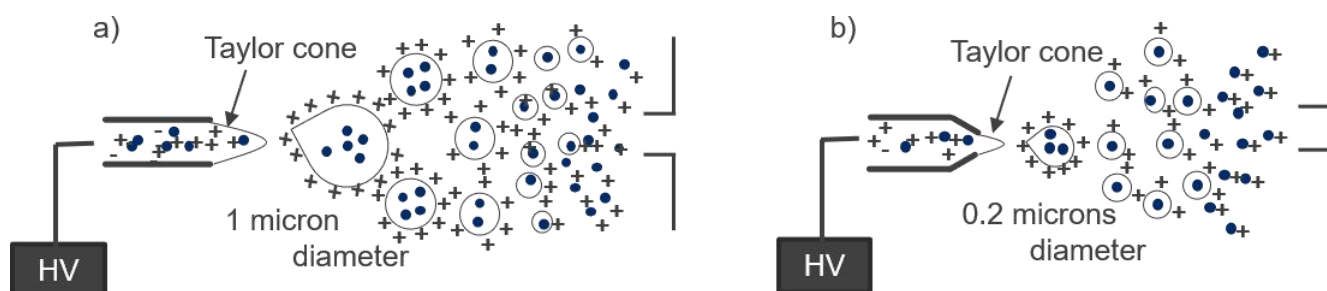
Figure 1. Diagram of (a) electrospray ionisation (ESI) and (b) nanoelectrospray ionisation (nESI) mass spectrometry.

1.6.4. Limitations & Ion supercharging

There are a few significant limitations with using electrospray ionisation. Firstly, the initial formation of relatively large ionic droplets can result in relatively low ion signal. In applying high flow rates (1-10 µL/min) and a constant high spray voltage (3-5 kV) from relatively large bore capillary emitters (e.g., >100 µm) results in the generation of large initial droplet sizes which do not readily desolvate.[61-62] Thus, signal can be detrimentally affected. nESI in which narrower emitter capillaries and lower flow rates are used, can reduce the spray voltage and flow rates to low or even sub-nL/min to substantially increase ionisation efficiency and signal intensity. Moreover, nESI can minimize ion suppression to produce high charge states and high abundance to maximize the sequence coverage of intact protein ions.

In addition, in protein analysis approaches, the extent of ion fragmentation can be significantly supressed by the formation of protein ions in relatively low charge states. Over the last decade, 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'.[63] Williams and co-workers first discovered that m-NBA (m-nitrobenzyl alcohol) can be used to increase protein ion charge states. m-NBA is a non-volatile chemical that has been shown to be useful for increasing the average charge states of the proteins when doped into the ESI solutions at relatively low concentrations. Since then, different superchargers have been discovered, such as dimethyl sulfoxide (DMSO), m-chlorophenol, sulfolane, 2-methoxyethanol, formamide, and cyclic alkyl carbonates such as 1,2-butylene carbonate (C2).[64-65] 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,[53, 66] and such highly charged protein ions are so reactive that they can protonate $Ar(g)$ and $N_2(g)$ in thermal, ambient temperature ion-molecule reactions.[67] The Kuster group reported that DMSO can be doped into the eluent in LC-MS/MS

to significantly boost the number of proteins and peptides identified in whole cell digests by 10-25%, resulting in an improvement in the sensitivity for protein analysis in bottom-up proteomics by up to 10 fold.[68]

In protein analysis using ESI MS, the Rayleigh equation (Equation 1) indicates that the higher the droplet's radius, the more charge can be accommodated by the droplet. Although alternative explanations have been reported, one likely contributing factor to the supercharging effect is that such additives can increase the surface tension of droplets formed from denaturing solutions compared to the same solutions without the additive, which can increase the extent of charging droplets can accommodate prior to complete ion desolvation, ultimately leading to the formation of higher charged ions. Donald and his co-workers have tested the additive, 1,2-butylene carbonate biomolecules with different sizes, such as ubiquitin (Ubq), cytochrome c (CytC), myoglobin (Myo) and carbonic anhydrase II (CAII). The most abundant charge state of protonated CAll (29,000 Da) increased from 36+ to 44+, and Ubq (8,600 Da) increased from 13+ to 17+. Therefore, by adding 1,2-butylene carbonate to the analyte solution at low concentration (5% v/v), intact protein ions are generated in higher charge states than when the samples are analysed without 1,2-butylene carbonate and other known supercharging additives. It has also been shown that the performance gain for extensive sequence coverage increased to 85% to 99% for the tandem MS of intact proteins with masses as large as 66 kDa.[53] The results obtained have shown improvement of charge states for all proteins tested, and the extent of the increase in charging increases as the masses of the proteins increases. In addition, the use of these additives in electrospray solutions can increase the tolerance of salt adduction. Williams and co-workers have shown that the addition of m-NBA in low concentration into native protein solutions with 1 mM sodium chloride, decreased the average number of sodium ion adducts to Ubq from 3.5 ± 0.1 to 1.2 ± 0.2, which led to improvement in mass accuracy for large proteins and protein complexes in native MS.[69] Thus, it is expected that utilising

supercharging additives with pulsed nESI MS will increase the charge states of proteins, compared to conventional nESI.

1.7. Direct current electrospray, alternating current electrospray & pulsed nanoelectrospray ionisation

1.7.1. Direct current ESI

There are two main methods for supplying high voltage to an ESI source: direct current (DC) and alternating current (AC). Direct current electrospray ionisation (DC ESI) is by far the more conventional technique than AC ESI for the analysis of a wide variety of biomolecules using ESI MS. DC ESI can supply a constant spray voltage to the solution flow without polarity switching, i.e., either positive mode or negative mode. The continuous supply of high voltage in DC ESI corresponds to a duty cycle of 100% (Figure 3a); i.e., ions are continuously formed for the entire duration of the measurement.[70]

1.7.2. Alternating current ESI

Alternating current electrospray ionisation (AC ESI) is a newer technique than DC ESI in which the applied voltage polarity switches from positive to negative (usually with a sine waveform) with a frequency range typically from 80 to 400 kHz, and which can increase the signal intensity by an order of magnitude for some analyte ions in ESI (Figure 3b).[71] Sarver et al. investigated the performance of AC ESI as a function of frequency from 80-400 kHz. The frequency can be tuned to optimize the signal for different analytes.[72] The proposed mechanism is somewhat complicated. Briefly, the Go and Chang groups proposed that low mobility protein ions can be charged and accumulated in the Taylor cone during the AC ESI process (Figure 2). Basically, when the polarity of the high voltage AC field is positive, high mobility protons rush to the Taylor cone to protonate proteins in solution prior to droplet formation, and ionic droplets

can be formed. When the polarity switches, ionic droplets are no longer formed and charged proteins remain in the Taylor cone as the protons rush back into the bulk of solution. Presumably, the rate that protein ions are ejected from the Taylor cone in ESI droplets is lower than the rate that proteins electrophoretically migrate to the Taylor cone resulting in preferential entrainment of analytes prior to ionisation, thereby boosting signal abundances relative to conventional DC ESI. Furthermore, the authors propose that as the AC frequency increases, the local pH in the Taylor cone decreases (from pH 4.1 to pH 2.75) as more protons are driven into the tip of the cone, resulting in an increase in the extent of protein ion charging (e.g., the most abundant charge state for CytC increases from +13 to +16 for a frequency of 50 to 350 kHz). Hence, AC ESI can enable more efficient transport of protein ions into the MS to increase protein ion signal intensity, and potentially forming higher protein ion charge states.[71]
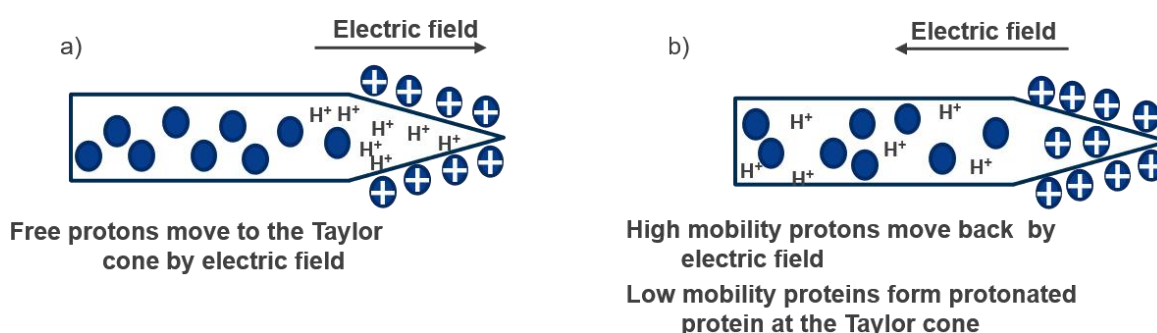
Figure 2. Diagram for rationalising the signal enhancement in AC ESI, highlighting some key processes that occur when the polarity switches from the (a) anodic half cycle to the (b) cathodic half cycle.

1.7.3. Pulsed nESI

Based on the mechanism of AC ESI, an aim of this MPhil project was to develop a new method called pulsed nESI in which the polarity switches from 0 V to a high positive voltage repeatedly (Figure. 3c). For example, the use of a 1 kHz waveform operating with a 50% duty cycle would yield high voltage pulses for 500 μs for each pulse and spaced by a 500 μs delay.

With a high voltage pulser, the duty cycle can be reduced such that the high voltage pulses are only nanoseconds long, while having microseconds between pulses. This added control over AC and DC ESI is expected to yield performance gains because charged droplets can be rapidly pulsed with a higher proton density than in either AC or DC ESI. We expected that extremely small droplets as low as a femtoliter can be formed by using the pulsed voltages because the initial sizes of ESI generated droplets should theoretically decrease as the ESI current decreases.[73] As a result, the power and sample solution consumption can be significantly decreased with increasing frequency and/or reducing duty cycle to improve ion signal.[74] Thus, pulsed nESI has significant potential advantages compared to more conventional approaches.
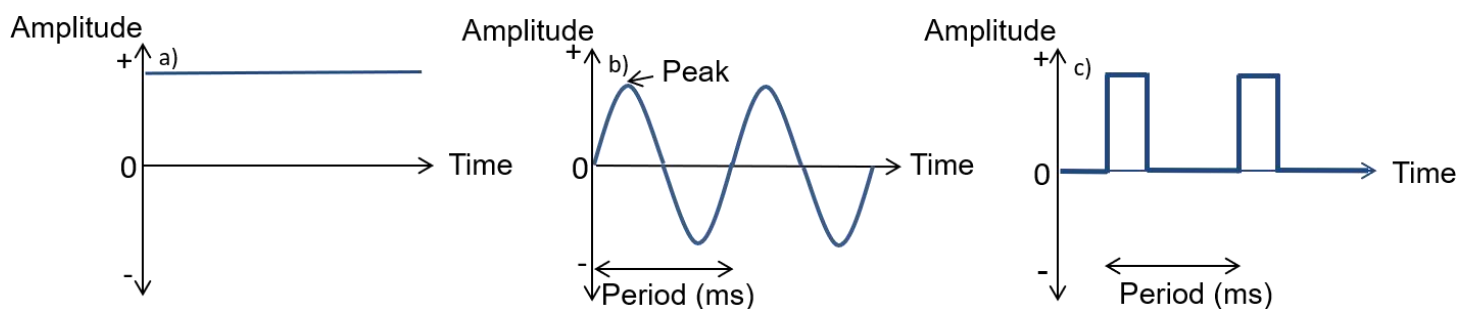
Figure 3. Diagram showing different high voltage waveforms that can be used to initiate and maintain nESI. (a) DC 100% duty cycle; (b) AC (2 cycles shown); and (c) pulsed (2 cycles shown) nESI.

