Development of an Ion Mobility Mass Spectrometer to Study Gas Phase Conformations of Biomolecules

Submitted in partial fulfilment for the degree of
Doctor of Philosophy

Bryan John McCullough
s9810125

PhD by Research
The University of Edinburgh
2007
Hamlet: Do you see yonder cloud that's almost in shape of a camel?

Polonius: By the mass, and 'tis like a camel, indeed.

Hamlet: Methinks it is like a weasel.

Polonius: It is backed like a weasel.

Hamlet: Or like a whale?

Polonius: Very like a whale.

William Shakespeare, *Hamlet*, Act 3 scene II
Declaration

This thesis is submitted as part requirement for PhD by research at the University of Edinburgh. The work is my own apart from where otherwise stated. This work has not been submitted for any other degree or professional qualification at this or any other university.

Bryan John McCullough
07/05/07
Acknowledgements:

Many people deserve thanks for their input (both direct and indirect) to this project. Firstly - and most importantly – thanks to Dr. Perdita Barran whose determination, inspiration and harassment drove this project forward from the beginning.

Thanks to our collaborators at UCSB (especially Paul Kemper who came over to help build the cell) and Waters (Bob, Steve, Jason, Kevin, Tony, Ian et al).

The help of the technical staff at Edinburgh both in Physics (Douglas and Jim) and Chemistry (Stuart, Davie and Donald) was invaluable. Thanks also to the other staff in Chemistry: Stores, cleaners, admin. etc... whose ‘behind the scenes’ work helps keep things ticking along.

A big thanks to PBRG colleagues and affiliates past and present for their input and help throughout the three years. Thanks must also go to the various parts of Team Defensin for their invaluable input into this work.

Thanks to my family and friends for their support throughout this work. Special thanks to the lovely Miss Laura Tracy for putting up with me.

Lastly, thanks to the mighty St. Johnstone F.C. who provide more ups and downs than a PhD and much like a PhD kept the best bit for the end of the three years (2-0, GIRUY TFOD!).
Abstract

Design, development and implementation of a new Ion Mobility capable Mass Spectrometer – the MoQToF – is presented. The instrument is a Micromass Q-ToF modified to include a temperature regulated drift cell. Initial testing of the instrument to measure cross sections of well characterised proteins (cytochrome C, ubiquitin and lysozyme) in a range of charge states is described, showing the apparatus to perform well in comparison with values reported by others on analogous instruments.

Ion mobility data are presented on a number of novel systems from small peptides to large proteins. The largest volume of work focuses on the study of β-defensins and related peptides. β-defensins are small anti-microbial peptides that form a vital part of the innate immune system of all mammals, they are characterised by the presence of six conserved cysteine residues connected via disulphide bonds. Characterisation of these bonds (number and topology) using mass spectrometry based techniques is presented. The ion mobility data presented here probes the influence of these disulphide bonds on conformational flexibility.

The mode of action of β-defensins is not known, two techniques designed to further understanding of this are described here. Firstly a mass spectrometry based technique in which the interaction between a defensin, DEFB107, and an artificial membrane is studied using hydrogen deuterium exchange revealing the N-terminal section of the peptide to interact favourably with the lipid bilayer. Secondly, a heparin binding assay is described revealing a relationship between heparin binding strength and anti-microbial activity. This interaction is further studied using the MoQToF and molecular modelling.
List of Abbreviations Used

AmAc - ammonium acetate
AMP - anti-microbial peptide
APCI - atmospheric pressure chemical ionisation
ATD - arrival time distribution
BIRD - black body infra red dissociation
BPTI - bovine pancreatic trypsin inhibitor
CaM - calmodulin
CD - circular dichroism
CE - capillary electrophoresis
CI - chemical ionisation
CID - collision induced dissociation
Cyt C - cytochrome C
DC - direct current
DIP - defensin inspired peptide
ECD - electron capture dissociation
EHSS - exact hard spheres scattering
EI - electron ionisation
Ek - kinetic energy
EM - electrophoretic mobility
ESI - electrospray ionisation
ETD - electron transfer dissociation
eV - electron volts
FAIMS - high field assymetric waveform ion mobility spectrometry
FTICR - Fourier transform ion cyclotron resonance (mass spectrometry)
FTMS - Fourier transform mass spectrometry
FWHH - full width half height
GAG - glycosaminoglycan
Glt - glutathione
GnRH - gonadotropin releasing hormone
List of Abbreviations Used

HDB - human b-defensin
HDX - hydrogen deuterium exchange
HPLC - high pressure liquid chromatography
IM - ion mobility
IMMS - ion mobility mass spectrometry
IMS - ion mobility spectrometry
IR - infra red
IRMPD - infra red multi-photon dissociation
LC - liquid chromatography
LD - laser desorption
LHRH - leutenising hormone replacing hormone
LPS - lipopolysaccharide
LU VET - large unilamellar vesicle
m/z - mass to charge ratio
MALDI - matrix assisted laser desorption/ionisation
MCP - microchannel plates
MDB - mouse b-defensin
MeOH - methanol
MoQToF - mobility quadrupole time of flight
MS - mass spectrometry
nano-ESI - nano electrospray ionisation/nanospray
nano-LC - nanoflow liquid chromatography
NMR - nuclear magnetic resonance (spectroscopy)
PA - projection approximation
PC - personal computer
PCB - printed circuit board
PLIMSTEX - protein ligand interactions by mass spectrometry titration and hydrogen deuterium exchange
Q - quadrupole
Q-ToF - quadrupole time of flight
RF - radio frequency
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>SID</td>
<td>surface induced dissociation</td>
</tr>
<tr>
<td>SLD</td>
<td>soft laser desorption</td>
</tr>
<tr>
<td>SRIG</td>
<td>stacked ring ion guide</td>
</tr>
<tr>
<td>TIC</td>
<td>total ion count</td>
</tr>
<tr>
<td>TM</td>
<td>trajectory method</td>
</tr>
<tr>
<td>ToF</td>
<td>time of flight</td>
</tr>
<tr>
<td>T-wave</td>
<td>travelling wave</td>
</tr>
<tr>
<td>TWIG</td>
<td>travelling wave ion guide</td>
</tr>
<tr>
<td>Ubi</td>
<td>ubiquitin</td>
</tr>
<tr>
<td>UCL</td>
<td>University College London</td>
</tr>
<tr>
<td>UCSB</td>
<td>The University of California Santa Barbara</td>
</tr>
<tr>
<td>UV</td>
<td>ultra violet</td>
</tr>
<tr>
<td>XRC</td>
<td>X-ray crystallography</td>
</tr>
</tbody>
</table>
# Table of Contents

Declaration.......................................................................................................................... i

Acknowledgements: .......................................................................................................... ii

Abstract............................................................................................................................. iii

List of Abbreviations Used.............................................................................................. iv

Table of Contents............................................................................................................ vii

1. Introduction................................................................................................................. 1

1.1 Mass Spectrometry.................................................................................................... 4
  1.1.1 Ionisation Sources.............................................................................................. 4
    1.1.1.1 MALDI ....................................................................................................... 5
    1.1.1.2 ESI............................................................................................................... 7
  1.1.2 Mass Analysers................................................................................................... 11
    1.1.2.1 Quadrupoles ............................................................................................. 12
    1.1.2.2 Time of Flight ........................................................................................... 13
    1.1.2.3 Fourier Transform Ion Cyclotron Resonance ........................................... 16
    1.1.2.4 Tandem Mass Spectrometry ..................................................................... 19
  1.1.3 Detectors............................................................................................................ 20

1.2 Ion Mobility Spectrometry...................................................................................... 23
  1.2.1 Theories and Concepts..................................................................................... 23
  1.2.2 Experimental Set-up......................................................................................... 27
    1.2.2.1 Linear Ion Mobility ................................................................................... 27
    1.2.2.2 High Resolution Linear Ion Mobility ....................................................... 35
    1.2.2.3 Travelling Wave Ion Mobility.................................................................. 36
    1.2.2.4 FAIMS ...................................................................................................... 39
  1.2.3 Mobility Calculations....................................................................................... 41

1.3 Studying Biomolecules by Mass Spectrometry & Ion Mobility............................. 46
  1.3.1 Mass Spectrometry........................................................................................... 48
    1.3.1.1 Primary Structure Determination.............................................................. 48
    1.3.1.2 Higher Order Structure Determination ..................................................... 50
  1.3.2 Ion Mobility Mass Spectrometry ..................................................................... 56
    1.3.2.1 Cytochrome C ........................................................................................... 57
    1.3.2.2 Bradykinin Dimer ..................................................................................... 62
    1.3.2.3 GnRH ........................................................................................................ 63
    1.3.2.4 Amyloid β-Protein .................................................................................... 64
    1.3.2.5 Complexes by IMMS................................................................................ 66
    1.3.2.6 Nucleotides ............................................................................................... 68
# Table of Contents

1.3.2.7 Analysis of Complex Mixtures ................................................................. 69  
1.3.2.8 Ligand Binding Studies ............................................................................. 71  

References: ................................................................................................................ 72

2. The MoQTof – a new ion mobility mass spectrometer ........................................ 76

2.1 Design and Construction ......................................................................................... 77
2.1.1 Micromass QToF I ........................................................................................... 77
2.1.2 Instrument Layout ............................................................................................ 81
2.1.3 The Cell ............................................................................................................ 83
2.1.4 Einzel Lens Design and SIMION Modelling ................................................... 92
2.1.5 Power Supply Design ....................................................................................... 99
2.1.5.1 Lens 2 - X-Y Steering ............................................................................. 100
2.1.6 Pulser Design .................................................................................................. 101
2.1.7 Pressure Measurement ................................................................................... 101
2.1.8 Temperature Measurement ............................................................................ 101
2.1.9 Gas Inlet ......................................................................................................... 102
2.1.10 Cell Hexapole ............................................................................................... 102
2.1.11 Quadrupole Upgrade .................................................................................... 103

2.2 Experiment Set-up ................................................................................................ 104
2.2.1 MS Mode ....................................................................................................... 104
2.2.2 IMS Mode ...................................................................................................... 105

2.3 From ATDs to Drift Times to Mobilities .............................................................. 108
2.3.1 Obtaining Good ATDs ................................................................................... 109

2.4 Initial Testing ........................................................................................................ 110
2.4.1 No Orifices ..................................................................................................... 110
2.4.2 Front Orifice 2 mm Aperture ......................................................................... 111
2.4.3 Front Orifice 2 mm Aperture, Rear Orifice 1.67 mm Aperture..................... 112
2.4.4 Front and Rear Orifices 1mm Apertures........................................................ 113

2.5 Mobility Experiments on Standard Compounds ................................................ 115
2.5.1 Cytochrome C ............................................................................................... 115
2.5.2 Ubiquitin ........................................................................................................ 120
2.5.3 Lysozyme ....................................................................................................... 121

2.6 Issues Affecting Operation of MoQTof ............................................................... 124
2.6.1 Source Issues ................................................................................................ 124
2.6.2 Software Issues .............................................................................................. 126
2.6.2.1 Data Resolution ....................................................................................... 126
2.6.2.2 Pulse Synchronisation ............................................................................. 129
2.6.2.3 Data Acquisition ..................................................................................... 129
2.6.2.4 Data Processing ....................................................................................... 130
# Table of Contents

2.7 Conclusions .......................................................................................................................... 132

References: .................................................................................................................................. 134

3. β-Defensins: Insight from Mass Spectrometry ........................................................................ 135

3.1 Accurate Mass of β-defensins ................................................................................................. 141
  3.1.1 Experimental ..................................................................................................................... 141
  3.1.2 Defr1 ................................................................................................................................ 141
    3.1.2.1 Results ......................................................................................................................... 142
  3.1.2 DEFBI07 .......................................................................................................................... 144
    3.1.2.1 Results ......................................................................................................................... 144
  3.1.3 Other Defensins studied ................................................................................................. 144

3.2 Mapping Disulphide Connectivity of β-Defensins .............................................................. 146
  3.2.1 Experimental ..................................................................................................................... 146
  3.2.2 Results: DEFB107 ......................................................................................................... 147
  3.2.3 Results: Defb14 .............................................................................................................. 148

3.3 Defensin Membrane Interactions ......................................................................................... 151
  3.3.1 Experimental ..................................................................................................................... 154
  3.3.2 Results ............................................................................................................................. 156

3.4 Defensin – Glycosaminoglycan Interactions ...................................................................... 160
  3.4.1 Materials and Methods .................................................................................................... 161
  3.4.2 Results ............................................................................................................................. 161

3.5 Conclusions .......................................................................................................................... 169

References: .................................................................................................................................. 170

4. Ion Mobility Studies of β-Defensins and Defensin-Related Peptides .................................... 172

4.1 DEFB107 .............................................................................................................................. 174
  4.1.1 Experimental: Large Apertures ......................................................................................... 174
  4.1.2 Experimental: Small Apertures ......................................................................................... 176
  4.1.3 Results ............................................................................................................................. 177
  4.1.4 Molecular Modelling ....................................................................................................... 178
  4.1.5 Discussion ......................................................................................................................... 181

4.2 Defr1 .................................................................................................................................... 182
  4.2.1 Experimental ..................................................................................................................... 182
  4.2.2 Results ............................................................................................................................. 183
  4.2.3 Discussion: Defr1 Dimers ............................................................................................... 183
  4.2.4 Discussion: Defr1 Monomers ......................................................................................... 186
Table of Contents

4.3 Defb14
4.3.1 Experimental ................................................................. 193
4.3.2 Results ............................................................................... 194
4.3.3 Discussion: Full Length Peptides ..................................... 194
4.3.4 Discussion: Defb14 DIPs .................................................. 197

4.4 Conclusions ........................................................................ 202

References ............................................................................... 203

5. Defensin – Glycosaminoglycan Interactions by IMMS .............. 205

5.1 Experimental ....................................................................... 205
5.2 Results .................................................................................. 206
5.3 Discussion ............................................................................ 206
5.4 Conclusions ......................................................................... 216

References ............................................................................... 216

6. Ion Mobility of Other Systems .............................................. 217

6.1 Trp-Cage
6.1.1 Experimental ................................................................. 218
6.1.2 Results ............................................................................. 220
6.1.3 Discussion ........................................................................ 222

6.2 Melittin
6.2.1 Experimental ................................................................. 225
6.2.2 Results ............................................................................. 227
6.2.3 Discussion ........................................................................ 229

6.3 Calmodulin
6.3.1 Experimental ................................................................. 234
6.3.2 Results ............................................................................. 235
6.3.3 Discussion ........................................................................ 236

6.4 Conclusions ......................................................................... 242

References ............................................................................... 243

7. Conclusions ......................................................................... 244

References ............................................................................... 245
Table of Contents

Appendix 1: MoQToF Drawings ................................................................. 247
Appendix 2: Amino Acid Structures and Masses ........................................ 261
Appendix 3: β-Defensin Sequences and Activities ...................................... 263
1. Introduction

Genome mapping projects, such as the human genome project, have provided an enormous amount of genetic information for proteome (PROTEin complement to a genOME) characterisation – known as proteomics. While genetic information gives a wealth of primary structure information, it can tell us nothing about the higher order protein structures which give rise to their function. The characterisation of these structures has been traditionally carried out in the condensed phase using x-ray crystallography\(^1\) and multi-dimensional NMR\(^2\).

The reasons for using these experiments are two-fold: firstly the condensed phase has greater molecular density and hence larger signals are obtained; secondly the condensed phase is thought to more closely resemble biologically relevant conditions\(^3\). While the first reason is undoubtedly valid, the second is perhaps less so. For example, it is not clear that the crystalline environment required for x-ray diffraction experiments can be directly related to the in vivo environment; proteins are often extremely flexible molecules and their conformations can be extremely sensitive to their environment (solvent, T, pH, salt conc. etc.) which can change dramatically throughout the body\(^4\). Similarly, it is often extremely difficult to perform NMR experiments in biologically relevant conditions.