In 2020 during the course of this project, Ninomiya and Hiraoka reported pulsed nESI MS results for peptides and proteins (gramicidin S, ubiquitin and cytochrome *c*) in denaturing solutions (water:methanol 50:50). A pulsing high voltage of up 4000 V and a DC bias voltage of ~880 V was applied to the solution with pulse widths ranging from 200 ns to 1 ms. The results indicated that the ion intensity of analytes decreased as the pulse width decreased, and there were only peptide peaks observed for the mass spectra obtained with the pulse width of 200 ns (i.e., no protein ion peaks were detected). 300 μs was the minimum pulse width required to detect protein charge states, and at this pulse width, the overall ion intensity was stronger than those obtained with shorter pulse widths.[9] In this work, the performance of pulsed vs

conventional is directly compared which was not done in the previous research. An additional objective is to better understand the ionisation mechanism. In this project, a high voltage pulse of up to 2500 V without a biasing voltage and with pulse widths ranging from 90 to 360 µs were used for the pulsed nESI MS analysis of biomolecules including small molecules and proteins.

1.7.4. Pulse width, frequeny and duty cycle relationship

The pulse width in pulsed nESI can be controlled over a wide range of frequencies and duty cycles. Equation 2 represents the relationship between applied duty cycle, frequency (f) and the pulse width (T). The pulsed waveform instrument can be used to control the pulse width for the ionisation source by adjusting duty cycle. Here higher duty cycles result in prolonged voltage durations while generating longer pulse widths. For example, by applying duty cycle of 40% and frequency of 1 kHz using the pulsed waveform instrument, the pulse duration is 1 ms and the pulse width is 400 µs of each pulse period.

Duty cycle % = Pulse width (sec) × frequency (Hz) × 100%   (Equation 2)

1.8. Native protein mass spectrometry and salt adduction

1.8.1. Introduction to native mass spectrometry

'Native' in the context of experiments involving proteins refers to the fully functional protein in its folded and/or assembled form.[75] Native MS has been developing over the last two decades, and refers to the identification and characterization of protein ions formed from native-like solutions, normally using nESI, such that the proteins largely retain their native-like structures and non-covalent interactions prior to detection by MS. Native MS can be used to study proteins and protein assemblies to define structure-function relationships, understand protein functions and complex cellular interactions.[76] Noncovalent interactions can be

maintained during the ESI process, leading to information regarding subunit stoichiometry, binding partners, complex topologies and binding affinities in native MS. Once the protein ions are inside the MS, they can be detected in their native-like intact state (if detected rapidly enough), and can also be fragmented by energy deposition.[77] In native MS, the solution conditions are the key to maintaining the protein in their native folded state, such as pH and ionic strength.[78] Compared with denatured protein analysis, native protein MS requires the use of volatile buffers at near neutral pH values. For example, ammonium acetate and ammonium bicarbonate, both can be used to increase the ionic strength to improve protein stability for measurements.[79] Such volatile buffers can also typically be used to 'desalt' protein ions at high buffer concentration, by competing with non-volatile salts for protein surface adduction during the ESI process.[79] In careful native MS studies, the general structures can be largely retained upon transfer from solution to the gas-phase on the timescale of the ion formation, transfer and detection processes such that: (i) protein-small molecule binding affinities can be accurately measured; and (ii) gas-phase ion collision cross sections can largely match those obtained from X-ray crystallography data.[80]

1.8.2.   Salt adduction in native MS

Salt ions play an essential role in stabilizing native protein conformations.[81] Somewhat low salt concentrations (12 mmol/L) can in some cases effectively mimic the intracellular environment, leading to improvements in charge-charge interactions in native proteins,[82] and result in high charge stability to support the protein in the folded state.[83] However, salts in protein solutions, particularly non-volatile salts, can cause adverse effects on the detection of proteins and protein complexes in the MS. Non-volatile salts can be readily enriched in ESI generated droplets and then bind to protein ions resulting in the formation of multiple salt adducts in the charge state distribution.[78] Salt in ESI solutions can also result in the formation

of abundant ion clusters that increase baseline noise, limiting protein ion detection.[84-86] High concentrations of non-volatile buffer salts are often used in protein solutions in biochemical laboratories and contain high ionic strengths. In ESI MS detection, too much salt in the sample solution can decrease vapour pressure and evaporation is inhibited via colligative effects.[23] Therefore, it is typically necessary to remove non-volatile salts from solutions to prevent such adverse effects. Normally, ammonium salt solutions are used in the native MS analysis of protein and protein complexes, such as ammonium acetate and ammonium bicarbonate.[84, 87] Although ammonium ions can adduct to protein ions in positive mode, ammonia can readily evaporate as a neutral species leaving the proton 'residue' behind. Protein solutions often require a minimum amount of ammonium salt to maintain the native structure and function of the protein.[88] Native MS is highly sensitive to the adduction of non-volatile salts.[89] Salt adduction and ion suppression in native MS can reduce the signal-to-noise ratio of analyte ions.[89] The distribution of some small signals such as salt adducts can reduce the performance of ESI MS peptide and protein analysis.[61] There are many salts which are common constituents of protein solutions, such as sodium chloride, that can adversely impact the performance of protein analysis by reducing the signal intensity of protein ion charge states, ultimately resulting in increased background noise and decreased S/N_C.[90] Therefore, significant amounts of protein desalting steps are often required to obtain quality native MS spectra.

1.8.3. Protein desalting methods

To reduce the side effects of extensive salt adduction on native MS performance, typically extensive sample preparation is required.[69] Several approaches are used in desalting proteins prior to native MS, including ion-exchange chromatography.[91-92] liquid chromatography[93], microdialysis[94] and nanoparticle-based microextraction,[95] However, there some disadvantages to such techniques for protein desalting. Firstly, the structure and

conformation of protein and protein complexes can strongly affected by removing even low-concentrations of salts.[96] Secondly, some proteins require specific salts or other non-volatile cofactors, e.g., Ca^{2+}, to stabilize their structures. For both soluble and membrane bound proteins, the inefficient removal of detergents that are sometimes used in their purification can cause substantial ion suppression.[97]

Alternative techniques have been introduced to improve measurements of proteins by MS. The use of nESI with nanoscale ion emitters can significantly reduce the extent of sodium adduction to protein ions in native MS.[61, 98-99] Nguyen et al. demonstrated that compared to the conventional larger-bore emitters, the binding affinities of six ligands can be measured simultaneously by using nanoscale emitters (~250 nm) since the salt adduction was significantly decreased to enhance resolution. Overall, the use of nanoscale emitters in nESI can increase tolerance to large protein and salt contamination and improve measurements of protein-ligand interactions in native MS.[61] It is hypothesised that by applying pulsed high voltage nESI, the use of lower duty cycles should reduce ESI current owing to the lower duty cycle for ion formation which is conventionally associated with the formation of smaller initial ESI generated droplets. Compared to larger charged droplets, smaller charged droplets lead to better solvent evaporation since there is not much solvent in the droplets to begin with, potentially resulting in the reduction of background noises.[100] Moreover, smaller initial droplets should contain less total salt, and upon solvent evaporation less salt adduction.[61]

1.8.4. Reducing salt adduction by using pulsed nESI MS

As mentioned above, pulsed nESI MS is an emerging technique for biomolecule analysis, which can be used to analyse protein and small molecules. Our hypothesis was that by applying shorter pulse widths to the protein solution, smaller droplets should be generated from the Taylor cone, and thus solvent evaporation should occur more readily resulting in less

background noise by reducing the formation of ionic solvent clusters, and lowering salt adduction. Also, the duty cycle and frequency can be optimized to potentially preference the detection of specific analytes of interest. Although our group has previously shown that the use of nanosecond high voltage pulses in dielectric barrier discharge ionisation can substantially improve MS performance for a plasma ion source, the mechanism of ion formation is very different for ESI. Thus, this project aimed to explore the effects of pulsed nESI on the performance of a spray based ion source under denaturing conditions. In addition, high concentrations of sodium chloride were added into the native protein solutions to investigate whether pulsed nESI has the ability to reduce salt adduction and improve the performance of native MS measurements.

1.9. Aims

The overall aim of this project is to integrate pulsed nESI source with MS to significantly improve the performance of nESI MS for protein and small molecule analysis. The primary objective is to examine whether pulsed nESI source can be used to improve biomolecular analysis by increasing signal-to-background chemical noise ratio (S/N_C). By comparing the technique with conventional nESI, it is hypothesised that reduced ionisation time of the analyte ions in pulsed nESI can facilitate better solvent evaporation leading to the decrease of background noise. In addition, the effects of supercharging additive on the protein charge states in denatured solution using pulsed nESI analysis will also be investigated. The following objectives will be addressed to fulfil the above aim:

i)   Investigate pulsed high voltage nESI for increasing ion intensity and S/N_C of peptide and protein analysis.

High ion intensity and the S/N_C can provide improved confidence in protein identification and quantitation, and broader protein coverage in complex mixtures, such as the contents of whole

cells. The pulsed nESI uses external DC voltage supplier to decrease the limiting of voltage consumption, thereby producing more stable ions entering the MS.

ii)   Investigate the effect of salt adduction and ion suppression using pulsed nESI. Applying pulsed DC high voltage can generate a strong electric field at the nESI narrow tip, and smaller charged droplets are sprayed out in bulk by the strong electric forces. Therefore, in Chapter 3, I have investigated the effects of pulsed nESI on the extent of salt adduction and ion suppression in native MS.