Researchers have also been studying biological molecule conformations in solvent free environments – ‘the gas phase’\(^5,6,7\). Again, it is not clear how comparable this environment is to the natural environment of a protein but these studies can often provide complementary information to condensed phase studies, especially with regard to conformational flexibility. Optical spectroscopy techniques are commonly used to determine molecular conformations\(^8,9\), however, the techniques are currently limited to comparatively small molecules (a nucleotide base pair, an amino acid...) leaving them lagging behind condensed phase techniques.
Until the mid 1980s, mass spectrometry (MS) methods struggled to analyse large molecules. This changed, however, with the advent of new, more advanced MS instruments and more importantly through the development of two new ionisation techniques: Matrix Assisted Laser Desorption Ionisation (MALDI) and Electrospray Ionisation (ESI). These soft ionisation techniques allow the transfer of extremely large, intact biomolecules into the gas phase with relative ease. ESI is particularly useful as it generates highly charged ions which appear in a mass spectrum at low m/z and can be analysed on an MS with a relatively small mass range. The application of ESI to biomolecules was first shown by Fenn who received the 2002 Nobel Prize in chemistry along with Tanaka - who pioneered soft desorption ionisation a precursor of MALDI – and Wuthrich, an NMR spectroscopist.

While it is clear that MS can be used to study the mass of a protein, it is not necessarily clear how MS can be used to study protein conformation. One, quite indirect method is to use charge state analysis whereby the degree of ‘foldedness’ of a protein can be estimated by studying the distributions of charge states; more highly charged protein ions tend to be more unfolded than lower charged protein ions. By analysing the abundances of higher and lower charged ions it is often possible to characterise gas phase unfolding transitions. Another, more direct method of analysing conformation by MS is hydrogen/deuterium exchange mass spectrometry, a technique which probes the solvent accessibility and hence structure of proteins in both the solution phase and the gas phase. These measurements, however, require some prior knowledge of the structure if they are to be useful. Therefore, they are mostly used to confirm the retention/loss of higher order structure in the gas phase.

A more direct method to study gas phase conformation is ion mobility spectrometry (IMS). The technique was developed by Cohen and Karasek in 1970 as a sensor. It has since been used to detect illegal drugs, chemical warfare agents, explosives and environmental pollutants. Ion mobility is a measure of how quickly a gas phase ion moves through a buffer gas under the influence of an electric field, this depends on two things: the rotationally averaged collision cross section of the ion and the charge present.
on it. By measuring the drift time of an ion through a known distance it is possible to determine its collision cross section which can then be used, in conjunction with computational studies, to obtain conformational information.

The use of IMS to elucidate structural information was pioneered by the groups of Bowers\textsuperscript{20} and Jarrold\textsuperscript{21} who used it to study conformations of gas phase atomic clusters. These methods have since been developed to provide structural information on biomolecules. In this work a new ion mobility mass spectrometer (IMMS) is developed in order to analyse gas phase conformation of biomolecules.
1. Introduction

1.1 Mass Spectrometry

A mass spectrometer is a device that analyses ions by mass-to-charge (m/z) ratio. The technique was first developed by J.J. Thomson in 1912 to determine elemental masses. Over a short period of time mass spectrometry progressed greatly, allowing larger molecules to be analysed more easily with each innovation. However, much MS has changed since the days of Thomson and his fellow pioneers, the basic elements of an MS experiment remain unchanged, namely: a source in which gas phase ions are produced; a mass analyser where ions are separated by m/z and a detector with which to record spectra. One useful addition to most spectrometers since the early days is a computer with which the data can be more easily recorded and analysed. The major innovations associated with each of the three main elements will be discussed in the next three sections.

1.1.1 Ionisation Sources

The first mass spectrometers used electron ionisation (EI) sources. EI requires volatile samples which can be transferred to the gas phase by heating. Once the sample is volatilised it is subjected to bombardment by a beam of electrons; these electrons ionise the analyte molecules by electronic interaction. The electrons used in EI are typically around 70 eV. Of this energy, 10-20 eV is transferred to the molecule during the ionisation process, however, 10 eV is enough energy to ionise most molecules and there is therefore up to 10 eV excess energy per molecule. This excess energy leads to the formation of fragment ions (useful for structure determination of small molecules) and is therefore termed a ‘hard’ ionisation technique.

Chemical ionisation (CI), or more commonly atmospheric pressure chemical ionisation (APCI), is a related technique in which firstly a carrier gas – usually methane – is ionised by EI, the resultant ions then interact with the analyte molecules causing them to ionise via a proton transfer reaction. CI is a much softer ionisation technique than EI and
therefore causes less fragmentation; it does, however, still require a volatile sample and is therefore only useful for small molecules.

For larger and less volatile samples, it was necessary to develop techniques in which less volatile samples could be transferred to the gas phase easily and ionised with the minimum amount of fragmentation. The resulting techniques fall loosely into two categories: desorption techniques in which a sample is desorbed from a surface by a laser or molecular beam; and techniques where samples are ionised directly from solution. Of these categories by far the most important techniques are matrix assisted laser desorption/ionisation (MALDI)\textsuperscript{11} and electrospray ionisation (ESI)\textsuperscript{12} respectively. MALDI will be briefly described in the next section, while ESI – the ionisation method used in most of this work – will be discussed in depth in the subsequent section.

1.1.1.1 MALDI

In the 1960s an ionisation process known as laser desorption (LD) was first described\textsuperscript{27}. This technique involved the ablation of a sample (usually solid) from a surface with a pulsed laser to create gaseous ions. This technique was extremely efficient but due to the energies required for the process, extensive fragmentation was seen for larger molecules (>500 Da). This problem was first overcome by Hillenkamp and Karas\textsuperscript{11} when they successfully demonstrated that the addition to the experiment of a small organic molecule - known as a matrix – which absorbs strongly in the wavelength of the laser used, allows much larger molecules to be ionised intact. This work, along with work on an analogous technique soft laser desorption (SLD) by Tanaka\textsuperscript{10} (for which he won the 2002 Nobel Prize for Chemistry), led to the development of MALDI.

A basic MALDI experiment is performed as follows: a solution of analyte molecule(s) is mixed with a saturated solution of the matrix molecule. The resulting mixture is spotted onto a target plate and allowed to dry – the resulting spot is said to be a ‘solid solution’ i.e. the matrix is in such excess that the analyte molecules are isolated from each other as if they are solvated by the matrix. The target plate is then placed in the source region of
I. Introduction

the mass spectrometer – this can be at atmospheric pressure or more commonly at relatively low vacuum – where it is irradiated by a pulsed laser to produce ions.

The matrix is chosen based on a number of criteria but must possess two key features: it must firstly absorb strongly in at the wavelength of the chosen laser (almost always a UV laser); secondly it must be easily sublimable. Most matrices are therefore small aromatic compounds.

The mechanism of MALDI is not fully understood but it is thought to involve two basic steps\(^2\)\(^8\),\(^2\)\(^9\),\(^3\)\(^0\):

1. **Matrix Excitation:** the LASER beam is focussed onto the sample-matrix mixture causing clusters of analyte and matrix molecules to be ablated from the surface. Most of the laser energy is transferred to the matrix molecules (not the analyte molecules) which are photoionised by the laser.

2. **Analyte Ionisation:** the subsequent ionisation of the analyte molecules is not fully understood but may involve a combination of processes including proton transfer, photoionisation, desorption of preformed ions and ion molecule reactions. The most widely accepted mechanism is gas phase proton transfer from matrix to analyte to produce protonated analyte molecules.

The resulting cluster of analyte ions (and residual matrix ions) is then be extracted into the mass spectrometer for analysis.

MALDI tends to produce low charge states i.e. ions carrying few charges \((z = 1, 2, 3)\) and therefore requires a mass spectrometer with a large mass range to be useful for the analysis of large molecules \((m/z > 10 \text{ kDa})\). While MALDI can be used with virtually any mass spectrometer the requirement for a large mass range coupled with the pulsed nature of the ion source means it is most effective when coupled with a time of flight (ToF) mass analyser as they have (theoretically) an infinite mass range and require a pulse of ions.
The great advantage of MALDI over standard LD is that the amount of energy transferred to the analyte molecules is low, meaning there is little or no evidence of fragmentation allowing MS analysis of molecules with molecular mass of up to 300,000 Da\textsuperscript{28}.

While whole protein MALDI on such large systems is extremely interesting, it is often difficult to identify an unknown protein by mass alone and it is in the identification of proteins (often from complex mixtures of several proteins/peptides) that MALDI has come into its own. A typical experiment might involve the extraction of a number of proteins from an organism and a subsequent enzymatic digestion (i.e. cleaving the proteins at specific point to form peptides using an enzyme such as trypsin) of those proteins. Analysis of the resulting mixture by MALDI-MS will produce an extremely complex mass spectrum, often containing several hundred different peaks, but due to the large amount of proteins identified by genome mapping projects it is possible – with the aid of bio-informatics – to assign the peptides to specific proteins and therefore identify them\textsuperscript{31}. The reasons MALDI is so well suited to this type of analysis are manifold but it is primarily due to the sensitivity of the technique; often only a few femtomoles of the peptide are required to be present for a successful analysis by MALDI-MS.

1.1.1.2 ESI

The electrospray phenomenon has been known about for several hundred years but it was not until the late 1960s that its potential as to be used in ionisation was realised when Dole invented electrospray ionisation (ESI)\textsuperscript{32}. It took almost twenty more years for the technique to be applied to biomolecules through the pioneering work of John Fenn\textsuperscript{12,13}, the co-recipient of the 2002 Nobel Prize for Chemistry for his work on ESI.

A quick search of the literature – or indeed a quick look around any modern mass spectrometry laboratory – reveals many different styles of ESI source\textsuperscript{33}, the main components, however, remain unchanged. The sample of interest is introduced into the source in a solution at a low flow rate (1-10 \(\mu\)l min\(^{-1}\)) from a syringe pump or from an
HPLC. It passes through the electrospray needle, to which a high potential difference (2-4 kV with respect to the counter electrode) is applied. This leads to the formation of a ‘Taylor cone’ at the end of the needle (see figure 1.1) from which a spray of highly charged droplets appears. The spray is often accompanied by a co-axial flow of inert gas (usually nitrogen) which helps to confine the spray within the source chamber.

![Figure 1.1: Schematic diagram of the ESI process. A potential difference is applied between the spray needle (capillary) and cone leading to the formation of a Taylor cone from which highly charged, analyte containing droplets are produced. The solvent evaporates, shrinking the droplet until the 'Rayleigh' limit is reached and coulombic explosion occurs. This process continues until free analyte ions are produced.

As the droplets are ejected from the needle the solvent contained in them begins to evaporate (this is usually assisted by filling the source region with a heated nitrogen) thus reducing the size of the droplet. This brings the charge on the surface of the droplet closer together therefore increasing the coulombic repulsion between the charges. Eventually the coulombic repulsion becomes so great the droplet fissures into a number of smaller, charged droplets of sample solution. The point at which this fission occurs is determined by the size of the droplet, the surface tension of the solvent and the charge on its surface – the relationship between these factors can be easily described by the
Rayleigh equation (equation 1.1) which can be used to reveal the diameter at which a droplet of given charge and solvent will fissure – the so called Rayleigh limit. In this equation \( q \) is the charge on the droplet, \( \varepsilon_0 \) is the permittivity of the environment, \( \gamma \) is the surface tension and \( D \) is the diameter of the droplet.

\[
q^2 = 8\pi^2 \varepsilon_0 \gamma D^3
\]

Equation 1.1

The use of this equation falls down, however, when you consider it relies on the droplets being spherical in nature. Gomez and Tang showed this not to be the case when they obtained photographs of the ESI process and showed them to be deformed thus reducing the effective diameter of the droplets in places, allowing fission to occur before the droplet has reached the Rayleigh limit.

The droplets that result from the fission go through the same process until only desolvated, charged analyte molecules are present. Figure 1.1 shows is a schematic representation of the ESI process. The charged ions produced by ESI are of the same polarity as the needle and are therefore repelled from it and attracted towards the counter electrode (typically a skimmer or a capillary) through which they are drawn into the mass spectrometer.

Initially two mechanisms were put forward for the final stage of the ESI process i.e. the production of a ‘naked’ ion. The first mechanism, known as the charged residue model (Dole et al\(^33\)), postulates that the droplet fission process occurs until there is no solvent remaining, leaving only a charged gas phase ion. The second mechanism, the ion evaporation model (Iribarne et al\(^36\)), proposed that once the droplets reached a certain size analyte ions were ejected from the droplet due to coulombic repulsion. It was initially thought that ions of molecular mass < 2000 Da were produced by ion evaporation whilst those of mass > 2000 Da were produced by the mechanism described by Dole. However, the ion evaporation model has now largely been discredited thanks to the research of Williams and all ions produced by ESI are believed to be formed via the charge residue mechanism\(^37\).
The true beauty of ESI is its ability to produce highly charged ions of low m/z meaning large molecules can be analysed easily even on instruments with small mass ranges. This is what makes ESI particularly useful for the study of biomolecules. A small protein such as ubiquitin has a mass of ~8,500 Da; with only one charge present this would be undetectable on a large number of mass spectrometers (e.g. most quadrupole instruments). However, ubiquitin has 11 basic residues (i.e. lysine and arginine) which can be protonated (i.e. charged positively) in positive ionisation mode; the large amount of charge present on a droplet produced by ESI means that it is possible to charge some or all of these basic residues thus reducing the m/z of the ions, meaning they are now detectable on almost any MS.

Another feature of ESI that makes it particularly useful for the analysis of biomolecules is that it is an extremely soft ionisation process; little or no fragmentation is seen for even the largest of molecule. This has led to the use of ESI-MS for the analysis not only large proteins but also large complexes of biomolecules of up to several MDa molecular mass. The advent of ESI has revolutionised mass spectrometry and opened up whole new field of study for MS users – this will be discussed in more detail in section 1.3.

Nanoelectrospray ionisation (nano-ESI)\textsuperscript{38} is a related technique which allows much lower volumes of sample to be used in the course of an experiment. In nano-ESI the sample solution is sprayed from a glass capillary which has been pulled out into a fine point of only a few micrometers diameter. The voltage (1-1.5 kV) can either be applied directly to the solution via a platinum wire or to the glass capillary itself by coating it in an inert metal (e.g. Pt, Au). The sample flow rate used for nano-ESI is at least an order of magnitude lower than that required for standard ESI (5 µl in a capillary can often last an hour or more). Aside from the obvious benefit of lower sample consumption, nano-ESI is also thought to be more sensitive (the droplets produced are smaller than for ESI and therefore require less desolvation) and more salt and contaminant tolerant than ESI. For these reasons, nano-ESI is the technique of choice for much of this work.
1. Introduction

1.1.2 Mass Analysers

The mass analyser is the heart of any MS experiment; it is where the ions are separated by m/z such that they can be detected. There are many different types of mass analyser from the relatively simple and cheap quadrupole, to the complicated and extremely expensive Fourier Transform Ion Cyclotron Resonance Mass Spectrometer (FTICR). Different types of mass analyser offer different features and each has its advantages and disadvantages that must be weighed up. Factors that may affect the choice of mass analyser include:

1. Resolution – the ability to differentiate between two ions with a small m/z difference
2. Mass accuracy – how well the m/z can be assigned
3. Mass range
4. Sensitivity – essentially a measure of how well the analyser transmits ions
5. Ease of use
6. Additional features – e.g. can it be used to trap ions?
7. Cost

While the choice of mass analyser is often dependent on what is available, given a free choice, it is usually a play-off between the quality of data required and the cost of analysing a sample. In general high resolution, high sensitivity instruments are more expensive – in terms of initial purchase and day to day running – than low resolution, low sensitivity instruments.

This section will focus on the three different types of mass analyser used in this work namely quadrupole, time of flight (ToF) and FTICR.
1. Introduction

1.1.2.1 Quadrupoles

Quadrupoles are probably the most common type of mass analyser in use today, due in no small part to their relatively low price and comparatively simple operation. They were developed by Wolfgang Paul\textsuperscript{39} (in tandem with his Nobel Prize winning work on the development of the quadrupole ion trap) in the early 1950s. They consist of four parallel (usually circular) rods to which a fixed DC potential (or offset) is applied along with an alternating RF potential. The rods are paired such that directly opposite rods are in phase with each other with regard to the RF, while adjacent rods are 180° out of phase i.e. when one rod is positive w.r.t the RF ground, the rods next to it are negative and vice versa.