Chapter 2: Experiment and materials

2.1.   Sample preparation

Seven different prototypical biomolecules were used in proof-of-concept experiments involving a small molecule, a lipid, a peptide, and four proteins. Specifically, these were dimethyl methylphosphonate (DMMP, ≥97%), phosphatidylcholine (PC, ≥90%); angiotensin II (Angio, ≥95%); ubiquitin from bovine erythrocytes (Ubi, ≥98%), myoglobin from equine heart (Myo, ≥90%) and carbonic anhydrase isozyme II from bovine erythrocytes (CAII, ≥3,000 W-A units/mg protein), which were all were purchased from Sigma Aldrich. Cytochrome C from equine heart (CytC, ≥90%) was obtained from Alfa Aesar. Methanol (≥99.9%) was obtained from Honeywell. 1,2-butylene carbonate (C2) was sourced from Tokyo Chemical Industry (Japan).

Table 1. Analyte's neutral mass, m/z values of the most abundant ion, and most abundant charge states of analytes used in the current study. The assignment of additional major ions formed by ESI MS of analytes are also listed.

Analyte	Mass (Da)	m/z of most abundant ion[a]	Most abundant charge state	m/z of additional major ions
DMMP	124	125	1+	111, neutral loss of CH2 from precursor ion
PC	759	760	1+	782 [M+Na]$^+$ of PC(34:2), and 810, [M+H]$^+$ of PC(38:4)
Angio	1,031	1,032	2+	517 ([M+2H]$^{2+}$)
Ubi	8,566	-	11+	Multiple charge states
CytC	12,361	-	14+	Multiple charge states
Myo	16,954	-	19+	Multiple charge states
CAII	29,855	-	30+	Multiple charge states

[a] Positive mode ionisation.

For most nESI experiments, samples were solubilized in a 50:50 by volume mixture of deionized water and methanol. By addition of 1% acetic acid and 5% v/v of 1,2-butylene

carbonate in methanol solution, intact protein ions can be generated in high charge states compared to ions generated from solutions without the additive. The final concentrations of the analytes in the ESI solutions ranged from 1 to 10 μM.

In native MS experiments, the proteins analysed in the experiment were the same as studied in the denatured protein experiments (see above). In this work, native protein sample solution consisting of 50 mM ammonia acetate (Chem Supply, ≥97%) and 5 to10 μM of protein of interest were used. Such solutions can be used to largely maintain native-like protein structures throughout the ESI desolvation process on the timescale of the ESI MS measurement. To investigate the salt adduction in pulsed nESI MS, two different concentrations of NaCl (Ajax Finechem, ≥99.9%), specifically 25 mM and 50 mM, were added into the protein solutions for Ubi, CytC and Myo. Because CAII is larger with a higher solvent accessible surface area and therefore more prone to salt adduction during ESI, a lower concentration of non-volatile salt, either 0.5 mM or 1 mM, was added to the CAII solution compared to the other proteins. To compare pulsed nESI and conventional nESI, the same sample solutions were used in both techniques.

2.2.   Mass spectrometry

Experiments were performed using a Linear Ion Trap LTQ mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) which is modified with an aftermarket electrodynamic ion funnel (Heartland Mobility, Wichita, KS, USA). The temperature applied to the stainless steel capillary entrance to the MS was 120 °C. The ion funnel conditions were optimized for 'maximal' signal. Specifically, the RF frequency and drive were tuned between 70-90 kHz and 10-20 kV. The entrance and exit electrode voltages for the ion funnel were set to 100-250 V and 100-250 V, respectively. The potential applied to the extraction lens was 1-20 V. A home-built nanoelectrospray ionisation (nESI) source was used to ionise a variety of test analytes

(Table 1). nESI emitters were pulled from glass capillaries (1.0mm o.d./0.78 i.d., 1.2 mm outer diameter/0.69 mm inner diameter, Harvard Apparatus, USA) using a Flaming/Brown micropipette puller (Sutter Instrument, Novato, CA, USA, Model P-97). The inner diameters of emitters are regularly confirmed in our lab by use of scanning electron microscopy measurements to calibrate the tip pulling conditions that are routinely used. For DMMP, Angio, Ubq, CytC, Myo and CAII, nESI emitters with an inner diameter of 500 nm were used while for PC, those with 300 nm inner diameters were used throughout in all experiments comparing the performance of DC and pulsed nESI-MS. The glass capillaries were then coated with Pd (Quorum, UK, SC7620) to ensure conductivity. A coated nESI emitter was positioned about 3 mm from the capillary inlet to the MS for all of the DC and pulsed nESI experiments. All data comparing the performance of DC and pulsed nESI-MS for a given test analyte was acquired on the same day keeping all conditions as identical as possible with the exception of the application of the nESI high voltage being either pulsed or direct current."

For the native MS experiments, a 0.005'' platinum wire (SDR Scientific, AU) was inserted into an uncoated nESI glass capillary (1.0 mm o.d./0.78 i.d., Harvard Apparatus, UK) filled with 30 μL of a sample solution. The pulsed voltage from the external DC high voltage supply was applied directly to the platinum wire. Strong electric fields can then be generated at the emitter tip by applying a high voltage (+0.8-1.5 kV) that is either pulsed or DC for initiating and maintaining pulsed and conventional nESI. Generation of the ultrashort, repetitive high voltage pulses is detailed below.

2.3. Generation of high voltage pulses for nESI

The nESI pulse setup consists 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, Wavesufer 3024, Teledyne Lecroy, USA), a waveform generator (20MHz; DG1022, Rigol, Beaverton, OR, USA), a stabilised power supply (model 272A, B W D Electronic, Melbourne, Australia), a control panel and a picoammeter (Keithley 6485 Picoammeter, Oregon, USA). An electrical circuit was used to generate high voltage pulses for performing pulsed nESI MS. As shown in Figure 4, a DC high voltage potential is applied to the internal circuit of the high voltage pulsar, which includes a logic control circuit, an isolated DC/DC converter (or supply), and a half-bridge leg. A positive 5 V is connected to the input of the isolated DC/DC converter and the logic control circuit. The isolated power supply generates two isolated voltages for the dual channel isolated gate driver which drives (turns on/off) the switching devices on a half bridge, S1 and S2. These two switches correspond to a complementary mode, only one switch can be turned on at any time, for example when S1 is on, S2 must be off and vice versa. When S1 is on and S2 is off, the output of the generator is connected to the positive rail of the HV DC power supply, resulting in the transmission of high voltage to the source. In contrast, when S1 is off and S2 is on, the output of the generator will connect to the ground, resulting in no voltage applied to the source. When ions are introduced into nESI source, applying high DC voltage to the nESI capillary and grounding the circuit forms a blocking circuit, where the potential gradient was applied between the nano-pulse generator and the nESI source. The HV power supply was used to apply voltage to nESI emitter from +1000 V to +2500 V, and the duty cycles applied ranged from 10% to 40% to form pulse widths from 90 to 360 µs at 1.1 kHz.
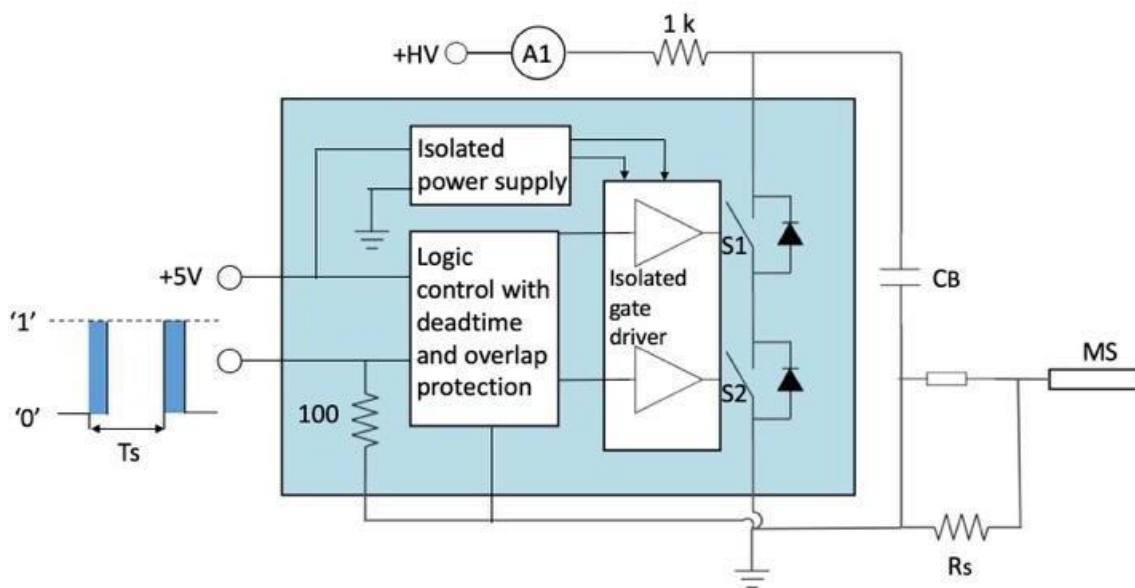
Figure 4. Electrical circuit for generated high voltage pulses for performing pulsed nanoelectrospray ionisation for mass spectrometry. *HV*, A, T, C and R correspond to high voltage, ampere meter, period of waveform (time), decoupling capacitor, and resistor.

2.4. Data analysis

Mass spectra were processed using XCaliburTM (Thermo Fisher Scientific, USA) and the average signal intensity was obtained by averaging the integrated ion abundance of the most abundant analyte ion for at least three experimental replicates. The raw data was exported to Excel for calculating the average background chemical noise (N_C), which refers to the average chemical background signal that is not assigned to signal arising from the analytes. The signal-to-chemical background noise (S/N_C) was obtained from the measured analyte signal divided by N_C. Average N_C and S/N_C values were obtained from at least three technical replicate mass spectra.[42, 101] For all comparisons between pulsed and DC nESI-MS, *p* values were calculated. If the *p* value is less than or equal to 0.05, the results are considered statistically significant.

Chapter 3: Results and discussion

In this chapter, seven analytes were tested in both conventional nESI and pulsed nESI in which ion abundances and S/N_C of all analytes were measured and calculated. By applying different duty cycles for pulsed nESI, the fraction of the time that ions are formed by nESI can be reduced from 100% (direct current nESI) to 10% of the time (with pulse width as narrow as 90 μs). Stable ion signals were not observed at lower duty cycles under these conditions. The impact of pulsed nESI on protein ion charge states formed from denaturing solutions was investigated including in cases in which a supercharging additive was introduced to facilitate the generation of high charge states. In the second part of the chapter, the effects of using pulsed nESI in native mass spectrometry was investigated, with an emphasis on solutions with high concentrations of non-volatile salts. Typically, nESI is considered incompatible with high concentrations of non-volatile salts such as sodium chloride because such ions can bind to protein ions resulting in the generation of salt adducts that broaden peaks and lower S/N_C values, in addition to the formation of ionic salt clusters, thereby decreasing sensitivity and efficacy of native protein analysis. Pulsed nESI under specific conditions can increase S/N_C values for solutions containing high concentrations of non-volatile salts.

3.1.   Pulsed nESI increases signal-to-background chemical noise ratios

To investigate the effects of pulsed nESI, mass spectra were obtained for DMMP, PC, Angio, Ubq, CytC, Myo, and CAII in the denatured solvent (MeOH:Water:ACOH, 50:50:1) at duty cycle from 10% to 40%. Duty cycles below 10% were not investigated because no ion signal could be readily detected with lower pulse widths under these conditions. For comparison, conventional and pulsed nESI mass spectra were obtained for same analyte

solutions (Figure 5). For each analyte, ions corresponding to protonated molecules can be readily detected with ion counts of 0.7×10^5 to 3.9×10^6 and 2.0×10^5 to 9.5×10^6, respectively by pulsed nESI and conventional nESI methods. For example, abundant ions corresponding to the protonated small molecule and lipid (DMMP and PC) were observed at m/z 125 and 761, respectively, by both the pulsed and conventional nESI methods. In both types of ionisation, protonated DMMP can fragment via the loss of CH_2 to presumably form protonated dimethylphosphite, which is a well-known fragmentation pathway for this test molecule in ESI and other soft ionisation methods.[42, 102] Interestingly, this fragment ion was 85% lower in abundance by using pulsed nESI with duty cycles from 40 to 10% compared to DC nESI. In the case of PC, an ion corresponding to the sodiated PC (m/z 783) was also measured under all conditions in addition to the protonated molecular ion. Pulsed and DC nESI MS of peptide and proteins (i.e., Angio, Ubq, CytC, Myo, and CAII) resulted in the formation of multiple charge states of between 3+ and 30+ that were broadly similar. Although the ion abundances obtained using pulsed nESI were either about the same or slightly lower than DC nESI such as for the smaller molecules (i.e., DMMP, PC) and Ubq, the use of pulsed nESI can significantly reduce background chemical noise. For example, the chemical background noise values of all analytes are obtained from the pulsed nESI ranged from 74 to 6900, which is up to 93% lower compared to that obtain by the conventional nESI approach (Table 2).

The mass spectra obtained for the proteins had some contaminant ions such as those at m/z 538 and 555 which were also formed by nESI of un-spiked solutions. The same contaminant peaks can be observed in all nESI spectra, regardless of the identity of the analyte. However, when the same samples were analysed using pulsed nESI, the intensity of the contaminant ions decreased by up to 74%. In addition, Myo is heme protein in which the heme group (m/z 616) is noncovalently bound but can readily dissociate in acidified denaturing

solutions (Figure 5f and 5m). The ion abundance corresponding to the heme group decreased by 27% in pulsed nESI MS to 1.9×10^5 from 2.6×10^5 in conventional nESI (Figure 5f and 5m).

Signal-to-background chemical noise (S/N_C) is a key parameter in analytical mass spectrometry.[42] Thus, to investigate the performance of the pulsed nESI, we calculated S/N_C by dividing the abundance of the ion of interest (S) by the average chemical background ion abundance (N_C), and compared these values with the S/N_C values obtained from the conventional nESI method. Although the overall signal intensity obtained by conventional nESI is slightly higher for some analytes (i.e., DMMP and Angio), the overall background chemical noise is significantly lower than that of the conventional nESI method (see above and Figure 6). As presented in Table 2, the highest S/N_C calculated by the pulsed nESI is 2200, which is up to 154% times higher compared to that of the conventional nESI method. For example, the S/N_C of DMMP and PC obtained by the pulsed nESI is 4,500 and 140 whereas the values obtained from the conventional nESI is 570 and 71, respectively. The S/N_C of the large molecules decreased significantly by both pulsed and conventional nESI methods. For example, the S/N_C ratio obtained for Ubq, CytC, Myo and CAII from the pulsed nESI ranges from 66 to 350, whereas those obtained from the conventional nESI ranges from 16 to 220 (Figure 7).

The formation of larger biomolecular ions such as proteins (Ubq, CytC, Myo, and CAII) were also investigated using both pulsed and conventional nESI. As presented in Figure 5 and Table 2, the larger molecules mostly resulted in increased ion abundances by using pulsed nESI compared to conventional nESI. For example, the ion abundance of CytC and Myo increased by 25% and 33%, respectively by the pulsed nESI compared to DC nESI, whereas ion abundance of Ubq and CAII decreased by 69% and 39% by pulsed nESI in contrast to the conventional nESI method. However, the use of pulsed nESI reduced background chemical

noises by 90%, 92%, 77%, and 58%, respectively for Ubq, CytC, Myo, and CAII by pulsed nESI compared to the conventional nESI (Table 2).

To investigate the effects of the pulse width, a range of duty cycles from 10% to 40% were investigated for the pulsed nESI at a fixed frequency of 1.1 kHz. To save power as well as to improve the performance of ionisation, applying pulse widths below half of the 100% duty cycle (constantly high voltage supply) can produce similar ion abundance as the conventional nESI. In contrast, lower duty cycles (i.e., below 10%) resulted in exceedingly limited ion signal. Therefore, applying the duty cycle ranging from 10% to 40% resulted in reliable and consistent sample analysis; i.e., stable ion signals. As presented in Figure 6, the background noise of peptide and protein ions decrease with reduced duty cycle values from 40% to 10% corresponding to a decrease of 81% to 97%. For DMMP, PC, and Angio, both the analyte ion abundance and the background noise values decreased at lower duty cycles (i.e., 10%) compared to that of higher duty cycles. For example, the background noise of Angio significantly decreased from 74 at 40% duty cycle to 2.0 at 10% duty cycle as the ion abundance reduced from 1.3×10^5 to 1.4×10^3, in which the ion abundance of background noise significantly decreases more than that of analyte ions. For other molecules, the pulsed nESI produces lower variations in terms of background chemical noise for different duty cycles (see Figure 6).

In principle, the nESI signal for the analytes of interest and the background chemical ions should both in principle increase as the duty cycle increases from 10-100% because ions are being formed for a greater fraction of the time at higher duty cycles than lower duty cycles. However, the response between the ion abundances and magnitude of the duty cycle clearly does not linearly increase. A number of factors can result in such a non-linear response with duty cycle. 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 droplets tend to be formed at higher ESI currents.[103] 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. Second, the electrophoretic mobility of ions in solution may be different. In such cases, the initial droplet compositions may be different and result in partial separation of analytes and contaminant ions during the initial droplet formation processes. Finally, 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 which may be beneficial to the detection of lower abundance ions and result in the signal not being linearly dependant on duty cycle.
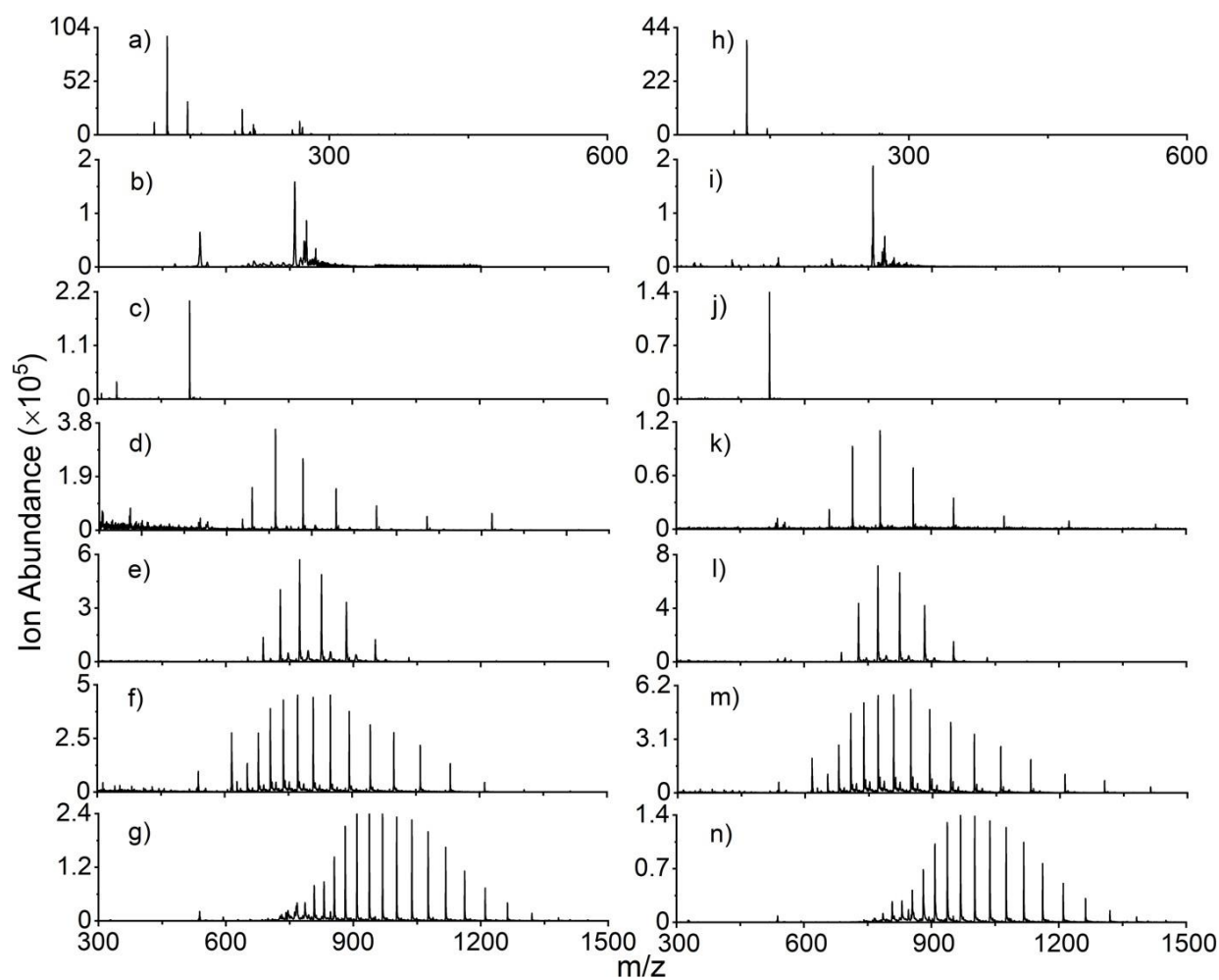
Figure 5. Conventional nESI (a, b, c, d, e, f, g) and pulsed nESI (h, I, j, k, l, m, n) mass spectra of 1 μM DMMP (a, h), 100 μM PC (b, i), 1 μM Angio (c, j), 1 μM Ubq (d, k), 1 μM CytC (e, l), 1 μM Myo (f, m), 5 μM CAII (g, n). The 'optimal' pulsed nESI duty cycles for each analyte were 40%, 40%, 40%, 10%, 10%, 30%, 10% for DMMP, PC, Angio, Ubq, CytC, Myo, and CAII, respectively.