The trajectory of an ion passing through a quadrupole is complex; it is dependent on the spacing of the rods (a fixed parameter), the amplitude of the applied potential and the frequency of the RF. The total electric field experienced by an ion travelling in the x-direction through a quadrupole is described in equation 1.2\textsuperscript{25} where $\Phi_0$ is the total potential on the rods, U is the DC potential, V is the zero-to-peak RF voltage and $\omega$ is the angular frequency of the RF where $\omega = 2\pi v$, where v is the RF frequency.

$$\Phi_0 = + (U + V \cos \omega t) \quad \text{and} \quad -\Phi_0 = -(U + V \cos \omega t) \quad \text{Equation 1.2}$$

Figure 1.2 is a schematic diagram of a quadrupole, the first potential described is applied to one pair of rods (labelled +) while the second is applied to the other pair (labelled -). From the equations it is clear to see that the potential applied to the rods varies sinusoidally with time $t$. This varying potential causes ions to pass through the quadrupole with an oscillating trajectory; for an ion of a given $m/z$ there is a set of values of U, V and $\omega$ that give rise to a stable trajectory allowing transmission of the ion. These values are specific to that $m/z$ and ions of all other $m/z$ to have unstable trajectories which do not allow transmission.
For most situations, quadrupoles will be set up to transmit ions within a given m/z range; this is usually achieved by scanning the RF amplitude (V). By detecting ions at the end of the quadrupole and relating their detection time to the applied potential it is possible to determine the m/z and therefore produce a mass spectrum.

Another common use of quadrupoles is as mass filters where they are set to transmit only ions of one specific m/z. This is particularly useful for tandem MS instruments in which an ion is selected in order to dissociate e.g. by CID.

### 1.1.2.2 Time of Flight

The concept of time of flight (ToF) mass analysis was first described by Stephens in 1946\(^{40}\) but it was not demonstrated practically until 1955 when Wiley and McLaren published their design of a ToF instrument which went on to become the first commercial ToF mass spectrometer\(^{41}\).
1. Introduction

In a ToF experiment, ions are accelerated over a short distance by a potential difference; they then enter a field free region through which they drift at a velocity dependent on the ions m/z. The time taken for an ion to pass through a set distance (flight time, $t_f$) can therefore be used to find its m/z. Equations 1.3, 1.4 and 1.5 describe the effect of the acceleration potential on the ions and how it is related to time of flight where: $E_k$ is kinetic energy, $m$ is mass, $v$ is velocity, $q$ is charge, $e$ is the charge on an electron, $z$ is the number of charges on an ion, $V$ is potential (voltage), $d$ is distance and $t_f$ is the flight time.

\[
E_k = \frac{1}{2}mv^2 \quad \text{Equation 1.3}
\]

\[
E_k = qV = zeV \quad \text{Equation 1.4}
\]

\[
t_f = \sqrt{\frac{m}{z}\left(\frac{d^2}{2eV}\right)} \quad \text{Equation 1.5}
\]

From equation 1.3 it is clear that for a given kinetic energy, the velocity of an ion is dependent on its mass. From equation 1.4 it can be seen that the kinetic energy given to an ion in the acceleration region (pusher) varies linearly with charge i.e. a $+2$ ion will receive twice as much kinetic energy as a $+1$ ion. Combining these two expressions and solving for time gives equation 1.5. This shows how measuring $t_f$ can yield m/z information.

The original ToF instruments used linear ToF analysers, figure 1.3 is a schematic of a linear ToF.
I. Introduction

Figure 1.3: Schematic diagram of a linear ToF where the green, red and blue circles are low, medium and high m/z ions respectively.

The far left of the diagram shows the pusher region where the ions are accelerated, the potential difference needed to cause this acceleration is brought about by periodically pulsing the far left plate with a high voltage. This accelerates a pulse of ions into the field free region where they separate by m/z before being detected at the far end. Once all the ions have been detected, the pusher can be pulsed again to start the next ToF experiment.

The potential experienced by an ion in the pusher will be dependent on its position within the pusher region; an ion adjacent to the first pusher plate will pass through a greater potential difference than an ion a few millimetres away. This difference in kinetic energy will result in two ions of the same m/z will result in them having slightly different flight times causing a loss in resolution.

To overcome this problem, two different methods can be used: (i) delayed extraction – this is most useful for pulsed ion sources such as MALDI; (ii) reflectron – a more widely applicable method.
Reflectrons were first described by Mamyrin in the mid-1970s\textsuperscript{42}. It is a series of grids and ring electrodes used to create a retarding field. In ToF MS a reflectron is positioned at the opposite end of the flight tube from the pusher/source and is used to reflect ions back through the flight tube to a detector near the source. The depth an ion will penetrate into a reflectron (and hence the time it will spend within the reflectron) is dependent on its kinetic energy – an ion with high energy will penetrate the reflectron further than one with lower $E_k$. By tuning the reflectron voltage correctly it is possible to bring ions of the same m/z with slightly different $E_k$ into focus at the detector. Figure 1.4 shows a schematic of this process.

**Figure 1.4**: A reflectron ToF instrument. The filled square and open square represent ions of the same m/z with different $E_k$ (filled square – high, open square – low). The high energy ion has a greater velocity and reaches the reflectron first; it spends longer in the reflectron than the low energy ion, exiting later (but with higher $E_k$). If the reflectron voltage is correctly, both ions reach the detector simultaneously giving higher mass resolution.

### 1.1.2.3 Fourier Transform Ion Cyclotron Resonance

Fourier Transform Ion Cyclotron Resonance Mass Spectrometry (FTICR-MS) was developed in the 1970s by Comisarow and Marshall\textsuperscript{43,44}. It works on the principle that in the presence of a magnetic field, ions will be subject to the Lorentz force which will cause them to process in a circular path perpendicular to the magnetic field in a direction dependent on the ions polarity. Figure 1.5 shows this motion for positive and negative
charges and equation 1.6 describes the Lorentz force where $F$ is the force, $q$ is charge, $v$ is velocity and $B$ is magnetic field strength.

\[ F = qv \times B \]  
Equation 1.6

The ions process at a frequency - known as the cyclotron frequency, $\omega_c$ - dependent on their m/z as described by equation 1.7.

\[ \omega_c = \frac{qB}{m} \]  
Equation 1.7

This frequency is the same for all ions of the same m/z, regardless of their kinetic energy. It is this cyclotron motion that is exploited in FTICR.

Figure 1.6 is a schematic diagram of an FTICR cell.
Figure 1.6: An FTICR cell showing the tapping plates (blue), excitation plates (green) and detection plates (yellow).

In an FTICR experiment, ions are injected into the cell through the front trapping plate and trapped within it by rapidly raising the voltage on the trapping plates.

The ions are detected on a pair of detection plates on opposite sides of the cell; when an ion passes a detection plate it induces an image charge on the plate and therefore sets up a potential difference between the two detection plates. The difference in charge between the plates produces a current which oscillates with the same frequency as the cyclotron motion. Measuring the frequency current will therefore allow the m/z of the ions to be determined. The magnitude of the current seen is dependent on the distance the ion is from the plate, the charge on the ion and the abundance of that ion.

Upon entering the cell the ions begin to exhibit cyclotron motion, however, the motion of ions of any one m/z is incoherent i.e. the ions are distributed randomly about their orbit and are therefore undetectable. Further to this, the radius of the ions on entering the cell is too small to allow detection – the ions must be excited to a greater radius in order for them to be detected. This excitation is achieved by applying a resonant frequency (i.e. a frequency equal to the cyclotron frequency of the ions of interest) to the pair of excitation plates. The excitation pulse has the added function of bringing all ions with the same m/z into resonance with each other creating a packet of ions precessing together. After the
excitation the ions are therefore in a coherent packet at a detectable distance from the
detection plates. Early ICR experiments used single frequency excitation, exciting one m/z at a time; this was an inefficient method of obtaining mass spectra as separate scans are required for every m/z resulting in a loss of resolution. By using broadband excitation (i.e. exciting a wide range of m/z simultaneously) it is possible to measure an entire spectrum in one experiment. The data output from the detection plates in this case is a superposition of the signals obtained for all ions present. To convert this complex, frequency scale data into discreet m/z data, a Fourier transform is performed.

The primary advantage of FTICR-MS over any other MS technique is its unsurpassed mass resolution. The main reason for the high resolution is the extreme stability of the magnets used; their fields vary by only very small amounts over a long period of time (reportedly parts-per-billion over many hours). Secondly, the orbital motion of the ions averages out any inhomogeneity in the magnetic field (c.f. spinning samples in NMR). This means that the magnetic field strength is known to an extremely high accuracy and, as the frequency can be measured to an equally impressive accuracy, the m/z of species in the cell can be calculated to extremely accurately. More importantly, there is little peak broadening hence the high resolution and mass resolving power.

1.1.2.4 Tandem Mass Spectrometry

Tandem mass spectrometry (MS) describes any MS method involving at least two stages of mass analysis, usually in conjunction with a dissociation process. This can involve the combination of two mass analysers (MS/MS) – using the first to isolate an ion of interest (parent ion) and the second to analyse product ions – such as quadrupole and time of flight; or the use of a single trapping mass analyser (Paul trap or FTMS) in which ions can be stored, dissociated and re-analysed a number of times.
I. Introduction

The most common dissociation technique used in these experiments is collision induced dissociation (CID). In this process a portion of the kinetic energy of the ions is converted to internal energy by collision with neutral molecules. The excess internal energy brings about fragmentation of the ions. For proteins and peptides this fragmentation occurs at the amide bond producing well defined fragment ions which can be used to sequence the molecule.

Other dissociation techniques used include surface induced dissociation (SID) – a technique that uses ion-surface collisions to induce fragmentation; electron capture dissociation (ECD) - where dissociation is mediated by interaction with a beam of electrons; and infrared multi-photon dissociation (IRMPD) – where dissociation is brought about through excitation with an IR laser.

1.1.3 Detectors

Clearly FTICR mass analysers also function as ion detectors, other mass spectrometers however, require a separate detector after the analyser. Most MS detectors use the incident ions to generate secondary electrons which are further amplified to create a detectable current. The three most common detectors are: (i) electron multiplier; (ii) photomultiplier; (iii) microchannel plate (MCP). These will be discussed in turn.

i. Electron multiplier: these consist of a series of dynodes held at sequentially increasing potential. Ions from the mass analyser are incident on the first (lowest potential) dynode causing the release of secondary electrons; these secondary electrons are incident on the next dynode where further electrons are produced. This process continues along to the end of tube by which point the signal has been amplified by \( \sim 10^6 \). The electrons are then detected as a current. Figure 1.7 is a schematic of an electron multiplier tube.
ii. Photomultiplier tube\textsuperscript{46}: this works on a similar principle to the electron multiplier. The ions are incident on a conversion dynode and generate electrons. These electrons are then incident on a phosphor screen causing the generation of photons which are then detected by a photomultiplier. Figure 1.9 is a schematic of a photomultiplier tube.

iii. Microchannel plate\textsuperscript{25}: ToF instruments often use microchannel plate (MCP) detectors; these consist of a glass plate with many channels each similar to an electron multiplier. An ion incident on one channel will produce over 1000 electrons at the other side of the channel. Figure 1.10 shows a schematic of an MCP detector.
Figure 1.10: Schematic representation of a microchannel plate
1. Introduction

1.2 Ion Mobility Spectrometry

This section will discuss the theories and concepts behind ion mobility spectrometry (IMS) and the types of instrumentation used, concentrating on the relatively recent development of ion mobility mass spectrometry (IMMS).

1.2.1 Theories and Concepts

The behaviour of ions moving through gases was thoroughly described by Mason and McDaniel in their excellent book “Transport Properties of Ions in Gases”\textsuperscript{51}. Much of the theory described here is adapted from that work.

In IMS, an electric field is used to separate ions in a buffer gas based on their mobility in that gas. A typical IMS experiment requires the injection of a pulse of ions into a chamber filled with a known gas at a known pressure; an electric field is then applied across the chamber and the time taken for the ions to pass through the chamber is measured. Upon injection into the chamber (drift cell), the ions experience an electrostatic force pulling them through the cell; this force is counteracted by collisions between the ions and the buffer gas. The velocity of the ion quickly reaches a constant known as the drift velocity, $v_d$ which can be determined by measuring the time taken for an ion to pass through the drift cell, $t_d$.

The behaviour of an ion moving through a gas under the influence of an electric field is dependent on its energy which is determined by the ratio of electric field strength to buffer gas number density, $E/N$.\textsuperscript{21,47} At low $E/N$ the ions are said to be in the low field limit, under this regime the ions have low velocities which are independent of the field strength. At higher $E/N$ the ions may align in the field and their motion becomes dependent on the field strength. In the low field limit, the motion of the ions can be described in more simple terms and most ion mobility measurements are therefore performed below the low field limit.
1. Introduction

Under these conditions it is possible to define the low field mobility, \( K \), as the constant of proportionality between drift velocity, \( v_d \), and electric field, \( E \) as shown by equation 1.8.

\[
v_d = KE
\]

Equation 1.8

Drift velocity of an ion depends on gas pressure, electric field, the charge on an ion and its shape. The mobility, \( K \), must therefore contain terms relating to molecular shape, charge and buffer gas pressure. In the limit of vanishing electric field and ion concentration, the mobility of an ion can be described by Nernst-Townsend-Einstein relation, which is obtained as follows:

Firstly, the ion flux density, \( J \), of an ion is stated using Fick’s law of diffusion:

\[
J = -D \nabla n
\]

Equation 1.9

Where \( D \) is the diffusion coefficient and \( n \) is the number density of the buffer gas. \( J \) can also be described in terms of drift velocity:

\[
J = n v_d
\]

Equation 1.10

Allowing Fick’s law to be stated in the following form:

\[
v_d = - \left( \frac{D}{n} \right) \nabla n
\]

Equation 1.11

Combining equations 1.8, 1.9 and 1.10 gives:
\[ J = nKE - D\nabla n \]  
\text{Equation 1.12}

To find an expression for \( \nabla n \) we take the equilibrium case where \( J = 0 \) and the ion distribution as described by a Boltzmann exponential:

\[ n = n_0 \exp\left( + \frac{zeE \cdot r}{k_bT} \right) \]  
\text{Equation 1.13}

Where \( z \) is the integer number of charges on the ion, \( e \) is the charge on an electron, \( r \) is the position of the ion and \( k_b \) is the Boltzmann constant. Differentiation over all \( r \) and rearranging yields:

\[ \frac{1}{n} \nabla n = \left( \frac{ze}{k_bT} \right) E \]  
\text{Equation 1.14}

Which combined with expression 1.11 at \( J = 0 \) gives:

\[ v_d = \left( \frac{zeD}{k_bT} \right) E \]  
\text{Equation 1.15}

Or:

\[ K = \frac{zeD}{k_bT} \]  
\text{Equation 1.16}

Chapman-Enskog theory\textsuperscript{52} allows mobility of an ion to be related to its rotationally averaged cross section, \( \Omega \), by expansion of the diffusion coefficient giving:
1. Introduction

\[ K = \left( \frac{3ze}{16N} \right) \left( \frac{2\pi}{\mu k_b T} \right)^{\frac{1}{2}} \left( \frac{1}{\Omega} \right) \]  
Equation 1.17

Where \( \mu \) is the reduced mass of the buffer gas and ion of interest, and \( N \) is the number density of the buffer gas.

From equation 1.17 it is clear that, in the low field limit, the mobility of an ion is dependent on its charge, cross section, temperature and mass, along with the pressure of buffer gas used in the experiment, but independent of the electric field as stated previously. For large molecules, such as peptides and proteins, the reduced mass approaches the mass of the buffer gas and the mobility is therefore essentially mass independent.

In an ion mobility experiment, all parameters apart from rotationally averaged cross section, can be determined directly. The mobility of the ion is therefore a measure of its collision cross section. For any one species, a compact ion with a small cross section (e.g. a folded protein) will have higher mobility than a more extended ion with a larger cross section (e.g. a denatured protein).

In order to make comparison across different instruments, where temperature and pressure may vary greatly, the mobility of an ion is usually expressed in terms of the reduced mobility, \( K_0 \) as shown in equation 1.18\(^{20,21}\) where \( p \) is the pressure in mmHg and \( T \) is the temperature in Kelvin.

\[ K_0 = \frac{p}{760} \frac{273.15}{T} K \]  
Equation 1.18
1.2.2 Experimental Set-up

Traditionally, IMS was used as a stand alone technique to monitor the gaseous environment in a variety of locations. The instruments used vary in size from the small, hand-held instruments used in the defence industry to detect the use of chemical weapons to the large scale instruments used in the semiconductor industry to monitor contaminant level. More recently, IMS devices have been coupled to mass spectrometers to allow simultaneous collection of mobility and mass data via ion mobility mass spectrometry (IMMS)\textsuperscript{20,21}.

Stand-alone IMS instruments can operate at a wide range of pressures but experiments are often performed at atmospheric pressure for ease of operation. Mass spectrometers, however, function at pressures below atmosphere (10\textsuperscript{-4} to 10\textsuperscript{-11} mTorr); IMS devices coupled with mass spectrometers are therefore usually housed within a vacuum chamber.

Several different types of IMS device have been described in the literature and each of these will be discussed in turn; the basic experimental set-up, however, is common to all IMS devices. Much like MS, IMS devices require three basic components: (i) an ion source; (ii) the mobility chamber (drift cell); and (iii) a detector.

Ion sources and detectors have been covered in detail in section 1.1; the different types of IMS will be covered in the following sections. Discussion of specific instruments will be restricted to IMMS instruments.

1.2.2.1 Linear Ion Mobility

This is the most common and the most well understood type of ion mobility spectrometry. A typical experimental set-up requires a drift cell which can be held at some elevated pressure relative to the vacuum chamber; the cell must also be designed such that a linear electric field can be applied to it. Providing the low pressure limit is
observed \((E/N \leq 2 \text{Td})\), ions in such a device will behave according to the equations outlined in section 1.2.1.

Figure 1.11 shows the linear ion mobility cell used in the Bowers group at UCSB\(^5\).

**Figure 1.11:** Linear ion mobility cell used in the Bowers group. (a) cooling line, (b) cell body, (c) buffer gas inlet, (d) cell end cap, (e) drift guard ring, (f) ion entrance hole, (g) ion focusing lens, (h) ceramic ring, (i) ion exit hole, (j) ceramic rod holding guard rings, (k) ceramic rods holding cell assembly.

The cell is constructed from a copper block, a copper end cap and a ceramic ring to separate the two. The block can be heated by electrical heaters and cooled with a flow of liquid nitrogen, the end cap has a separate temperature control. The drift field is provided by a set of ring electrodes connected by a set of precision 1 MΩ resistors. The total drift length is 4.503 cm, the cell temperature can be varied between 80 K and 800 K. Typical buffer gas pressure is 4-5 Torr allowing for drift voltages of 2 to 20 V/cm. Higher temperature and pressure is possible with some modification to the cell.
1. Introduction

This cell design has a resolution of around 20 and has been used in a number of instruments, the most recent of which is shown in figure 1.12.

Figure 1.12: (Top) Cross-sectional view of entire instrument as viewed from the top. (Bottom) Perspective cross-sectional view of source, funnel and cell. (a) and (b) vacuum chambers, (c) pump ports, (d) source flange, (e) ion funnel, (f) drift cell, (g) quadrupole mass analyser, (h) conversion diode, (i) detector, (j) capillary heating block, (k) insulator, (l) funnel first section, (m) funnel second section, (n) funnel third section, (o) funnel flange, (p) hat flange, (q) second pump stage, (r) cell body, (s) cell end cap, (t) ceramic ring, (u) guard rings, (v), (w) and (x) ion optics.

This instrument consists of an ESI source, ion funnel and drift cell followed by a quadrupole mass analyser. The ion funnel is a high transmission RF ion guide which has two functions: firstly, it compresses the divergent stream of ions produced by the source into a small diameter; secondly, it transports the ions from the source to the cell at low voltage thus avoiding high energy ion-neutral collisions that may affect the ions of interest.
In a typical experiment, the ions are created in the ESI source and pulled through the ion funnel towards the drift cell; they are then trapped in the final section of the ion funnel and pulsed into the drift cell to start an ion mobility experiment. The ions are then mass analysed by the quadrupole and detected on the detector – this detection event also stops the IMS experiment.

Several similar instruments (IM followed by MS) have been described in the literature\cite{55,56,57,58}, including those used in the Clemmer group at The University of Indiana. This group has been responsible for a number of exciting advances in the field of IMMS including the development of multidimensional ion mobility (IMS\textsuperscript{9}). They were the first group to demonstrate the use of a ToF MS as a back end for an IMS device\cite{59}.

The original instrument consisted of an IMS device followed by a ToF, it was subsequently improved upon by the addition of a quadrupole mass analyser followed by an octapole collision cell between the drift cell and the ToF\cite{60} and finally the addition of an ion trap prior to the drift cell; this set up allows IMS-MS-MS experiments to be performed. A diagram of this instrument is shown in figure 1.13\cite{61}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{HPLC-ESI-IMS-Q-o-ToF_instrument.png}
\caption{(HPLC)-ESI-IMS-Q-o-ToF instrument.}
\end{figure}
1. Introduction

The challenge of this technique is that drift times and mass spectra of several different ions must be measured simultaneously. This is possible because ToF flight times are very much shorter than drift times (μs as oppose to ms), therefore flight times can be 'nested' within the time windows of the ion mobility spectra. Ions are injected into the drift cell at a rate of 10-30 Hz and into the ToF flight tube at a rate of $10^4$ Hz, or approximately 1000 times for every injection into the drift cell, therefore it is possible to observe all m/z present in a drift time window using a ToF MS.

In this set-up ions are produced by the ESI source and stored in the ion trap before being injected into the drift cell where they are separated. The ions then pass through the quadrupole to the ToF where they are sampled at a pre-defined rate, mass analysed and finally detected on the MCPs. The quadrupole is typically set to transmit, it can be 'parked' on an individual mass to allow a specific ion to be dissociated in the collision octapole.

The arrival times, $t_a$, measured on the MCPs are a composite of the time the ions spend in the drift cell, $t_d$, the flight time in the ToF, $T_f$ and the time spent in the rest of the instrument (ion optics etc.) – the dead time, $t_d$. By accurately measuring $t_a$ and $T_f$ it is possible to determine $t_d$ and hence calculate $t_d$.

Clemmer et al first reported this method as a technique to simultaneously measure conformations of different charge states of peptides, a technique also used by Hill and co-workers. The method has also been used to separate and analyse complex mixtures.

The Clemmer group have recently reported a variation on this instrument using a new type of linear IMS device which uses a split-field drift tube to separate and fragment ions within the same device. A schematic diagram of the instrument is shown in figure 1.14.
1. Introduction

Figure 1.14: Diagram of the Clemmer group split-field drift tube instrument. Ions are created in the ESI source and transferred to the linear trap from where they are pulsed into the drift tube and separated before detection using the ToF MS. Figure taken from reference 65.

The drift cell differs from a standard mobility cell as the later portion of it can be operated at both high and low drift fields. With this portion at low drift field, the device is used as a standard IMS drift tube; with the latter part of the drift tube at high drift field, dissociation of the ions occurs causing the formation of CID type fragments. This set-up allows mobility separated conformers to be dissociated thus revealing difference in the covalent structure of the conformers studied.

This technique has been furthered by the advent of multidimensional IMS in the same group\textsuperscript{66,67}. In IMS-IMS, two drift cells are coupled together separated by an ion funnel and ion gate. An instrument schematic is shown in figure 1.15\textsuperscript{66}. 


1. Introduction

Figure 1.15: Schematic diagram of the Clemmer IMS-IMS-ToF instrument. The drift regions (D1 and D2), ion funnels (F1-F3), ion gates (G1 and G2), ion activation regions (IA1-IA3), and resolution (R) of specific components are labeled.

This instrument offers four modes of operation:

(i) Both drift tubes are used together: ions are separated in the first drift tube; refocused by the ion funnels into the second drift tube where they are further separated. This allows high resolution (80-160) mobilities to be determined.

(ii) IMS-IMS: ions are separated in the first drift tube but only a selected window of these ions is allowed to enter the second drift tube. This allows ions with a specific mobility to be selected for further analysis.

(iii) Activation of mobility selected ions: ions are mobility selected in the first drift tube as in (ii) before activation/dissociation in the ion funnel (IA2). The resultant ions are then analysed in the second drift tube.

(iv) Parallel dissociation: ions are dissociated in IA2 as described in (iii); further dissociation is carried out after the second mobility separation in the post cell ion funnel (IA3).

Clearly this development represents a new and exciting avenue for IMMS offering an extremely versatile instrument capable of performing a number of different experiments.
1. Introduction

Not all groups use the IMS followed by MS set up used in the instruments described above. Several groups use MS-IMS\textsuperscript{68} or MS-IMS-MS\textsuperscript{69} set-ups. The MS-IMS-MS approach was pioneered by Bowers and Kemper\textsuperscript{70} who used a double focussing mass spectrometer (MSI) before the drift cell with a quadrupole mass analyser after the drift cell. Figure 1.16 is a schematic diagram of the instrument\textsuperscript{70}.

![Figure 1.16: The Bowers and Kemper MS-IMS-MS instrument.](image)

The advantage of this sort of set-up over and IMS-MS set-up is the ability to mass select ions prior to IMS separation thus allowing greater experimental control. This sort of set-up has since been used in conjunction with other mass analysers including FTICR, quadrupole, quadrupole ion trap and ToF. Weis et al.\textsuperscript{71} report an experimental configuration where a ToF is used to pre-select the ions, this is a particularly useful front end as the ToF has no theoretical upper mass limit and it requires short (<1µs), intense bundles of ions which are ideal for injection into the cell.

One disadvantage of MS-IMS-MS (and MS-IMS) instruments is that they are generally restricted to relatively low pressure (3-10 Torr) ion mobility devices due the pumping requirements for the pre-cell MS.
1. Introduction

1.2.2.2 High Resolution Linear Ion Mobility

The resolution of an ion mobility measurement is limited by the diffusion of the ion packet as it passes through the tube. It is defined by equation 1.19 where $t_d$ is the drift time, $t_{1/2}$ is the peak width at half height and $V_d$ is the voltage drop across the cell (drift voltage).

$$\frac{t_d}{t_{1/2}} = \frac{1}{4} \left( \frac{ze}{k_b \ln 2} \right)^{1/2} \left( \frac{V_d}{T} \right)^{1/2}$$  Equation 1.19

Clearly, the resolution of an instrument can be increased by decreasing the temperature of the drift cell. This, as will be shown later, may have an effect on the conformations adopted by the ions being studied. It is therefore more practical to increase the drift voltage. In order to keep the ion motion within the low-field limit it is necessary to increase the number density of the gas (i.e. the pressure) along with the drift voltage.

Increasing the buffer gas pressure presents a number of practical problems, most notably at high pressure it becomes more difficult to get the ions into the drift tube due to the quantity of buffer gas flowing out of the drift cell. To overcome this it is necessary to increase the ion injection energy, as some of this energy is converted to internal energy, increasing the injection energy too much can bring about fragmentation of the ions. In the set-up developed by Dugourd et al, this is overcome by housing the source and drift cell together allowing the ions to be transferred directly into the drift region. Figure 1.17 shows a schematic of this instrument.

The resolution of a drift tube is defined in equation 1.19 where $\Delta t_d$ is the drift time spread and $V_d$ is the drift voltage across the tube.
1. Introduction

Figure 1.17: A schematic representation of the high resolution IMS instrument developed by the Jarrold group. It uses a laser vaporisation/desorption source to produce ions which are introduced directly into the drift cell via an ion gate. The drift cell is 63cm long and is typically held at 500 Torr, at such pressure it can be used with voltage drops up to 15000V. The ions are then mass analysed and detected using a quadrupole MS.

High resolution IMS-MS instruments have also been reported by the Hill group including an ESI instrument\textsuperscript{55} and an ambient pressure drift cell instrument\textsuperscript{74}.

1.2.2.3 Travelling Wave Ion Mobility

A new ion mobility separation technique has recently been launched commercially by Waters Micromass Technologies (Manchester, UK)\textsuperscript{75}. It uses an elevated pressure stacked ring ion guide (SRIG)\textsuperscript{76} as a mobility cell\textsuperscript{77}. A SRIG is an ion guide consisting of a stack of thin ring electrodes arranged in a sequence with opposite phase RF applied to consecutive electrodes as shown in figure 1.18\textsuperscript{73}. The RF provides a potential well which keeps the ions radially confined within the device.
1. Introduction

Figure 1.18: An RF only stacked ring ion guide.

In order to propel the ions through the device, a travelling wave DC voltage is superimposed on top of the RF voltage. This voltage is applied sequentially to pairs of ring electrodes providing a potential which can push ions through the device as shown by figure 1.19.

Figure 1.19: Pulse sequence for travelling wave SRIG mobility device.
1. Introduction

At low pressure the travelling wave provides an effective method of transmitting ions through the SRIG. At elevated pressure (~1 mBar) the ions can be separated based on their mobility: ions are pulsed into the cell and remain in the first region until they experience a travelling wave; the ions then 'surf' on the wave as it passes through the cell. As in other ion mobility methods the progress of the ions through the cell is retarded by collisions with the buffer gas, because of this the ions will eventually fall off the back of the wave into the potential well behind it where they remain until they experience the next wave. The distance an ion travels before it falls off the wave is mobility dependent; a high mobility ion will 'surf' on the wave for longer than a low mobility ion. The device can therefore be used to separate ions by mobility.

It is not clear, however, whether mobility values (and hence cross sections) can be measured directly using this method. While the ions are reported to be in the low field limit in this device, their motion is complex and non-linear. The equations outlined in section 1.2.1 are therefore not applicable to this device.

In order to obtain cross sectional information from this device, a calibration technique was described by Thalassinos et al. They related the arrival times measured in this device to cross sections obtained from linear ion mobility measurements in order to provide a calibration equation; they showed good agreement with literature for a number of peptides.

The main advantage of this device over a linear mobility device is its high transmission efficiency. The ions are very effectively constrained by the RF field and are efficiently transferred by the travelling wave, addition of this device results in almost no loss of sensitivity.
1.2.2.4 FAIMS

High-field Asymmetric Waveform Ion Mobility Spectrometry (FAIMS) was developed in Russia in the 1980s\textsuperscript{79,80} and launched commercially by Ionalytics Co. (Ottawa, Canada) as an atmospheric pressure mobility device for front end separation of ions prior to MS analysis\textsuperscript{81}.

It is a two electrode system that uses the difference in the mobilities of ions at high and low field to separate them. This is achieved by applying a square wave potential to one electrode consisting of a high positive voltage for a short time followed by a lower negative voltage for a longer time. The nature of the collisions with the gas at high field strength can affect the mobility of the ion making it either more or less mobile than at low field. In FAIMS the ions are carried through the device not by the electric field but by a flow of carrier gas; the applied electric field is perpendicular to this gas flow and it is in this direction they are separated.

Figure 1.20 shows the waveform used in a FAIMS experiment.

![Waveform used in FAIMS experiments. DV is the dispersion voltage – the amplitude of the high field pulse. The waveform is set such that the voltage-time integral for the high field pulse is equal to that of the low field pulse.](image)

Figure 1.21\textsuperscript{82} shows the paths of three different ions through a FAIMS device. The far left ion has higher mobility at high field than low field causing it to move towards and
eventually collide with the bottom electrode. The far right ion has higher mobility at low field than high field causing it to move towards and eventually collide with the top electrode. For the centre ion, the mobility high and low field is balanced such that the mobilities at high and low field resulting in the average ion position being unchanged for a cycle of the FAIMS pulse — this ion is therefore transmitted out of the FAIMS device.