Table 2. Background chemical noise values (N_C) and signal-to-background chemical noise (S/N_C) for all analytes measured by conventional nESI and pulsed nESI mass spectrometry. Refer to Figure 5 for the corresponding mass spectra.

Analytes	S/N_C				N_C			
	nESI	Pulsed nESI	% increase		nESI	pulsed nESI	% decrease	
DMMP	567 ± 15	4488 ± 23	691%	<0.000010	168.6 ± 5.7 ×10²	28.22 ± 0.97 ×10²	93%	<0.000010
PC	70.9 ± 4.4	143 ±17	101%	0.029	23.6 ± 2.3×10²	12.8 ± 2.9×10²	46%	0.13
Angio	878 ± 77	2229 ± 17	154%	0.038	2.04 ± 0.29 ×10²	0.740 ± 0.040 ×10²	64%	0.015
Ubq	16.8 ± 1.3	66 ± 6.4	318%	0.0012	22.9 ± 4.7 ×10²	2.33 ± 0.33 ×10²	90%	0.0054
CytC	224 ± 12	449 ± 12	100%	0.000021	12.2 ± 1.3 ×10²	1.01 ± 0.15 ×10²	92%	0.000031
Myo	137 ± 14	167 ± 12	22%	0.0080	34.5 ± 1.3 ×10²	7.77 ± 0.51 ×10²	77%	<0.000010
CAII	197 ± 12	230 ± 30	17%	0.39	13.5 ± 1.1 ×10²	5.65 ± 0.64×10²	58%	0.00079

[a] p value of < 0.05 corresponds to the increase/decrease being statistically significant

Figure 6: Average N_C values for conventional nESI and pulsed nESI at 10%, 20%, 30% and 40% duty cycles for DMMP (□), PC (○), Angio (▲), Ubq (▼), CytC (◊), Myo (◄) and CAII (►). Error bars correspond to ± the standard error of the mean for three technical replicates.

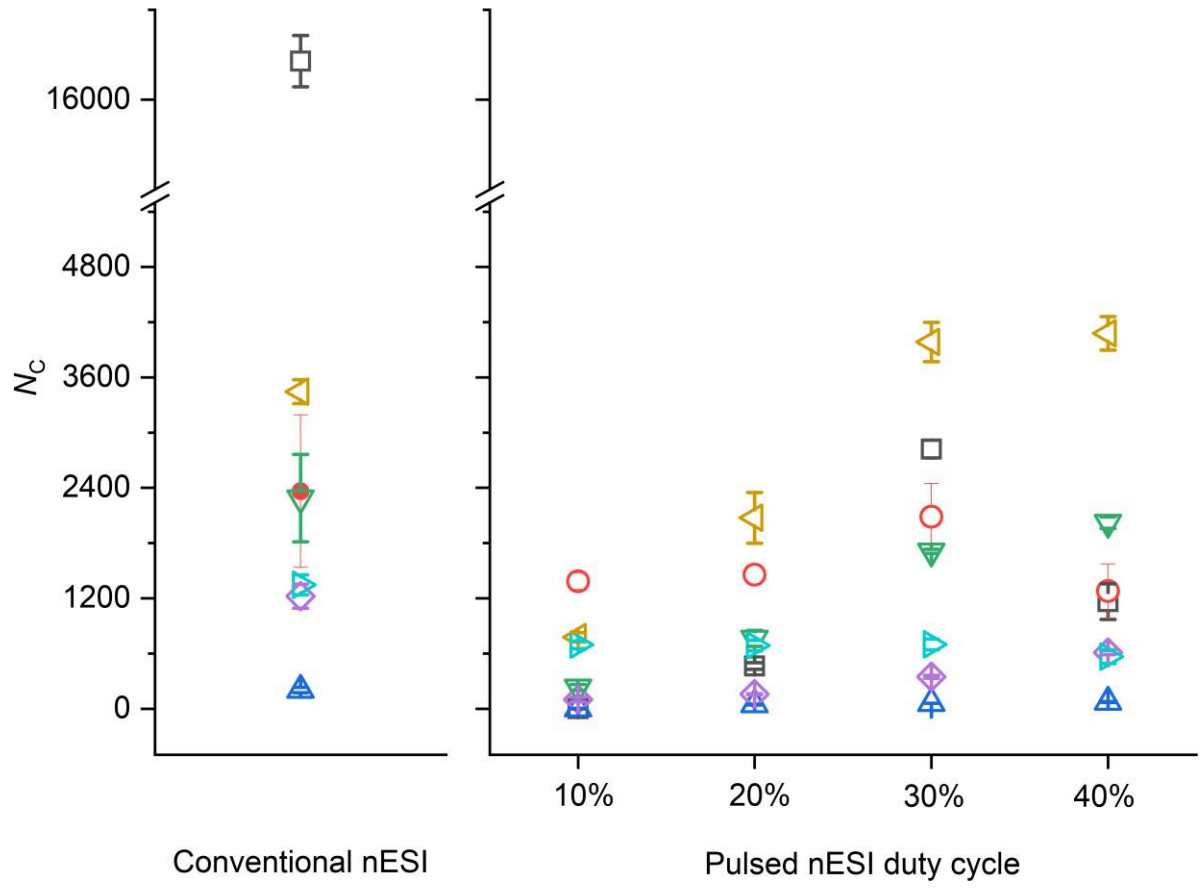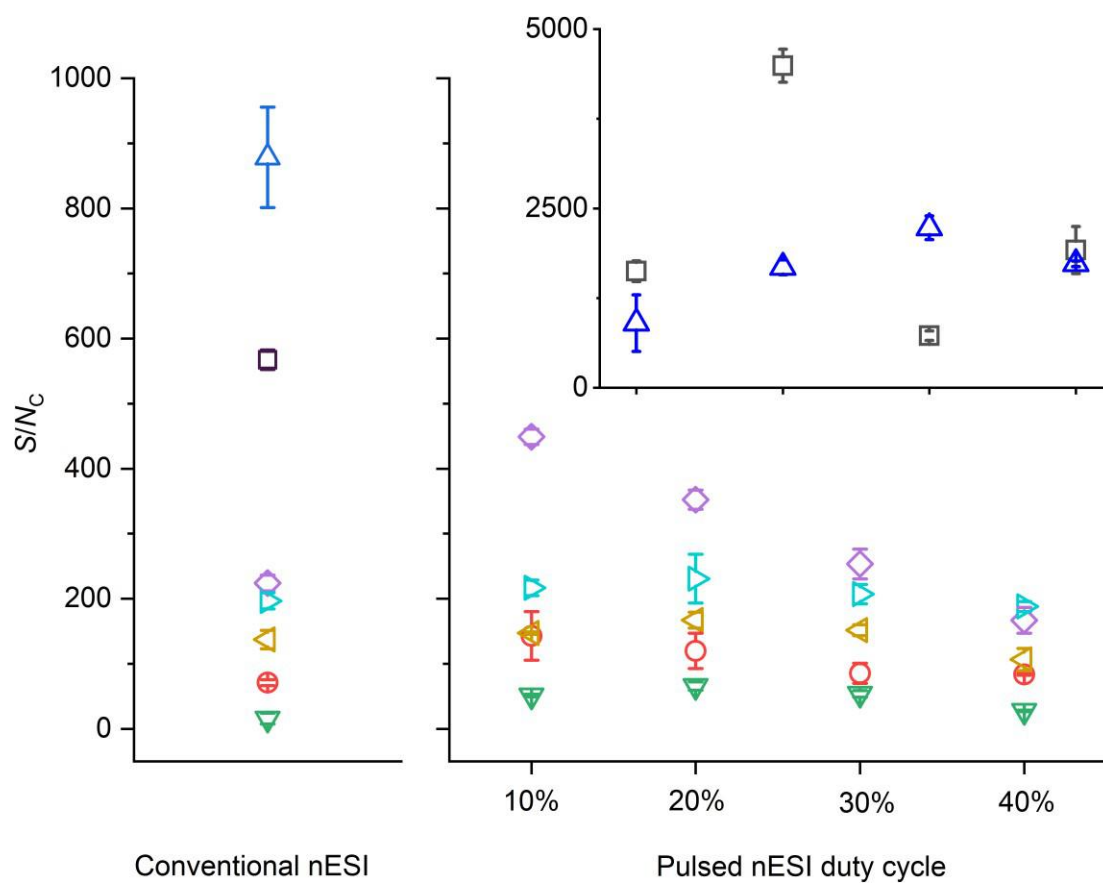Figure 7: Average S/N_C values for conventional nESI and pulsed nESI at 10%, 20%, 30% and 40% duty cycles for DMMP ($\square$), PC ($\circ$), Angio ($\blacktriangle$), Ubq ($\blacktriangledown$), CytC ($\lozenge$), Myo ($\blacktriangleleft$) and CAII ($\blacktriangleright$). Error bars correspond to ± the standard error of the mean for three technical replicates.

3.2. Supercharged protein ions with pulsed nESI

To further investigate the effects of the pulsed nESI on the charge state distributions of proteins with an emphasis on highly charged ions, a supercharging additive (1,2-butylene carbonate) was doped into the nESI solutions to form higher charge states. First to reproduce previous results and ensure we are obtaining similar spectra with supercharging as reported in the literature, the charge states obtained using conventional nESI with 1,2-butylene carbonate were obtained. Table 3 and 4 show that the charge states of proteins (Ubq, CytC, Myo and CAII) with and without supercharging additive, were 10.7, 14.3, 19.7 and 30.5, respectively at 40% duty cycle in pulsed nESI analysis, which then increased to 14.7, 18.7, 32.0 and 48.0 with the addition of supercharging additive into the protein solutions. The increase in protein ion charging has been attributed to: (i) increasing the surface tension of the ESI generated droplets compared to the same solutions without the additive (see Chapter 1); and (ii) the additive acting as a surfactant, which can hinder the ion evaporation of charge carriers from ESI generated droplets.[56]

Next, the effects of pulsing the nESI on the charge state distributions of supercharged protein ions was investigated. The charge states of proteins with a supercharging additive in pulsed nESI at 40% duty cycle is similar to the charge states of proteins analysed using conventional nESI (Table 3). However, decreasing the pulse width from 40% to 10% duty cycle results in a lowering of the charge states; i.e., the charge states of Ubq, CtyC, Myo, CAII was 14.7, 18.7, 32.0 and 47.9, respectively which decreased to 12.1, 15.9, 30.6 and 45.5 as the duty cycle was reduced from 40% to 10%. An explanation for the decreased charging at lower duty cycles is that highly charged protein ions are formed earlier in the ESI process outside the instrument at higher pressures than by used of higher duty cycles. Lowering the duty cycle should lower the ESI current, which should correspond to the formation of smaller initial droplet sizes that desolvate rapidly and at higher pressures than by forming larger droplets.

Highly charged protein ions can readily transfer protons to N_2 (g) and Ar (g) in thermal ion-neutral reactions.[67] Thus, if they are formed outside of the MS and/or at higher pressures earlier on in the transfer to under vacuum, then they can more readily be charged reduced by reactions with N_2 (g) and O_2 (g) from the atmospheric pressure ion source.

A key issue with doping in high concentrations of supercharging additives is that the background chemical noise can be quite high because of the formation of protonated and sodiated monomers, dimers and trimers of the chemical additives such as shown in the mass spectra for Myo and CAII (Figure 8c and 8d). Because the pulsed nESI can be used to reduce background chemical noise, it was hypothesized that pulsed nESI may be beneficial for protein supercharging experiments. Therefore, the use of pulsed nESI analysis result in significant reduction of background noise, where background noise decreased by 89%, 88%, 84% and 85% for Ubq, CytC, Myo and CAII, respectively (Table 5). Therefore, S/N_C values are expected to be higher in pulsed nESI compared to conventional nESI. Table 4 also show that S/N_C ratios of CytC, Myo and CAII analysis in conventional nESI are 100.2, 11.4 and 14.2 respectively, which by using pulsed nESI analysis can increase to 179.2, 64.9 and 105.0. However, the S/N_C ratio of Ubq in pulsed nESI analysis is the same or slightly lower than that in conventional nESI.

Table 3. In comparison of average charge state for protein with supercharging additive between conventional nESI and pulsed nESI at 40%, 30%, 20% and 10% duty cycle.

Analytes	nESI	Pulsed nESI				Decrease in charge states	
		40	30	20	10		
Ubq	15.26 ± 0.25	14.72 ± 0.12	14.436 ± 0.054	13.526 ± 0.051	12.067 ± 0.021	3.19	0.000020
CytC	19.38 ± 0.34	18.736 ± 0.012	17.93 ± 0.25	16.67 ± 0.24	15.91 ± 0.31	3.48	0.00022
Myo	32.044 ± 0.030	31.999 ± 0.023	31.959 ± 0.063	31.826 ± 0.055	30.61 ± 0.14	1.43	0.000026
CAII	49.27 ± 0.28	47.95 ± 0.41	47.24 ± 0.11	46.55 ± 0.54	45.50 ± 0.55	3.77	0.0027

[a] p value of < 0.05 corresponds to the increase/decrease being statistically significant

Table 4. Comparison of average charge state for denatured protein between conventional nESI and pulsed nESI at 40%, 30%, 20% and 10% duty cycle.

Analytes	nESI	Pulsed nESI				Decrease in charge states	
		40	30	20	10		
Ubq	10.84 ± 0.12	10.746 ± 0.013	10.67 ± 0.11	10.637 ± 0.037	10.401 ± 0.020	0.44	0.0048
CytC	14.4895 ± 0.0032	14.31 ± 0.013	14.355 ± 0.069	14.217 ± 0.047	14.158 ± 0.021	0.33	0.000026
Myo	20.055 ± 0.066	19.73 ± 0.25	19.970 ± 0.058	19.77 ± 0.30	19.205 ± 0.050	0.85	0.000074
CAII	30.862 ± 0.044	30.548 ± 0.063	30.354 ± 0.047	30.258 ± 0.043	30.241 ± 0.026	0.62	0.000068

[a] p value of < 0.05 corresponds to the increase/decrease being statistically significant

Table 5. Background chemical noise values (N_C) and signal-to-background chemical noise (S/N_C) for proteins with supercharging additive (1,2-butylene carbonate) measured by conventional nESI and pulsed nESI Mass Spectrometry. Refer to Figure 8 for the corresponding mass spectra.

Analytes	S/N_C				N_C			
	nESI	pulsed nESI	% decrease/ increase		nESI	Pulsed nESI	% decrease	
Ubq	545 ± 27	497 ± 14	-9%	0.0051	9.97 ± 0.51 $\times 10^3$	10.94 ± 0.48 $\times 10^2$	89%	<0.000010
CytC	100.2 ± 1.8	179.2 ± 8.9	79%	<0.000010	5.529 ± 0.089 $\times 10^3$	6.55 ± 0.18 $\times 10^2$	88%	0.044
Myo	11.44 ± 0.83	64.9 ± 6.5	467%	0.0025	101.2 ± 7.2 $\times 10^3$	$157 \pm 17 \times 10^2$	84%	0.000036
CAII	14.22 ± 0.52	105 ± 11	644%	0.00055	8.34 ± 0.53 $\times 10^3$	12.17 ± 0.27 $\times 10^2$	85%	0.0014

[a] p value of < 0.05 corresponds to the increase/decrease being statistically significant
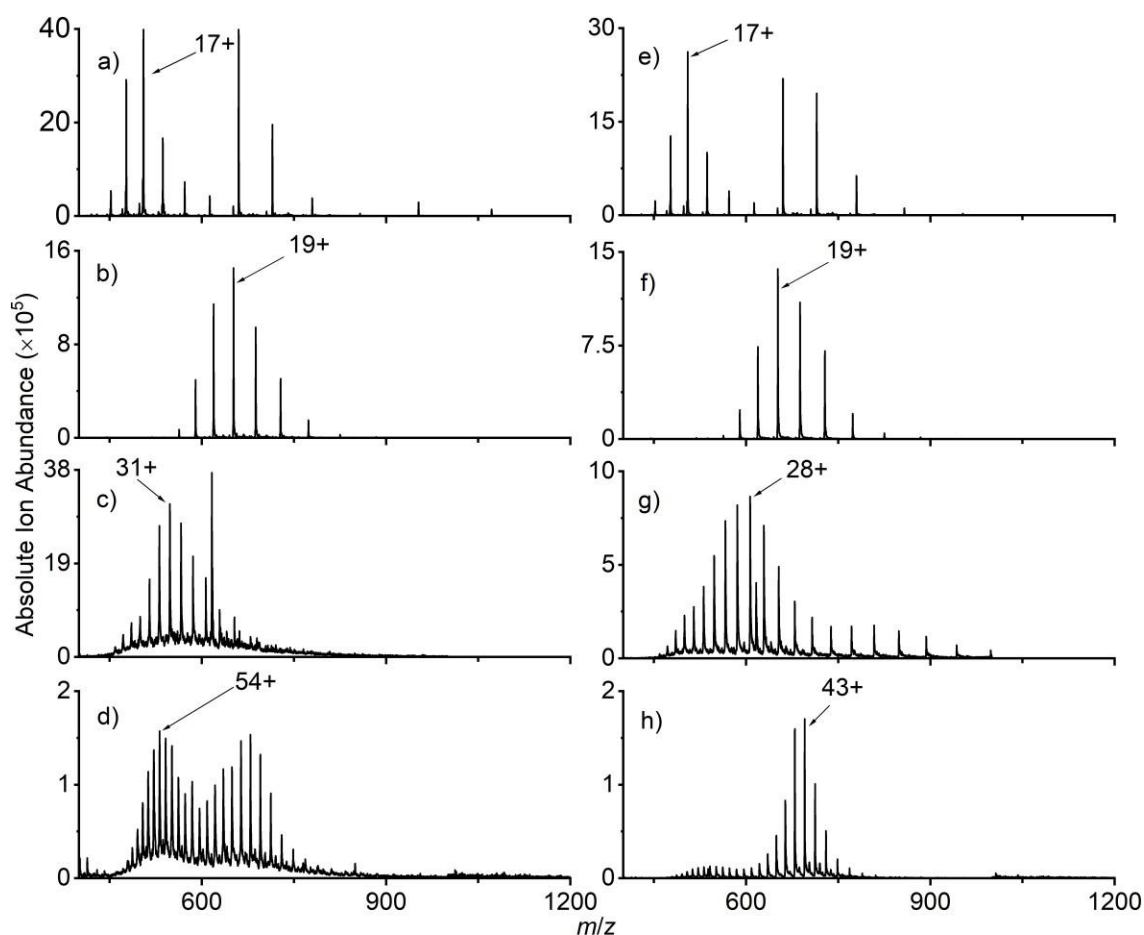
Figure 8. Conventional nESI (a, b, c, d) and pulsed nESI (e, f, g, h) mass spectra for analyte with supercharging additive (BC), 5 µM Ubq (d [Ubq, 17H]$^{17+}$, k [Ubq, 17H]$^{17+}$), 5 µM CytC (e [CytC, 19H]$^{19+}$, l [CytC, 19H]$^{19+}$), 5 µM Myo (f [Myo, 31H]$^{31+}$, m [Myo, 28H]$^{28+}$), 10 µM CAII (g [CAII, 54H]$^{54+}$, n [CAII, 43H]$^{43+}$). The 'optimal' pulsed nESI duty cycles for each analyte were 40%, 40%, 10% and 10% for Ubq, CytC, Myo and CAII respectively.

3.3. Effects of pulsed nESI in native MS

To investigate the effects of pulsed nESI in native MS, pulsed nESI MS spectra of proteins (Ubq, CytC, Myo and CAII) in buffered, near neutral aqueous solutions were obtained, and the signal intensity obtained from conventional nESI MS was compared. S/N_C values were also obtained from the pulsed nESI MS spectra and compared to those obtained from conventional nESI MS method. Ubq, CytC, Myo and CAII were individually dissolved in 50 mM aqueous ammonium acetate buffer solution to make a final individual concentration of 5 μM and sprayed into the MS by means of pulsed and conventional nESI methods. The aqueous ammonium acetate buffer solution at near neutral pH was used to maintain the proteins' native-like structures. As presented in Figure 9, such a concentration resulted in the generation of protein ions without detrimentally affecting the ion signal by using pulsed nESI compared to conventional nESI. The use of the platinum wire lowered the voltage (~0.8-1.5 kV) required to detect the native protein solutions compared to that of using metal coated tips (~1-2.5 kV), which may be beneficial to preventing 'electrothermal' activation and the formation of higher protein ion charge states that are less native-like.[104] A platinum wire was incorporated because it directly touches the solution to increase the conductivity of analytes ions, and can result in higher consistency between samples, unlike using coated tips in which the metal coating can readily flake away and potentially affect the electrical conductivity. The signal intensity obtained for all native protein solutions (5 μM) ranged from 1.8×10^3 to 1.9×10^4 by both ionisation techniques. Lower concentrations were used to ensure that the ion trap was not overly filled, which can make it challenging to determine the effects of different ionisation conditions. For each protein ion charge state distribution, the number of charge states were narrow (Figure 9) compared to the nESI MS of the denatured solutions, which is a characteristic of native MS. For example, native MS of Myo and CAII resulted in formation of only one predominant peak corresponding to charge states of 8+ and 9+, respectively under native

conditions whereas Ubq and CytC resulted in two dominant peaks corresponding to 6+ and 5+; and 7+ and 6+, respectively. Therefore, pulsed nESI resulted in similar ion abundances as that of conventional nESI for all proteins (Ubq, CytC, Myo and CAII).