![Ion Hits Lower Plate](image1.png) ![Ion Hits Upper Plate](image2.png) ![Ion in 'Balanced' Condition](image3.png)

**Figure 1.21:** Passage of ions of different mobility through a FAIMS device.

In order to select ions of different mobility, a weak DC field known as the compensation voltage (CV) is applied between the electrodes. CV can be used to compensate for an ion’s drift towards either electrode, bringing it back to the centre of the device and allowing it to be transmitted.

The commercial version of FAIMS does not use the linear geometry shown above but rather a cylindrical geometry as shown in figure 1.22.

---

40
Several groups have shown this device to be useful for conformational selection of ions\textsuperscript{84,85}, spectrum simplification\textsuperscript{86} and background subtraction\textsuperscript{87}. The device does not, however, allow low field mobility values – and therefore cross sections - to be determined.

### 1.2.3 Mobility Calculations

Clearly, from linear mobility (and calibrated t-wave mobility) experiments it is possible to determine the mobilities of different ions. Equation 1.17 can then be used to obtain cross section values from these mobility values. It is not clear, however, how these cross sections can be used to give structural information about the ions.

This is not a simple process, requiring many complex computational calculations. The start point for these calculations is obtaining candidate structures for the ions of interest. For smaller molecules <50 atoms, it is possible to perform precise quantum mechanical calculations on the molecules using programmes such as Gaussian\textsuperscript{88}. Large molecules such as proteins and peptides, however, are too complex for such high level calculations and candidate structures are normally generated using molecular mechanics techniques such as AMBER\textsuperscript{89}.
1. Introduction

These calculations use force fields – tables of atom types, parameters (bond length, bond angles etc.) and equations – from which the energy and geometry of a molecule can be calculated.

\[ U(r) = \sum_{bonds} k_b(b - b_0)^2 + \sum_{angles} k_\theta(\theta - \theta_0)^2 \]

\[ + \sum_{torsions} k_\psi [\cos(n \psi + \delta) + 1] + \sum_{non-bonded \ pairs} \left( \frac{q_i q_j}{r_{ij}} + \frac{A_{ij}}{r_{ij}^{12}} - \frac{C_{ij}}{r_{ij}^6} \right) \]

Equation 1.20

Where the first term describes the stretching of the bonds, the second term describes the bending of the bonds, the third term describes the twisting of the bonds and the final term describes the non-bonded interactions between atoms namely the electrostatic interaction and the van der Waals interaction.

The molecular modelling programs use this equation to model the potential energy surface of the molecule of interest. Candidate structures are generated by sampling this surface and minimising the energies of the molecules in order to sample its minima.

This can be done by a number of methods; in this work a technique called simulated annealing is used\(^9^0\). In a typical simulated annealing calculation, an initial candidate structure is built within the molecular modelling program either by simply joining amino acids in the correct order and minimising the energy or by using a previously published structure from NMR or X-ray crystallography studies. The molecule is then heated to a temperature at which the molecular structure is lost (e.g. 800 K); dynamics are performed at this temperature for a set time (e.g. 30 ns) before the molecule is cooled to 0 K and
I. Introduction

minimised. This process is carried out a number of times to produce a library of low energy structures.

These calculations can be carried out in a number of different environments but in the case of calculations for ion mobility they are generally calculated in the gas phase.

Once a library of gas phase structures has been created, the rotationally averaged cross sections of each can be calculated. This can be done by three different methods: (i) the projection approximation (PA); (ii) exact hard spheres scattering method (EHSS); and the trajectory method (TM). Each of these will now be discussed in turn.

i. The Projection Approximation: this method treats all the atoms within a polyatomic molecule as hard spheres and assumes the interactions between the molecule and the buffer gas are simple hard sphere collisions. The collision cross section of the molecule is obtained averaging the cross section of the molecule over all possible orientations. The method was developed by Mack in 1925\textsuperscript{91} before the advent of computational techniques; the measurement was performed manually using beeswax models of the molecule of interest and determining the cross section based on the shadow cast by the model. This technique has since been applied computationally by a number of groups.

The method has been updated somewhat by Bowers et al\textsuperscript{92} who included a temperature dependent term into their hard sphere modelling equations to allow some approximation of the long range interactions involved in the collisions. Variations in temperature can cause fluctuations in atomic radii which can in turn affect the collision cross section. This can be accounted for by the use of a (12, 6, 4) potential to model the ion-buffer gas interactions. The Bowers group have made available a program, Sigma, which will calculate collision cross sections for candidate structures using this method\textsuperscript{93}. 

43
ii. Exact Hard Spheres Scattering\textsuperscript{94}: the collisions between the ions of interest and the buffer gas in IMS cannot be completely described by the hard sphere scattering alone; in reality the temperature of the interaction and the nature of the collisions must be taken into account.

The method described above accounts well for temperature effects for small molecules (<200 atoms) but falls down for larger systems as it does not fully describe the nature of the collisions. In reality, the collisions between the ions and buffer gas involve a degree of scattering that is not described by PA. To fully describe these collisions, the trajectories of the ion and buffer gas must be described before and after collision. Shvartsburg and Jarrold have described a method to account for this: firstly, the momentum transfer integral is calculated by averaging a function of the scattering angle over the impact parameter and collision geometry. This collision integral is then calculated by averaging the momentum transfer cross section over the relative velocities of the ion and buffer gas.

iii. Trajectory Method\textsuperscript{95,96}: the EHSS method must be further refined to fully consider the long range interactions between the ion and buffer gas. To achieve this a potential must be described that includes all the interactions between the buffer gas and all atoms in the polyatomic ion including Lennard-Jones type interactions and ion-induced dipole interactions.

Table 1.1 is a reproduction from the excellent ion mobility tutorial by Clemmer and Jarrold\textsuperscript{97}. It shows theoretical cross sections obtained by each method for \((C_{60})_2\), \((C_{60})_{13}\), bovine pancreatic trypsin inhibitor (BPTI) and cytochrome C. The trajectory method has been reported to be the most accurate method for calculating theoretical cross sections for polyatomic molecules and values in the table are therefore also shown normalised to the TM values.
Table 1.1: Theoretical cross sections of four different polyatomic molecules obtained from three different calculation methods. Values in parenthesis indicate the collision cross section divided by the value obtained from the trajectory method.

It can be seen that as the molecular size increases – \((C_{60})_2\) being the smallest, cytochrome \(c\) the largest – the values obtained by TM and PA become more divergent with the projection approximation underestimating the value by nearly 20% for cytochrome \(c\). The agreement between TM and EHSS is good for all molecules studied and is seen to improve marginally for the larger molecules. This suggests two things: firstly, the largest source of error in the PS approach is its failure to account for scattering effects; secondly, the long range interactions included in the TM are of less importance for larger molecules.

The TM is, however, the most computationally expensive method and is therefore unsuitable for very large molecules due to the time taken for each calculation\(^{93}\). EHSS is a less demanding calculation and – as shown above – gives comparable values to TM; EHSS is therefore the most appropriate method for calculating collision cross sections of very large molecules.

Once candidate geometries have been generated for a molecule, the theoretical cross section can then be determined for each geometry by the most appropriate method. By comparing the theoretical values with those obtained from experiment it is possible to propose likely gas phase structures for ions.
1.3 Studying Biomolecules by Mass Spectrometry & Ion Mobility

The term biomolecule is a fairly loose way of characterising the myriad of molecules found in living organisms ranging from the small building blocks – amino acids, nucleic acids, mono-saccharides – to the very large – proteins, DNA, poly-saccharides and complexes of each. The structures formed by these molecules have long been of interest to scientist who use the information to gain insight into the function of biomolecules.

This work will focus on the study of the structure of proteins and peptides using gas phase techniques. Proteins and peptides have four levels of structure:

1. Primary Structure: the sequence of amino acids that form the polymer.
2. Secondary Structure: regions of repetitive structure such as α-helix and β-sheets.
3. Tertiary Structure: the arrangement of the 2° structure
4. Quaternary Structure: the arrangement of polypeptide chains (identical or different) to form oligomers (dimers, trimers etc.).

As stated previously, protein and peptide structure has traditionally been studied in the condensed phase by techniques such as X-Ray crystallography (XRC), circular dichroism (CD), fluorescence spectroscopy and NMR.

Table 1.2 considers some of the advantages and disadvantages of these ‘traditional’ techniques.
Table 1.2: Advantages and disadvantages of traditional biomolecular structural probes.

Clearly each technique has pluses and minuses; NMR and XRC provide greater structural detail about the molecules but require high volumes of sample and often require unusual solvent conditions; fluorescence on the other hand can be performed on very low volumes of sample (as little as one molecule!) but only provides structural information on lower level structure e.g. folded or unfolded.
Mass spectrometry and ion mobility have only relatively recently been applied to biomolecules. The combination of these techniques can provide a wealth of structural information for biomolecules from primary to quaternary structure. The great advantage of these techniques is the low sample volumes required; using nano-electrospray ionisation it is possible to gain structural information from attomoles of sample. Both techniques, especially MS, are also extremely fast especially in comparison to XRC and NMR.

As discussed above, early ionisation techniques such as EI were too harsh to transfer intact biomolecules into the gas phase; similarly, early mass analysers had insufficient mass range to analyse biomolecules. The advent of MALDI, ESI and more advanced analysers has provided a means of observing intact biomolecules in the gas phase.

This section will focus on the study of biomolecules using mass spectrometry and ion mobility mass spectrometry.

1.3.1 Mass Spectrometry

1.3.1.1 Primary Structure Determination

In its simplest form, primary structure determination by mass spectrometry requires only an accurate mass measurement. If the sequence of a protein is known, the mass can be calculated accurately from the molecular formula of the ion.

Determining the mass of an intact protein by mass spectrometry is a relatively simple task best performed by ESI-MS. ESI-MS typically produces a series of multiply charge ions, the m/z ratio of an ion with n charges is related to its mass, M by equation 1.21 where $m_i$ is the mass of the charge carrier (e.g. a proton).
From a high resolution mass spectrum it is possible to determine the charge on an ion based on the spacing between the isotopes which is equivalent to $1/z$ i.e. for an ion carrying one charge the isotopes are separated by 1 m/z unit; for an ion carrying three charge the isotopes are separated by $1/3$ m/z units. From a low resolution mass spectrum the spacing between consecutive charge states can be used in conjunction with equation 1.21 to find the charge state of each ion. Once the charge on each ion is known the mass of the molecule can be determined.

For unknown proteins, a more complicated procedure must be used whereby the molecule of interest is broken apart to determine its sequence. This can be achieved by either peptide mass mapping or tandem mass spectrometry.

i. Peptide mass mapping: in this so called 'bottom up' approach to primary sequencing, the protein of interest is digested using a proteolytic enzyme such as Glu-C, chymotrypsin or trypsin. These enzymes cleave proteins at specific points hence producing predictable peptides. Trypsin, for example, cleaves peptides at the c-terminus of lysine and arginine (unless followed by a proline).

In a typical experiment a protein (or mixture of proteins) is digested for a set period of time to generate a set of peptides. The peptide mixture is then analysed by mass spectrometry – most commonly MALDI-MS but also ESI-MS. Genome mapping projects such as the human genome projects have produced large databases of gene sequences from which protein sequences can be predicted. The peptide masses obtained from the MS are characteristic of the protein(s); the masses obtained are therefore compared to the protein sequences obtained from genome mapping in order to identify the protein present. This is done on a
statistical basis using programs such as MASCOT (Matrix Science Ltd., UK) which assign a probability score based on a number of factors including number of peptides identified, sequence coverage and the presence of missed cleavages.

ii. Top down sequencing\textsuperscript{25}: in top down sequencing tandem mass spectrometry is used to determine the primary sequence of the protein of interest. The intact protein is introduced to the mass spectrometer via ESI-MS. One ion is then selected and dissociated by CID, SID, IRMPD, BIRD, ETD or ECD. Much like bottom up sequencing, the masses produced are characteristic of the protein sequence and can be used to identify it from an online database.

1.3.1.2 Higher Order Structure Determination

Determination of higher order structures (2\textdegree, 3\textdegree and 4\textdegree) of proteins is most reliably performed by XRC and NMR spectroscopy. However, many proteins cannot be studied by these techniques due to difficulties in producing crystals, insufficient sample etc. These sorts of proteins are, however, often suitable for MS studies.

Higher order structure determination by MS can be performed by four main methods: (i) chemical cross linking; (ii) hydrogen/deuterium exchange; (iii) controlled fragmentation; and (iv) charge-state analysis.

Chemical cross linking uses small organic compounds – designed to bind to specific amino acid residues – in order to create proximity maps of proteins. The technique was first reported in the mid-1950s by Zahn and Meienhofer to identify the dimeric structure of insulin\textsuperscript{101,102}. The cross-linking reagents are used as molecular rulers to determine the distance between residues ranging from no separation to 20 Å\textsuperscript{99}. In order for the cross linking to occur on a native structure the reaction(s) are carried out under non-denaturing conditions. The specific residues to which the cross linkers are attached can then be determined by a combination of proteolysis, LC separation, MS and MS/MS.
Once the cross-linked locations are assigned a distance map for the protein(s) can be created. A number of groups have used this technique to characterise the $3^\circ$ and $4^\circ$ structures of a number of proteins and complexes including scallop myosin subfragment$^1$ and the 19s proteosome particle$^2$.

Hydrogen/deuterium exchange mass spectrometry (HDX) is a probe of solvent accessibility that can be applied in the solution or gas phase. Initial studies into the reaction between deuterium oxide (D$_2$O) and organic molecules showed that certain hydrogen atoms on a structure would exchange preferably for deuterium$^3$. For heteroatoms (type I hydrogen: -OH, -SH etc) the exchange rate was shown to be rapid while hydrogens attached to carbon atoms (type III hydrogen) do not exchange.

![Figure 1.22: Schematic diagram of a peptide with the three different types of hydrogen assigned. Type I – side chain hydrogen; type II – backbone hydrogen; and type III – aliphatic hydrogen.](image)

For proteins and peptides, a third type of hydrogen exists – the backbone amide hydrogen (type II hydrogens). As with other heteroatoms, type II hydrogens exchange for deuterium rapidly. Figure 1.22 is a schematic of a segment of peptide (sequence FVN) with the three different hydrogen types labelled.
1. Introduction

Solution protein structure can be probed by exposing the protein of interest to D₂O. The exchangeable hydrogens (types I and II) will then exchange for deuterium at a rate dependent on the solvent accessibility. Hydrogens involved in hydrogen bonding interactions are somewhat protected from exchange and hydrogens in regions of the protein sequestered from the solvent in the core of the proteins can be completely protected from exchange. The rates of exchange of different hydrogens can therefore indicate how well folded a protein is. This can be monitored by MS as the exchange of one hydrogen for one deuterium results in a mass increase of approximately 1 Da.

The kinetics of the HDX process can be monitored by time course experiments where the mass of the sample is measured at different times after exposure to D₂O, the degree of exchange with time can give information about the structure of the molecule. The sites of exchange can be identified by proteolytic digest with pepsin and MS/MS experiments.

The type of experiment described above is known as direct infusion HDX, specific assignments of exchange sites and the exact amount of exchange is difficult to determine precisely by this method due to a combination of back exchange to hydrogen and deuterium scrambling (movement of the deuterium from one site to another).

Gross and co-workers have recently described a new method which probes only the exchange of backbone hydrogens. The method is called protein ligand interaction by mass spectrometry titration and hydrogen/deuterium exchange or PLIMSTEX. This method exploits two factors of H/D exchange rates. Figure 1.23 shows the exchange rate of backbone amide hydrogens at different pH and temperature. The room temperature exchange rate at pH 2.7 can be seen to be four orders of magnitude slower than that at pH 7.0, reduction of the temperature to 0 °C produces a further order of magnitude reduction in the exchange rate.
In PLIMSTEX, the protein is exposed to D₂O as before, after a chosen time an aliquot of the sample is removed and the exchange is quenched with acid and placed on ice. This then gives a longer time window (~2 hours) to analyse the sample.

### Figure 1.23: Amide backbone exchange rates and exchange half-life (t½) at different temperatures and pHs.

<table>
<thead>
<tr>
<th>kₑₓ (sec⁻¹)</th>
<th>t₁/₂ (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 7.0, 25°C</td>
<td>00:00:00.69</td>
</tr>
<tr>
<td>pH 2.7, 25°C</td>
<td>00:01:09</td>
</tr>
<tr>
<td>pH 2.7, 0°C</td>
<td>00:11:33</td>
</tr>
<tr>
<td>pH 2.7, 0°C</td>
<td>01:55:33</td>
</tr>
</tbody>
</table>

Note: The table shows the exchange rates (kₑₓ) and exchange half-lives (t₁/₂) for different pH and temperatures conditions.
Figure 1.24: Relative exchange rates of side chain and backbone hydrogens at different pHs.

Figure 1.24 shows the relative exchange rates of backbone hydrogens and side chain hydrogens (type I). It can be seen that the exchange rate of type I hydrogens is higher than that of type II hydrogens.

In PLIMSTEX the quenched sample is loaded onto a reversed phase guard column – C18 for peptides, C8 or C5 for proteins – and washed with low pH, low temperature aqueous solution, this back exchanges the side chain groups from deuterium to hydrogen but leaves the backbone hydrogen exchanged i.e. backbone exchange remains quenched.

The sample is then eluted from the column into a mass spectrometer where it is mass analysed. As the wash stage has back exchanged the side chain hydrogens but left the backbones exchanged, any mass increase is only due to backbone exchange. This is a more sensitive probe of peptide structure primarily because the data obtained are easier to analyse as only one hydrogen can exchange per residue. The dynamics of exchange are also probed more easily by this method as shorter time scales are available due to the
quenching process. In PLIMSTEX quenching can be performed almost instantly; in direct infusion HDX the shortest time scale available is approximately 1 minute. This method has been used to study calcium binding to porcine calmodulin.\(^{108}\)

HDX can also be employed in the gas phase using ion trap type mass spectrometers (quadrupole ion traps and FTICR). These experiments can be used to probe the structure of gas phase ions by exposing trapped ions to D\(_2\)O vapour. Monitoring mass increase with time can give insight into the ‘foldedness’ of the gas phase ions.\(^{109,110}\)

Controlled fragmentation employs dissociation techniques such as CID and ECD to probe the structure and stoichiometry of protein assemblies i.e. quaternary structure. A large number of groups have shown that it is possible to transfer non-covalent complexes to the gas phase intact using ESI-MS. This is possible because the interactions that hold these complexes together – ionic, hydrophobic, hydrogen bonding and van der Waals – are, with the exception of hydrophobic interactions, preserved in the gas phase if the right experimental conditions are used.\(^{111,112}\)

By employing mass spectrometry – and knowing the masses of the species studied - it is possible to determine the stoichiometry of protein complexes on mass grounds alone. However, a loss of mass accuracy has been reported when studying large complexes due to poor desolvation; the species observed contain solvent and buffer molecules along with the protein complex ions.\(^{113}\) This lack of mass accuracy can make absolute confirmation of stoichiometry difficult. This can be overcome by the use of harsher desolvation conditions (higher cone voltage etc.) to bring about the dissociation of the complex. This allows accurate analysis of the components of the entire system.\(^{104}\)

Robinson and co-workers report the use of dissociation methods to characterise the quaternary structure of complexes.\(^{114,115,116}\) In these experiments they have been able to use CID to bring about the stepwise dissociation of extremely large complexes such as intact ribosomes, antibody:antigen complexes and GroEL.\(^{117}\) The dissociation
products obtained can be used to build up a contact map for the complex and hence give information on its quaternary structure.

Charge state analysis of proteins has been used by a number of groups to estimate the solvent accessible area of a protein or protein complex.\textsuperscript{99,118,119,120} Under standard ESI conditions (49:49:2 H$_2$O:MeOH:CH$_3$COOH) the m/z values of most proteins fall on the region of 800-2500 m/z regardless of mass. Under ‘native’ conditions (e.g. 10 mM ammonium acetate) a shift to the lower charge states is often observed. Kaltashov and co-workers\textsuperscript{99} report a method whereby the solvent accessible surface of a protein can be estimated using the charge observed on the protein. Using this method it has been demonstrated that protein surface area increases with charge in a fairly consistent manner i.e. the ion unfolds with increasing charge. By comparing the surface areas calculated using this method with those observed for solved structures it is possible to gain information on the gas phase structures adopted by protein ions. The method reveals that the lower charge states observed in native ESI experiments give solvent exposed surface areas similar to that calculated for solution and crystal structures. This indicates the conformations seen under these conditions are native-like.

1.3.2 Ion Mobility Mass Spectrometry

In section 1.2 it was demonstrated that ion mobility spectrometry can be used to determine collision cross sections of ions and that, with the aid of computational techniques, gas phase structures of ions can be determined.

This application of ion mobility was pioneered by the groups of Bowers and Jarrold with their work on determining the structure of atomic clusters such as carbon clusters,\textsuperscript{121,122,123,124} silicon clusters\textsuperscript{125} and sodium chloride crystals\textsuperscript{126}. The technique has since been used to study a range of biomolecules from small peptides to large bimolecular complexes. This section will summarise the work done in this area.
1. Introduction

1.3.2.1 Cytochrome C

Cytochrome C is perhaps the biomolecule that has been most widely studied by IMMS having been studied on a variety of instrumentation by a number of authors\textsuperscript{56,78,127,128,129,130,131}. Much of the important work on this peptide was performed by Jarrold and coworkers\textsuperscript{127}. Figure 1.25 shows the arrival time distribution (ATD – a plot of ion intensity as a function of drift time) obtained for +7 cytochrome c.

\begin{figure}[h]
\centering
\includegraphics[scale=0.5]{cytochrome_c.png}
\caption{Drift time distribution recorded for the +7 charge state of bovine cytochrome c with nominal injection energy of 130 eV. Arrows show the expected drift times for a variety of cytochrome c conformations: (a) the native structure (~1100 ps); (b) a partially unfolded structure with an open haem crevice (1680ps); (c) an unfolded coil that retains the \(\alpha\)-helices (2070ics); (d) a typical random coil with no secondary or tertiary structure (~2880 ps); and (e) a near-linear conformation (~3425 ps).}
\end{figure}
1. Introduction

The ATD shows three clear peaks: a small, narrow peak at \(~1600\) μs; a more intense broad peak at \(~1800\) μs; and an intense, sharp peak at \(~2000\) μs. The figure also shows the computed drift times for a number of candidate geometries ranging from the compact native structure (a) to the near-linear conformation (b). It can be seen that none of the geometries adopted by +7 are as compact as the NMR structure; a degree of unfolding has occurred. Neither are the conformations adopted as extended as the computed near-linear structure. In other words, +7 cytochrome c retains some degree of higher order structure with the most intense peak in good agreement with computed structure c, an unfolded coil which retains the native α-helices.

Figure 1.26: Drift time distributions recorded for the +7 charge state of cytochrome c as a function of drift tube temperature. The drift time scale has been converted into a cross section scale so that distributions recorded at different temperatures can be easily compared. The dashed line shows the distribution recorded at high injection energy (2100 eV). The other distributions were recorded with low injection energy (350 eV).
The work also shows the influence of temperature on the conformations adopted by cytochrome c. Figure 1.26 shows the drift times of +7 cytochrome c at different temperatures ranging from 28 °C to 300 °C.

It can be seen that drift time, and hence collision cross section, increases with temperature indicating an unfolding with temperature. At lower temperatures, the ATD peak is broad indicating the presence of a number of unresolved conformations. At higher temperature (200 °C upwards) the peak is far sharper indicating the presence of a single conformer.

Bowers and co-workers\textsuperscript{132} have shown that by carefully measuring the temperature of the drift cell it is possible to use this type of experiment to calculate energy barriers between conformers. If two distinct conformations exist at a given temperature, it can be said that the energy barrier between the two conformations is higher than the thermal energy of the system; if the conformations then merge into one at some higher temperature it is clear that the energy barrier has been overcome. Accurate determination of the temperature at which this occurs allows the barrier height to be determined. Examples of systems studied using this technique are di- and tri-nucleotides and the trimer of PET.

Increasing the temperature of the experiments increases the thermal energy of the system and hence the energy of the ions. The energy of the ions can also be increased by raising the injection voltage. Figure 1.27 shows the drift times of +7 cytochrome c at various injection energies ranging from 175 eV to 1750 eV.
Figure 1.27: Drift time distributions recorded for the +7 charge state of bovine cytochrome c as a function of injection energy. The dashed line shows the expected distribution for native conformation cytochrome c.

The dotted line on the figure corresponds to the calculated drift time of the NMR structure. At low injection energy, the drift time of the ion is slightly less than expected for solution phase native conformation of cytochrome c. In the solution phase, the side-chains extend out into the solvent to maximise their interactions; in the gas phase,
Intramolecular interactions dominate and the side-chains collapse onto the protein surface resulting in a collapsed native conformation. As the injection energy is gradually increased, the drift time of the ions increases indicating an unfolding of the peptide due to collisional heating.

The data discussed so far have only shown the +7 charge state of cytochrome c. In section 1.3.1, it was shown that charge state analysis can be used to determine the solvent exposed surface area of a protein revealing an unfolding with charge. The data available from this data are low resolution and rather indirect. As IMS gives a direct measure of conformation, the data obtained from IMMS are of higher quality than that obtained from MS alone. Figure 1.28 shows the experimental collision cross sections obtained for +3 to +20 cytochrome c.

![Figure 1.28: Plot of the collision cross sections for the features observed in the drift time distributions for the +3 to +20 charge states of cytochrome c. The dashed lines show cross](image)
sections calculated for the native conformation and an extended string. Filled circles show cross sections obtained at high injection energy while open circles show cross sections obtained at low injection energy.

As expected, there is an increase in cross section with charge. Low charge states \((z<6)\) exist in collapsed native conformations. As the charge is increased, the structure becomes more unfolded; this is attributed to increased Coulombic repulsion (due to the charging protons) overcoming some of the intramolecular interactions which keep the peptide folded.

At high injection energy (filled circles) two conformations are observed for the +6, +7 and +8 charge states; all other charge states exhibit a single cross section. The region from +5 to +9 corresponds to the steepest increase in cross section with charge, indicating a shift of conformation from the compact native and collapsed native conformations observed at +3 to +6 to the more extended conformation seen for +9 onwards.

### 1.3.2.2 Bradykinin Dimer

If an ion is undergoing a reaction from one species to another during the course of its flight through the drift tube then, providing there is a change in cross section from A to B (where \(A \neq B\)), the kinetics of the reaction can be studied by IMS. The ensemble of reacting ions gives a cross section distribution ranging from A (no reaction) to B (early reaction). An example of such a reaction is the dissociation of a dimer.

Singly protonated peptides have been shown to have a propensity for the formation of non-covalently bound aggregates of the form \([nM + nH]^+\). The m/z of a singly protonated monomer is the same as that of a doubly protonated dimer and so on; therefore these oligomers will appear as one peak in a mass spectrum. IMS can be used to separate and study these species.
Bowers and co-workers\textsuperscript{132} used IMS-MS to study the bradykinin dimer. Figure 1.29 shows IMS data for bradykinin at different temperatures. At 441K two distinct species are present, the monomer and the dimer; at 463K the area between the peaks begins to fill-in indicating the occurrence of a reaction; at 510K the only the monomer is present. An Arrhenius plot of the data can be used to find the dimer binding energy.

Figure 1.29: Arrival time distributions measured for bradykinin at different cell temperatures. The dimer present at lower temperatures dissociares into two monomer units as the temperature is raised. (a) 441K shows singly protonated monomer and doubly protonated dimer, (b) 463K shows dissociation of dimer into two monomer units (filling in between peaks), (c) 520K shows all dimer dissociated to monomer.

1.3.2.3 GnRH

Barran and co-workers\textsuperscript{133, 134} have used a combination of IMMS, molecular modelling and ECD to demonstrate the influence of minor sequence changes on the gas phase structures adopted by the small peptide GnRH. Four different species are studied: the mammalian form; the Cional form; and two variants thereof. One of the more
interesting mutations was the substitution of L-Ala for the D enantiomer in the Ciona form. This subtle change allows the peptide to form a II$^1$-turn in a manner similar to the mammalian GnRH. This suggests the L-Ala form imparts some steric hinderance on the structure.

1.3.2.4 Amyloid β-Protein

Bernstein et al have recently used IMMS to probe the aggregation propensity of the peptide Aβ, a neuropathic agent involved in Alzheimer’s disease$^{135}$; aggregation of this peptide to form amyloid ‘plaques’ in the brain tissue is believed to be the cause of the disease. The peptide exists in various forms but is most commonly found in its 40 and 42 amino acid forms (Aβ40 and Aβ42) the latter of which has been shown to be the predominant species in the ‘plaques’.

Figure 1.30$^{135}$ shows the ATDs obtained from nano-ESI studies of Aβ42. At low injection energy Aβ42 aggregates to form hexamers and di-hexamers. At higher injection energy the hexamer can be dissociated to form tetrarmers and dimers. The hexamers are believed to form the basis of the amyloid plaques.
The authors report that the hydrophobic core of the protein is an important factor in the aggregation process. To study this, a central phenylalanine residue was substituted for a proline in order to disrupt the hydrophobic core of the peptide. The aggregation propensity of this peptide, [Pro^{19}]Aβ42, was then studied by IMMS. Figure 1.30 also shows the ATDs obtained for this peptide.

While some aggregation is evident the largest oligomer seen is a tetramer, even at low injection energy; this is consistent with the fact that [Pro^{19}]Aβ42 does not form amyloid fibrils. This underlines the assertion that the hydrophobic core of the peptide is an

Figure 1.30: Arrival time distributions for the peptides [Pro^{19}]Aβ42 (a-c) and Aβ42 (d-e) for the -5/2 charge states at the injection energies indicated. The letter designations given for the features are D = dimer, Te = tetramer, and H = hexamer.
important feature in the formation of the higher order aggregates needed to form the amyloids.

1.3.2.5 Complexes by IMMS

Some groups are now beginning to use ion mobility to study large non-covalent complexes.

Guo et al report the use of a modified triple quadrupole instrument to measure the cross sections of the metallothionein MT-2A with heavy metals and As(III) containing molecules\(^{136}\). Metallothioneins have been shown to be induced in the liver by the presence of heavy metals and have been shown to bind to Cu(I), Ag(I), Zn(II), Cd(II), Hg(II) and As(III) in the form of arsenite, monomethylarsonous acid (MMA) and dimethylarsonous acid (DMA). Through this work they were able to show that the binding of these species to MT-2A stabilises the peptide. Sequential binding of MMA to MT-2A for example shows a decrease in collision cross section of the 6+ species with increasing numbers of MMA bound. With 0 MMA bound the cross section is 1090 Å\(^2\), with 10 MMA bound the cross section is 900 Å\(^2\), a decrease of \(~18\%\). This is interpreted as a stabilisation of the structure through increased cross-linking between cysteine residues.

Joseph Loo and co-workers have used a ion mobility technique known as GEMMA (gas phase electrophoretic mobility macromolecule analyser) to characterise the 28 subunit 20s proteosome complex\(^{137}\). They first characterised the stoichiometry of the complex by ESI-MS and CID. The GEMMA experiment uses an ESI source followed by a charge neutralisation chamber (which uses \(\alpha\)-radiation to reduce the charge on the droplets produced by ESI) then a differential mobility analyser (DMA – essentially linear FAIMS) to separate the ions prior to detection. This technique gives so-called electrophoretic mobilities (EMs) for the ions which are interpreted as an EM diameter.