To further investigate the performance of the pulsed nESI method, we calculated signal-to-background chemical noise ratio of native protein charge states and compared to those obtained from conventional nESI technique. All individual protein solutions were analysed by pulsed nESI at four different duty cycles (i.e., 10%, 20%, 30% and 40%). In Figure 10, the S/N_C of tested proteins comparison of conventional nESI and pulsed nESI are given. Pulsed nESI with a 40% duty cycle can result in similar spectra as conventional nESI. However, the use of lower duty cycles, particularly 10%, results in lower S/N_C. In contrast to denatured protein analysis, S/N_C values of native proteins at higher duty cycle (40%) are larger than at lower duty cycle (10%) (Figures 5 and 10). The results indicate that the S/N_C of native proteins with longer pulse widths can be increased significantly compared to DC nESI, whereas lowering the duty cycle decreases the S/N_C ratio. This can be attributed to the generation of a higher electric field for a longer duration ($\geq$30%) which can efficiently ionize the analytes. In contrast, the electric field generated at the lower duty cycle ($\leq$20%) is not sufficient to efficiently ionize the proteins. Presumably there is a competition between the positive effects of forming smaller droplets with lower duty cycles (which should enhance sensitivity and decrease ion suppression) and forming ions for less of the total time as duty cycle decreases. Under native like conditions, the concentration of protons are also far lower, which could result in the requirement of higher duty cycles to obtain higher S/N_C values. In addition, ions with different electrophoretic mobilities appear to have ideal nESI pulse widths at which they ionise most efficiently.
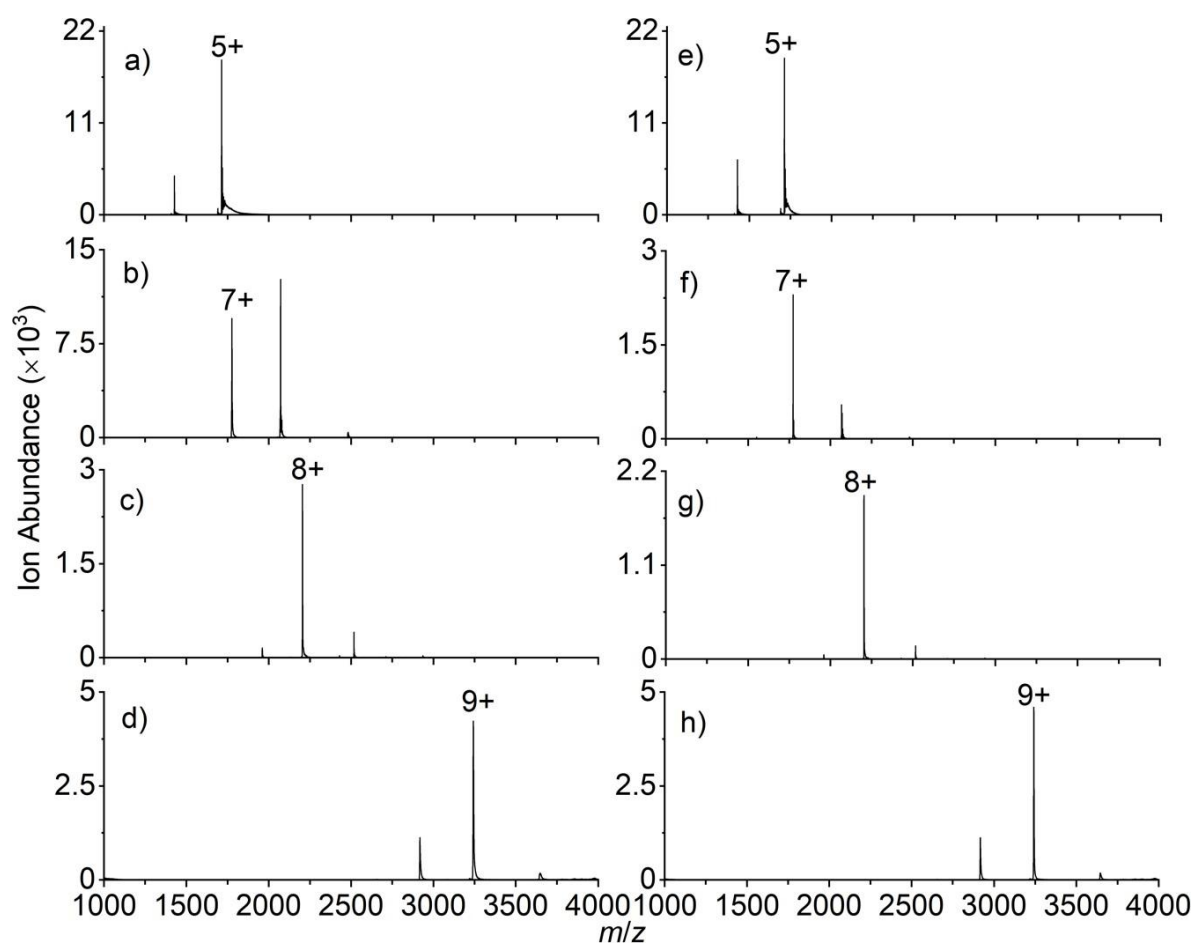
Figure 9. Conventional nESI (a, b, c, d) and pulsed nESI (e, f, g, h) mass spectra for analyte dissolve in 50 mM ammonium acetate, 5 μM Ubq (a,e), 5 μM cytC (b, f), 5 μM Myo (c, g), 5 μM CAII (d, h). The 'optimal' pulsed nESI duty cycles for each analyte were 40%, 40%, 40% and 40% for Ubq, CytC, Myo and CAII respectively.
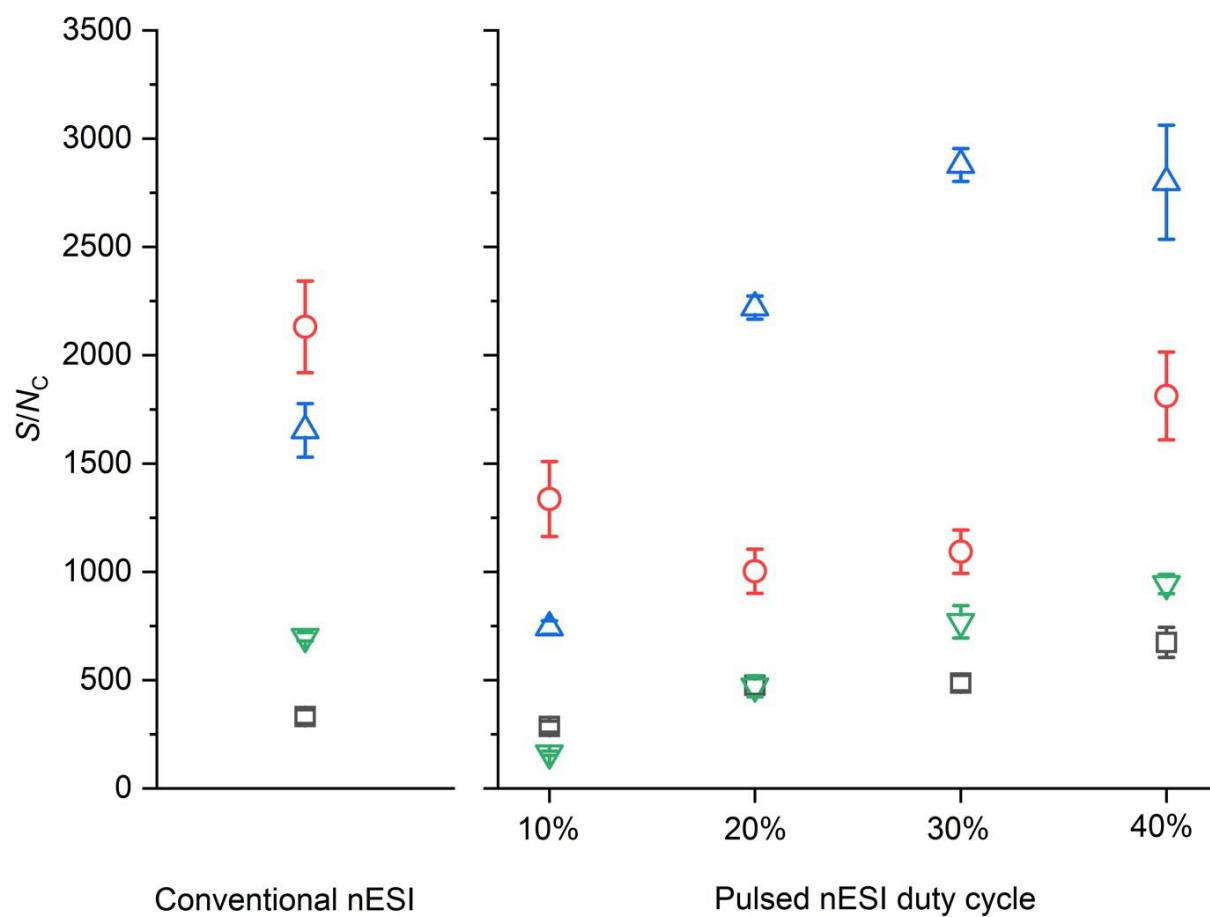
Figure 10. Average S/N_C values for comparison of conventional nESI (left) and pulsed nESI (right) at duty cycle 10%, 20%, 30% and 40% of 5 µM Ubq (□), 5 µM CytC (○), 5 µM Myo (▲) and 5 µM CAII (▼). Error bars correspond to ± the standard error of the mean for three technical replicates.

3.4. Effect of pulsed nESI on salt adduction in native protein MS

To determine the effect of pulsed nESI on the extent of salt-adduction in native MS, the S/N_C for native proteins (Ubq, CytC, Myo and CAII) using a pulsed potential was compared with that obtained from conventional nESI (Figure 11). Proteins (Ubq, CytC and Myo) were added into native-like aqueous solutions that contained NaCl (25 mM or 50 mM) and ammonium acetate (50 mM). Lower salt concentrations were used for the larger protein (CAII) (0.5 and 1 mM) because it has a higher solvent accessible surface area that favours salt adduction in ESI. Figure 11 indicates that native MS analysis of this protein sample resulted in mass spectra with reduced S/N_C values in pulsed nESI analysis compared to conventional nESI. Compared to conventional nESI, the S/N_C of proteins in pulsed nESI analysis decreased up to 77% at 10% duty cycle. However, at 40% duty cycle, pulsed nESI detection performs similar S/N_C values to conventional nESI. The S/N_C values for protein ions formed from solutions with high concentration of NaCl (50 mM for Ubq, CytC and Myo, 1 mM for CAII) were significantly lower than that obtained from samples with lower concentrations of NaCl (25 mM for Ubq, CytC and Myo, 0.5 mM for CAII). The addition of high salt generated more noise and adversely affected the detection of native proteins since low or no ion signal was observed by the use of pulsed nESI. For example, Myo was not detected at 50 mM NaCl when its duty cycle below 30% in pulsed nESI. Therefore, calculation of S/N_C of Myo was beyond the capability for the pulsed nESI at 20% and 10% duty cycle. In this work, by using pulsed nESI to analyse native protein can generate negative results, where S/N_C of protein samples decreases compared to the conventional nESI method. The origin of this effect remains unclear.