Figure 1.31\(^{137}\) shows the measured EM diameters for the full complex and one 7 subunit ring of the full complex along with the crystal structure of the full complex.
The EM diameters obtained for each of these species are in good agreement with the dimensions of the crystal structure suggesting a general retention of the solution phase structure into the gas phase.

Thomas et al. have reported the use of GEMMA to analyse the EM mobilities of intact viruses.

Ruotolo et al. have recently reported the use of travelling wave ion mobility to study the Trp RNA binding protein TRAP. Using MS techniques they were able to show that the protein exists as an 11-mer. The combination of molecular modelling and ion mobility allowed them to identify the structure of this oligomer to be ring like at low charge (+19, +20), this structure was then seen to collapse to a more compact oligomer at higher charge (+21, +22). Figure 1.32 shows the structures assigned different charge states of this complex at different activation energies.

**Figure 1.31**: ESI-GEMMA of the α7 and α7β7β7α7 20S proteasome complexes from *M. thermophila*. The structure (right) shows the dimensions as measured by X-ray crystallography.
1. Introduction

Figure 1.32: Cross sections obtained for the TRAP 11-mer complex at +19, +21 and +22 as a function of activation energy (175, 125, and 50 V) applied in the high-pressure, sampling-cone region of the instrument. The dashed lines (green and blue) represent the collision cross sections for the calculated collapsed and ring structures.

Woods et al report the use of a MALDI-IM-ToF-MS instrument to analyse phosphorylated and non-phosphorylated angiotensin II with acetylcholine\textsuperscript{140}. While the authors do not make any attempt to assign structures to the species seen they do report that the IMS stage allows simplification of the mass spectra seen; by looking at trends in the mobility of different species (e.g. phosphorylated vs. non-phosphorylated) it is possible to assign adduct peaks effectively increasing the sensitivity of the measurement.

1.3.2.6 Nucleotides

Bowers and co-workers have recently used IMMS to study the interactions of DNA nucleotides\textsuperscript{141,142,143}. In one study they look at the structure of deoxyguanosine complexes\textsuperscript{141}. G-rich areas of DNA have been shown to form structures known as G-quadruplexes, structures comprised of stacked G-quartets (subunits of four co-planer guanines).
In these experiments the deoxy form of guanosine, dG, was prepared in aqueous solution and allowed to anneal at 80 °C in order to allow aggregation to occur. The solutions were then analysed by nano-ESI-IMS-MS. The predominant species seen in the mass spectra produced correspond to 4 and 6 nucleotides with evidence of less abundant higher oligomers (11, 14, 16, 17 and 23 dG with differing amounts of ammonium adducts).

Analysis of the 4 dG containing peak by IMMS revealed the presence of three species: \([4\text{dG} \pm \text{NH}_4]^+\); \([8\text{dG} + 2\text{NH}_4]^{2+}\); and \([12\text{dG} + 3\text{NH}_4]^{3+}\). This again shows the ability of IMMS to identify the presence of oligomers that are unresolvable by MS alone. Molecular modelling of these three species shows the theoretical structures that correspond to the measured cross sections. The structures correspond to quadruplex structures (stacked rings with interstitial ammonium ions) as oppose to globular structures.

Analysis of the 6dG peak reveals the presence of 6, 12, 18 and 24 dG ‘under’ the peak. For 6 dG, the cross sections obtained correspond to a globular type structure. The cross sections for the 12, 18 and 24 dG ions, however, correspond to G-quadruplex type structures.

### 1.3.2.7 Analysis of Complex Mixtures

A common technique in analytical chemistry is the coupling of a separation device (e.g. HPLC) to a mass spectrometer. The separation device essentially ‘cleans up’ the mass spectrum of a complex mixture, allowing each component to be mass analysed individually. IMS can be similarly used to separate complex mixtures of molecules in the gas phase before MS analysis. This technique was first reported by Guevremont et al\(^{44}\) but it has been pioneered for biomolecules by the group of Clemmer who coupled IMS to TOF MS\(^{59}\).
1. Introduction

Clemmer and co-workers have since added an LC (or nano-LC) separation stage prior to the IMIMS instrument, thus providing an extra degree of separation\(^{67}\). The technique has been applied to a number of systems including a combinatorial library of tetrapetides\(^{145}\) and the proteome of *drosophila melanogaster*\(^{63}\).

The benefit of using IMMS over MS alone is the extra separation stage; a complex mixture of ions obtained from a protein digest, for example, can be separated by IMS yielding more simplified spectra. This is of course analogous to pre-fractionation of mixtures using liquid phase separations such as HPLC and CE. The great advantage of using a gas phase separation is the time scale of the separation. For a complex mixture of peptides HPLC separation times range from minutes to hours, IMS separation times range from \(\mu\)s to ms allowing much higher sample throughput.

IMMS is, however, limited by poor sensitivity due to the poor ion transmission often seen for IM cells. It is also limited by low peak capacity, essentially a measure of the separation power of a technique. McLean *et al*\(^{146}\) report a peak capacity of their MALDI-IM-TOF instrument to be \(5.5 \times 10^5\) as compared to \(6 \times 10^7\) for an LC-FTICR experiment; they do, however, report the peak capacity production rate of the IMMS experiment to be two orders of magnitude higher than that obtained for LCMS. The combination of HPLC with IMMS gives an increased peak capacity of \(4 \times 10^5\) but demonstrates a four orders of magnitude drop in peak capacity production rate as compared to the MALDI-IM-TOF experiment.

Much of the fundamental studies in this area have been carried out by the Russell group at Texas A&M University (TX, USA). They have demonstrated the influence of field strength\(^{147}\), solvent\(^{148}\), buffer gas\(^{149}\) and instrument design on ion separation\(^{150}\).

Tang *et al* have reported the combination of a FAIMS separation with a linear IMS separation for complex mixture analysis\(^{151}\). With this set up they report a peak capacity of \(-5 \times 10^6\), this is comparable with that reported for LC/MS by McLean *et al* but has the advantage of being a much more rapid separation.
The recently launched Synapt instrument (Waters Micromass Technologies, Manchester, UK)\textsuperscript{75} may have a significant impact in this area. The TWIG mobility device used in this instrument has very good transmission efficiency and its availability on an "off the shelf" commercial instrument will open make mobility separation available to many more research groups.

1.3.2.8 Ligand Binding Studies

It is possible to use IMS-MS to determine thermochemical parameters relating to an equilibrium providing the reactants and products have different m/z. A typical type of equilibrium studied is a ligand binding equilibrium such as ligation of metal ions and solvation of ions\textsuperscript{20}. The experiments are performed by injecting ions into a drift tube in which neutral ligand molecules are present at a known pressure. By varying the temperature of the cell and studying the abundance of reactant and product ions using MS, $\Delta H^0$ and $\Delta S^0$ values can be obtained. To ensure equilibrium has been reached, the drift voltage is varied and the ratio of product to reactant is studied, if this ratio is found to be constant then it can be said that equilibrium has been reached.

This method has been applied to the hydration of protonated arginine methyl ester $[(\text{argOMe}) + H]^+$. In the experiment, the ions are formed by ESI and injected into a cell filled with a known pressure of water (0.1-2 Torr). An equilibrium is established of the type shown in equation 1.23.

$$[(\text{argOMe}) + H]^+ + H_2O \Leftrightarrow [(\text{argOMe})H^+ \bullet H_2O$$

Equation 1.23

The product and reactant ions are analysed by MS at the exit of the drift cell. The equilibrium constant for the reaction is obtained at a number of temperatures using equation 1.24 (where $p(H_2O)$ is the water pressure). A van’t Hoff plot is then generated and used to calculate the reaction energies.
1. Introduction

\[ K_{eq} = \frac{[\text{arg OMe}H^+ \cdot (H_2O)_n]}{[\text{arg OMe}H^+ \cdot (H_2O)_{n-1}]p(H_2O)} \]  

Equation 1.24

The plot for the addition of the first water molecule to \([\text{arg OMe}+H]^+\) yields values of \(\Delta H^0 = -9\text{ kcal mol}^{-1}\) and \(\Delta S^0 = -17\text{ cal K}^{-1}\text{ mol}^{-1}\) for the reaction.

References:

14. www.nobelprize.org
1. Introduction

34 www.chm.bris.ac.uk/ms
35 J.W.S. Rayleigh, Philos. Mag., 1882, 14, 184-186
36 J.V. Iribarne and B.A. Thomson, J Chem. Phys., 1976, 64, 2287
38 M. Wilm and M. Mann, 1996, Anal. Chem., 68, 1
39 W. Paul and H.S. Steinwedel, Z. Naturforsch, 1953, 8a, 448
66 www.waters.com
67 Bahr R, Gerlich D, Teloy E, Verhandl., DPG (VI), 1969; 5, 131
1. Introduction

79 M.P. Gorkhov, USSR Inventors Certificate No. 966583, 1982
82 www.faims.com
90 E. Mack, J. Am. Chem. Soc., 1925, 47, 2468
92 http://bowers.chem.ucsb.edu/computing/index.shtml#sigma
98 I.A. Kaltshov and S.J. Eyles, in Conformation and Dynamics of Biomolecules, Wiley, Hoboken NJ, 2005
100 H. Zahn and J. Meinhofer, Makromol. Chem., 1958, 26, 126-152
104 K.F. Bonhoeffer and R. Klar, Naturwissenschaften, 1934, 22, 45
106 www.hdx.com

74
1. Introduction


119 R. Grandori, *J. Mass Spectrom.*, 2003, **38**, 11-15


144 R. Guevremont, K.W.M. Siu, J. Wang and L. Ding, 1997, **69**, 3959


2. The MoQToF – a new ion mobility mass spectrometer

Coupling of ion mobility to mass spectrometry is fast becoming the technique of choice for gas phase structure determination of molecules. The number of papers citing the use of ion mobility mass spectrometry (IMMS) in its various forms has increased more than five fold in the last ten years*. This has been partly due to the launch of commercial systems such as FAIMS which can easily be coupled to most mass spectrometers; however, most IMMS instruments – and all linear mobility IMMS devices - currently in use are home built. In line with this, we have developed a new linear ion mobility mass spectrometer known as the MoQToF (Mobility Quadrupole Time of Flight). This project has been carried out in collaboration with Dr. Robert Bateman’s group at Waters Micromass MS Technologies (Manchester, UK) and Prof. Mike Bowers’ group at The University of California, Santa Barbara (UCSB; Santa Barbara, CA, USA).

* Source: Scifinder Scholar search for “ion mobility mass spectrometry”
2. The MoQToF – a new ion mobility mass spectrometer

2.1 Design and Construction

2.1.1 Micromass QToF I

The starting point for the design of the apparatus was a Micromass QToF I mass spectrometer (Micromass, Manchester, UK). This instrument was originally launched in the late 1990s as part of the first wave of commercial tandem mass spectrometers. A schematic of the instrument is shown in figure 2.1. The apparatus has three major components: a z-spray ion source; MS 1, a quadrupole mass analyser and MS 2, a time of flight (ToF) mass analyzer. Each of these components will now be described in turn.
Figure 2.1: Micromass Q-ToF I schematic – (1) z-spray ion source; (2) first vacuum chamber containing source hexapole; (3) second vacuum chamber containing: (4) quadrupole mass analyser; (5) hexapole collision cell and (6) pre-ToF hexapole; (7) third vacuum chamber (ToF flight tube) containing: (8) pusher; (9) point detector; (10) reflectron and (11) MCP detector.

Z-spray ion source: the z-spray system was developed by Micromass in the late 1990s as an improvement to their on-axis ESI source. The basic design is shown in figure 2.2. The source consists of a sample capillary (APCI, ESI, nanospray etc.), a sample cone with a 4 mm diameter aperture orthogonal to the capillary, an extractor cone orthogonal to the first cone (located within the source block) followed by a 20 cm hexapole ion guide (located in the first vacuum chamber). The ion guide consists of two parts; the hexapole itself and a DC only ‘top hat’ lens at the end of the rods. The top hat serves two
2. The MoQToF – a new ion mobility mass spectrometer

functions namely to act as an electrostatic lens to aid extraction from the hexapole and to aid the vacuum - the top hat seals the hole between chambers, with only a 6 mm orifice between the chambers through which the ions can pass. For electrospray operation a high voltage (3-4kV for the ESI, 1-2kV for nanospray) is applied to the capillary setting up a potential difference between the capillary and the cone. Sample is passed through the capillary and ions are pulled into the mass spectrometer by this potential difference. The sample cone is held at 30-100V higher than the extractor cone; this voltage determines the kinetic energy of the molecules on entering the MS, this is one of the more important parameters when tuning for signal using this source (higher cone voltages are required for larger molecules but tend to fragment smaller molecules). The extractor is held at, or close to, the DC offset of the hexapole which is also applied to the top hat. Each of these voltages ‘float’ (i.e. rise and fall with) on top of the voltage applied to the collision cell (effectively the ground voltage for all supplies ‘upstream’ of the collision cell) and therefore the actual applied voltage varies depending on the experiment being performed.

(ii) MS 1 – quadrupole mass analyser: the quadrupole is located in the second vacuum chamber along with the hexapole collision cell.

a. The quadrupole can be used in transmission mode for normal MS experiments - where the quadrupole is set to scan across a fixed m/z range – and in mass selected range for MS/MS experiments – where the quadrupole is set to transmit a chosen m/z. In the original design of the Q-ToF I the upper limit of the quadrupole was set to 4000, however, our instrument has been modified to allow transmission of ions up to 32,000 m/z. As with the elements described above, the DC offset applied to the quadrupole floats on top of the collision cell voltage.

b. The quadrupole is followed by a short transmission hexapole leading to the hexapole collision cell. This is an 12.5 cm hexapole enclosed in aluminium to allow the pressure inside to be raised with an inert buffer gas
(usually to 10-15 PSI with Ar, He or N₂) in order to perform CID experiments without greatly affecting the background pressure of the vacuum chamber. CID experiments can be performed on a range of m/z ions by using the quadrupole in transmission mode or on a single m/z ion by using the quadrupole in mass selective mode. The ions are fragmented by increasing the collision voltage until fragmentation occurs. The collision cell is followed by a further short hexapole to focus the ions into the final vacuum chamber.

(iii) MS 2 – Time of Flight mass analyser: the final vacuum chamber is the ToF flight tube it consists of four main components: the point detector; the pusher; the reflectron and the MCP detectors. It is positioned orthogonal to the two other vacuum chambers in the vertical plane.

a. The point detector is a photomultiplier tube used as the detector for the quadrupole when in tune mode and when acquiring quadrupole spectra. It is located at the top of the flight tube, in line of sight of the ions entering from the quadrupole chamber.

b. The pulse of ions required for the ToF experiment is provided by a pusher, an electrostatic device which in this case accelerates the ions through 7.2 kV over 10 mm. The pusher is pulsed at a set frequency (dependent on the m/z range required) with each pulse starting a new ToF experiment.

c. The ToF has a v-geometry reflectron.

d. The ToF detector is a set of micro-channel plates (MCP).
2.1.2 Instrument Layout

From the literature it can be seen that an ion mobility cell can be placed in a number of positions within a mass spectrometer; before\(^1\) and after\(^2\) the mass analyser, between mass analysers on tandem MS instruments\(^3\), before\(^4\) and after\(^5\) a collision cell. The positioning of the cell, along with the types of mass analyser used will partially determine the types of experiments that can be performed on an instrument. Returning to figure 2.1 it is clear that there are two places a drift cell can be easily accommodated: (i) in place of the hexapole collision cell; or (ii) in a new vacuum chamber between the source hexapole and the quadrupole.

Each of the positions has certain advantages over the other e.g. placing the cell after the quadrupole mass analyser would allow mass selection prior to IMS separation. Ultimately it was decided that the cell should be housed in a new chamber between the source hexapole and the quadrupole. The reasons for this were manifold but included:

1. The pre-cell hexapole can be used to store ions prior to pulsing them into the IMS cell.
2. Housing the cell separately from the quadrupole (and away from the ToF) will allow the instrument to operate at near normal pressures.
3. The hexapole collision cell and hence MS/MS capability can be retained.
4. Designing a new vacuum chamber rather than modifying an existing one means the dimensions are less constrained.