We also noted that the pulsed nESI method has low or no effect on desalting in native protein analysis. With the salt solution added in the native solution, the predominant protein ions shifted from predominantly protonated to heavily salt adducted (Figure 12). For example, the Ubq generated a charge state of 5+ corresponding to m/z of 1713.8 when no salt was added,

whereas 25 mM salt added into the solvent of Ubq resulted a primary 5+ peak with an m/z of 1737 corresponding to five sodium ions adducted (i.e., $[M+5Na]^{5+}$). The same Ubq sample with 25 mM of NaCl were tested in pulsed nESI, generating the ions $(M+5Na)^{5+}$; i.e., the salt adduction was not reduced compared to that of conventional nESI (Figure 12b and 12c). Reasons for these negative results for pulsed nESI MS are unclear. It is possible that the sodium ions in solution have mobilities more comparable to the proteins than the protons and can be entrained in the Taylor cone. More research is needed to elucidate this phenomenon, such as the use of different cations including the use of larger monovalent metal ions and multivalent metal ions.
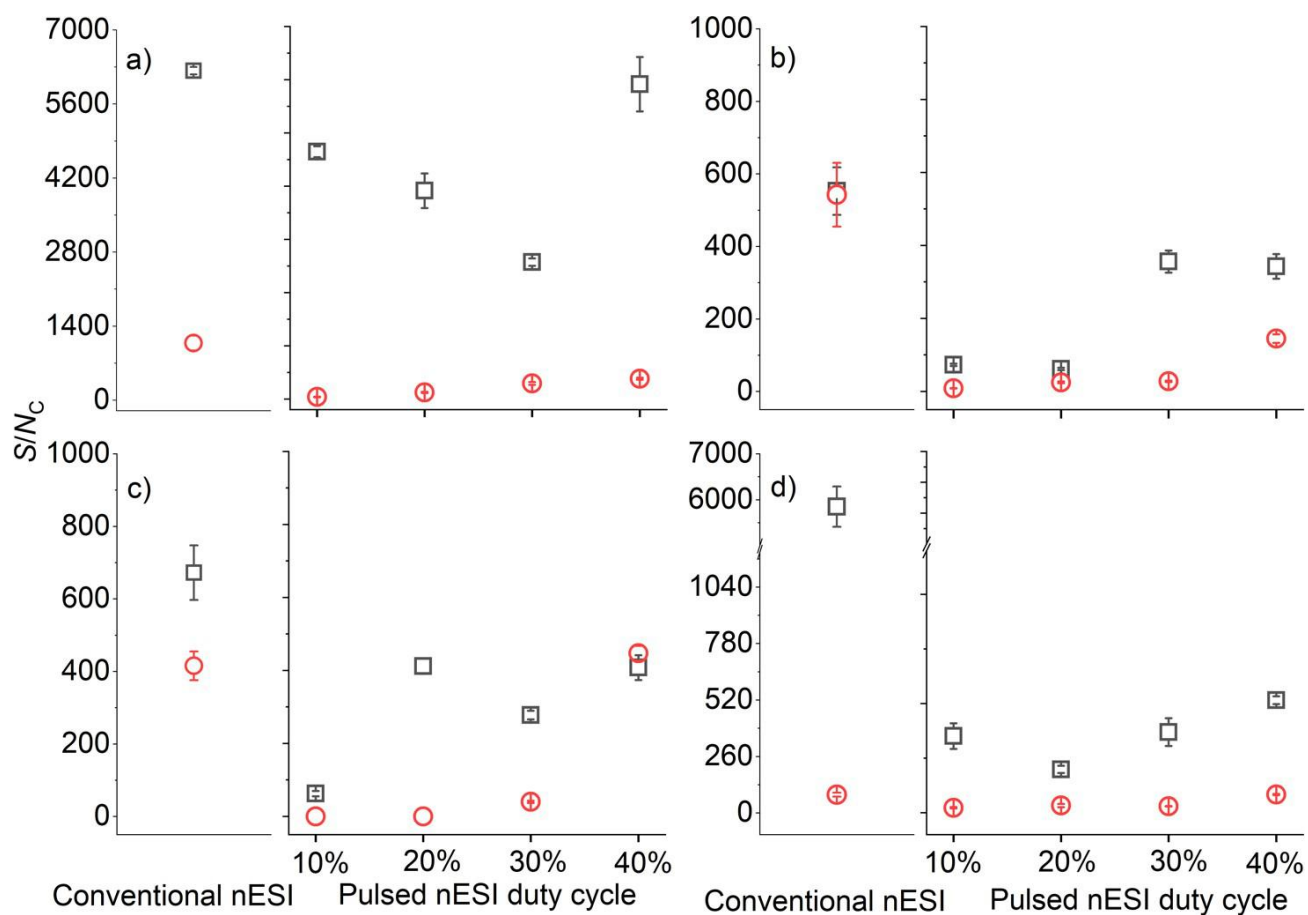
Figure 11. Average S/N_C values for comparison of conventional nESI (left) and pulsed nESI (right) at 10%, 20%, 30% and 40% of 5 µM Ubq (a), CytC (b) and Myo (c) with 25 mM (□) and 50 mM (○) NaCl, 5 µM CAII (d) with 0.5 mM (□) and 1 mM (○) NaCl. Error bars correspond to ± the standard error of the mean for three technical replicates.
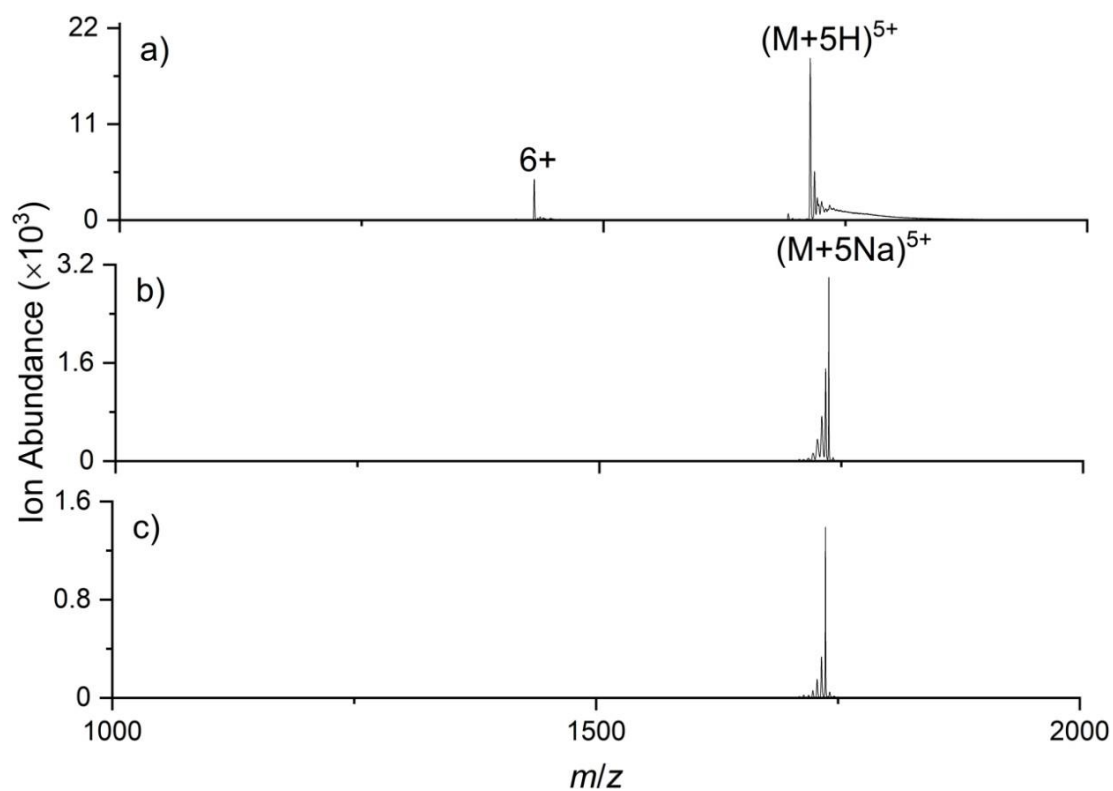
Figure 12. the mass spectra of 5 μM Ubq in 50 mM ammonium acetate a) no salt; b) 25 mM NaCl in conventional nESI; c) 25 mM NaCl in pulsed nESI at 40% duty cycle.

Conclusion

In this project, a customised ionisation source was developed for improving the performance of biomolecule analysis by MS, which resulted in a significant decrease in background noise and increased S/N_C of up to 93% and 691% in pulsed nESI analysis compared to conventional nESI. By applying short high voltage pulses with widths in the low microsecond range (360, 270, 180, and 90 μs) to reduce the time of ionisation, potentially leading to the formation of smaller initial droplets and more efficient desolvation processes to enhance signal. Moreover, such an approach can lower background noise. Pulsed nESI also can be used to address some limitations in the ESI MS of whole proteins. Specifically, adding a supercharging additive into an ESI solution can increase the protein ion charge states, although with a large chemical background noise level from the additive. Application of pulsed nESI led to slightly lower protein ion charge states formed using supercharged denaturing solutions, possibly owing to the formation of highly charged protein ions at higher pressures (due to more efficient ion desolvation) which then transferred charge to atmospheric gases.[67] However, by using pulsed nESI, very high S/N_C ratios were obtained compared conventional nESI in supercharging experiments.

The performance of pulsed nESI MS was also evaluated for native MS, with an emphasis on solutions containing high concentrations of non-volatile salts. Although pulsed nESI does not reduce the extent of salt adduction under these conditions, it can increase the sensitivity by reducing background chemical noise and improving the S/N_C ratio of native proteins with no NaCl added. In conclusion, these experiments demonstrate that pulsed nESI can be used to reduce some of the detrimental effects of ion suppression frequently observed in conventional nESI. In future work, pulsed nESI should be beneficial in many other applications of MS for biomolecule analysis.

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