Figure 2.2 shows one of the first design drawings for the apparatus. In order to ease the transition back into the original instrument, a short hexapole has been included in the chamber, this will transmit and focus the ions exiting the drift cell and also provide a vacuum seal via the top hat.
Figure 2.2: Design drawing of MoQTof including new chamber and post cell hexapole.
2. The MoQToF – a new ion mobility mass spectrometer

2.1.3 The Cell

Of the three types of ion mobility described in chapter 1, only linear ion mobility provides absolute conformational information as described by equation 1.17. Our interest in ion mobility stems from a wish to measure gas phase conformations of molecules and therefore linear ion mobility was chosen for our apparatus. As outlined previously there are two different categories within linear mobility; the low resolution mobility of Bowers\(^1\) and others and the high resolution mobility of Clemmer and co-workers\(^6\). The development of our instrument is part of a collaboration with the Bowers group at UCSB, their experience is in the design and construction of low resolution drift cells which can be temperature controlled.
2. The MoQToF – a new ion mobility mass spectrometer

Figure 2.3: Cell Drawings for MoQToF drift cell. (I) 3D section through cell; (II) section through cell viewed from side; (III) front elevation; (IV) rear elevation. Parts are labelled as follows: A – baratron connection; B – Gas in; C – Drift rings; D – Exit lens (L4); E – End cap (C2); F – Cell body (C1); G – Einzel lens (L1, L2 and L3); H – Heater terminal block; I – Mounting brackets; J – Heaters; K – Cooling line inlets; L – Feedthrough to drift rings; M – Molybdenum orifice; N – Thermocouple mounting; O – Cell screws

The cell chosen was based on the design used in the Bowers group as shown in figure 1.11. In order to incorporate the cell into the apparatus some significant redesigning of
the ion optics (see section 2.14), cell mounting and feedthroughs (power and gas supplies etc.) was required. Figure 2.3 shows the final cell design. The figure shows two cross section views of the cell (I and II) and the front and rear elevations of the cell.

The cell is made from a copper block and a copper end-cap separated by a ceramic ring giving a cell of the following dimensions: 65.5 mm x 88.9 mm x 88.9 mm (L x W x H).

The cell contains five drift rings (30.5 mm o.d. x 15.2 mm i.d. x 3.2 mm) spaced 5.2 mm apart, separated by ceramic spacers. The drift field is achieved by applying a potential difference between the cell body and the end cap; the field is kept linear using six 1 MΩ resistors connected in series along the drift rings (the first resistor between the cell body and the first drift ring, the second resistor between the first and second drift rings and so on). The cell orifices are cut into molybdenum discs (30.5 mm o.d. x 1.27mm); the orifice size can be chosen at the discretion of the user but is typically 1mm in diameter for the work shown here. The two orifices are held in place by copper retainer rings (30.5 mm o.d. x 15.2 mm i.d. x 1.6 mm).
Figure 2.4: Cell end cap and drift ring stack. A – End cap cooling lines (1/4” Swagelok VCR fitting); B – Exit lens (L4); C – Cell screw external insulator; D – End cap; E – Drift ring mounting ceramics; F – Cell screw internal insulator; G – Drift ring separating ceramics; H – Drift ring; I – Orifice retaining ring; J – Molybdenum orifice.

Figure 2.4 shows how the drift ring (H) stack is built up from the end cap (D) on a set of 6 ceramic rods (E). The figure also shows how the cell exit lens (L4 - B) mounts from the exterior of the end cap.
Figure 2.5: Cell body (C1). A – feedthrough for baratron (1/4" Swagelok VCR); B – Gas inlet (1/8" Swagelok VCR); C – Ceramic spacer ring; D – Orifice retainer ring (orifice not shown); E – Cell body; F – Ceramic mounting rods; G – Cell mounting brackets; H – Heater terminals; I – Cell cooling line feedthroughs (1/4" Swagelok VCR).

Figure 2.5 shows the cell body construction. The retainer ring (D) is used to keep the front orifice in place, once this is in place the end cap and drift ring stack can be inserted through the ceramic spacer ring (C) and located in the retainer ring (D). The end cap is then kept in position by a set of 12 hex-screws insulated from the end cap by a set of ceramics (Figure 2.X – C & F). Each ceramic heater is attached in series to the heater terminals (H) – the end cap and cell heater voltages are supplied separately.
The cell and end cap can be heated via a set of ceramic heaters. These consist of 71 mm ceramic rods threaded with Tantalum wire. The cell body contains 10 of these rods (five either side) while the end cap contains two shorter ceramic rods (50 mm). Applying a potential to the heaters causes the wire to resistively heat and hence heat the cell. The cell body and end cap can be cooled using a stream of nitrogen gas which can be passed through cooling channels in the copper. The cell temperature can be monitored via a set of three thermocouples - one on the cell body, two on the end cap.

The cell entrance lens (see section 2.1.4 for design details) is mounted on a set of ceramic rods from the front of the cell body (figure 2.X – F). A detailed drawing of the lens stack is shown in figure 2.6. The three lenses (L1 - A, L2 - B & C and L3 - D) are stainless steel discs with the following dimensions: 40.6 mm o.d. x 22.4 mm i.d. x 3.8 mm. L2 (B & C) is split into four sections to allow x-y steering of the ions – voltages are supplied separately to each lens and each pair of lenses (x and y) can be biased relative to each other using a variable resistor. The final lens (L3 - D) is tapered to a final i.d. of 7.6 mm to aid focusing into the cell.

![Figure 2.6: Cell entrance lens stack. A – L1; B – L2Y; C – L2X; D – L3; E – Ceramic spacers.](image-url)
In the Bowers instrument\textsuperscript{7}, the cell is mounted on a set of horizontal bars, employing this in our instrument would have made removal of the cell an extremely time consuming activity requiring the removal of the source and first vacuum chamber to gain access to the cell. The cell was therefore designed such that it could be inserted and removed vertically.

![Cell with mounting rods and gas feedthroughs attached. A - Plastic supports; B - Mounting rods; C - Cell body; D - End Cap; E - L4](image)

The cell is mounted on four vertical, stainless steel rods attached to brackets on each corner of the cell body (figure 2.3 - I; figure 2.5 - G) and to two plastic supports at the other end; the plastic supports are in turn attached to the top of an aluminium vacuum flange. Figure 2.7 shows the fully constructed cell with the plastic supports (A) and
mounting rods (B) attached. N.B. In the hose attachments for the various gas feedthroughs are attached.

Figure 2.8 shows the cell vacuum chamber (E & F), cell (I, J, K, L) mounted from the top flange (E) and post cell hexapole (H2 – H, TH2 – G). The chamber has an iso-160 flange to the bottom allowing the mounting of a 500 l/s Pfeiffer TMH520 turbomolecular pump (Pfeiffer Vacuum Ltd., Newport Pagnell, UK).

Figure 2.8: Cell chamber, cell and post-cell hexapole. A – Gas inlet; B – Cooling lines; C – Baratron feedthrough; D – Voltage feedthroughs; E – Cell chamber top flange; F – Cell chamber bottom flange; G – Post cell hexapole top hat (TH2); H – Post cell hexapole (H2); I – L4; J – End cap; K – Cell body; L – Einzel lens; M – Pre cell top hat (TH1); N – Cell mounting rods
The voltages applied to the cell and the pre-cell hexapole (H1) and top hat (TH1) are supplied via the voltage feedthroughs (D). The top flange contains three voltage feedthroughs; one for applying voltage to the cell components, one for supplying voltage to the heaters and one for connecting thermocouples. Figure 2.9 shows the final design drawing of the cell and cell chamber and its location in the instrument (N.B. the ToF has been removed from the drawing).

Figure 2.9: MoQToF final design drawing.
2. The MoQTof – a new ion mobility mass spectrometer

Figure 2.10 shows the MoQTof instrument schematic.

![Diagram](image)

**Figure 2.10:** The MoQTof. A – Z-spray ion source; B – H1; C – TH1; D – einzel lens; E – Drift cell; F – L4; G – H2; H – TH2; I – quadrupole vacuum chamber; J – ToF vacuum chamber

### 2.1.4 Einzel Lens Design and SIMION Modelling

In order to obtain the best possible ion transmission – and therefore sensitivity – the ions must be efficiently and tightly focussed into the cell. In order to achieve this it was necessary to design some ion optics for the front of the cell in the form of an einzel lens.

An einzel lens is a three element electrostatic lens used to focus ions without increasing their energy. The first and last lenses are held at the same potential with the central lens biased positive (acceleration) or negative (deceleration) with respect to these lenses. The degree of focussing seen is dependent on the lens geometry and the potentials applied.
The lens system was designed and tested using the electrostatic modelling program SIMION, a package developed by David Dahl; the program and its capabilities have been extensively reviewed by him\textsuperscript{9}.

The elements modelled were the pre-cell hexapole top hat (TH1), the three einzel lens components (L1, L2 and L3), the cell body and drift rings (C1) and the cell end cap (C2). The geometries of the lens elements were adjusted and optimised to give the potential arrays shown in figure 2.11 wherein the top panel shows the array used to model the drift cell and the bottom panel shows the array used to model TH1, the einzel lens and the cell entrance; the cell entrance is featured in both potential arrays so that they can be modelled separately or together.
Figure 2.11: SIMION arrays for Drift Cell and Einzel lens. A – 3D representation of drift cell; B – Cell Entrance (C1); C – Drift Rings; D – Cell Exit (C2); E – 3D representation of einzel lens; F – Pre-cell hexapole top hat (TH1); G – Lens 1 (L1); H – Lens 2 (L2); I – Lens 3 (L3); J – Cell Entrance (C2).

Figure 2.12 shows the potential energy view of the lens. The centre lens, L2, is held at a lower potential than the other lenses. This creates a potential well that focuses the ions; the degree of focussing is dependent on the potentials placed on each lens element. The black lines through the centre of the lens show the trajectories of a group of 51 parallel ions (\( m/z = 100, 50 \text{ eV}, \) starting 0.1 mm apart) passing through the cell. It is clear that
the ions are being focussed to a convergence point outside the lens array i.e. inside the drift cell.

**Figure 2.12**: Potential energy view of Einzel lens. A – TH1; B – L1; C – L2; D – L3; E – C1

The cell array was set such that the potential varies linearly across the elements from C1 to C2 with C2 at ground. Combing the two potential arrays gives the potential energy representation of the system as shown in figure 2.13. This combined potential array was used to estimate the transmission efficiency of the lens and cell. To achieve this a program was written to simulate the presence of gas in the drift cell part of the array. The program allowed for the mean free path of the ions to be varied along with the mass of the collision gas. Upon collision the ions randomise their trajectory and the trajectory line changes colour. The simulation was carried out at various mean free paths (and hence cell pressures) and the transmission efficiency was calculated by counting the number of ions passing out of the cell. Figure 2.14 shows the trajectories of a group of 51 parallel ions (100 m/z, 50 eV, starting 0.1mm apart) passing through the cell with a mean free path of \(4 \times 10^{-3}\) mm. The inset images show a magnified view of the cell exit and an end on view of the cell which shows the divergent trajectories of the ions when exiting the cell. Closer inspection of the cell exit shows that a number of ions reach the...
aperture at the cell exit but do not exit the cell. This is because they collide at or just before the exit of the cell where they are essentially in a field free region (see figure 2.13). These ions therefore stop at this point and are not transmitted. In practice this will not be an issue as there is sufficient gas flow from the cell to the vacuum chamber to push the ions out. However, it was decided that an exit lens (L4) should be included in order to accelerate the ions out of the cell into the post-cell heaxapole (H2).

Figure 2.15 shows a plot of the number of ions transmitted as a function of mean free path (for clarity the number of ions that collided with the cell, lenses and drift rings and were not transmitted is also plotted). The total number of ions included in each study was 2050 from 50 simulations of 51 ions (100 m/z, 50 eV).

As expected, the simulations show almost complete transmission at high mean free path and reducing transmission at lower mean free path (corresponding to higher pressure in the cell). This is due to collisions with the gas causing the ions to be deflected off axis away from the exit orifice. At around $1 \times 10^{-3}$ mm mean free path, there is a slight increase in transmission efficiency. This is thought to be due to collisional focussing; in
2. The MoQToF – a new ion mobility mass spectrometer

Figure 2.14: Simulation of ion paths through lens stack and cell at 4 \times 10^3 \text{ mm mean free path}

This regime, the ions are kept on axis by collision with the gas, thus increasing the number of ions transmitted, at higher pressure than this the increased number of collisions occurring reduces the influence of this effect. The mean free path of a 600 Å² ion in the cell at 5 Torr is approximately $1 \times 10^{-2} \text{ mm}$ corresponding to a transmission efficiency of \~25\%.
2. The MoQTof – a new ion mobility mass spectrometer

![Plot of Ion Transmission vs. Mean Free Path](image)

**Figure 2.15:** Plot of ion transmission as a function of mean free path obtained from SIMION modelling

It should be noted that SIMION has been reported to model collisions poorly\(^{10}\), it does not take into account effects such as diffusion which will cause the ion packet to spread out in all directions and therefore reduce ion transmission and it does not allow ions to gain energy from a collision. The transmission efficiency reported here will therefore be an over estimate and in practice the transmission efficiency of the device will be lower.

In the final design the lens L2 was split into four sections to allow x-y steering of the ions.
2.1.5 Power Supply Design

A bespoke DC power supply was designed and constructed in-house by the School of Physics Electronic Workshop. The elements that require a voltage supply are: H1, TH1, L1, L2 (four inputs), L3, C1, C2 and L4. The post-cell hexapole and top hat (H2 and TH2) are supplied via the instrument. The required voltages and their grounds are shown in the schematic below (figure 2.16).

![Schematic of Voltages](image)

**Figure 2.16:** Schematic of Voltages. Horizontal lines indicate the ground for each voltage i.e. for TH1 the ground voltage is CV (the collision voltage). Voltages indicated in blue are supplied from the instrument and controlled via the software.

As can be seen from the figure, each voltage in the supply is set with reference to the collision voltage (the voltage applied to the hexapole collision cell with respect to ground); all other voltages float on top of this value i.e. increasing the collision voltage by 5 Volts will cause all other voltages to increase by 5 Volts. Further to this L4 is referenced to the end cap voltage (C2) and all voltages to the source side of the cell body are referenced to C1. Table 2.1 shows the voltage ranges of each supply and the total voltage applied.
2. The MoQTof - a new ion mobility mass spectrometer

<table>
<thead>
<tr>
<th>Element</th>
<th>Voltage Range</th>
<th>Output Voltage</th>
</tr>
</thead>
<tbody>
<tr>
<td>CV</td>
<td>0 V to 200 V</td>
<td>CV</td>
</tr>
<tr>
<td>TH2</td>
<td>0 V to -5 V</td>
<td>CV + TH2</td>
</tr>
<tr>
<td>H2</td>
<td>0 V to -5 V</td>
<td>CV + H2</td>
</tr>
<tr>
<td>L4</td>
<td>+50 V to -50 V</td>
<td>CV + C2 + L4</td>
</tr>
<tr>
<td>C2</td>
<td>0 V to 75 V</td>
<td>CV + C2</td>
</tr>
<tr>
<td>C1</td>
<td>0 V to 200 V</td>
<td>CV + C1</td>
</tr>
<tr>
<td>L3</td>
<td>+50 V to -50 V</td>
<td>CV + C1 + L3</td>
</tr>
<tr>
<td>L2</td>
<td>0 V to -350 V</td>
<td>CV + C1 + L2</td>
</tr>
<tr>
<td>L1</td>
<td>0 V to -350 V</td>
<td>CV + C1 + L1</td>
</tr>
<tr>
<td>TH1</td>
<td>0 V to 200 V</td>
<td>CV + C1 + TH1</td>
</tr>
<tr>
<td>H1</td>
<td>0 V to 200 V</td>
<td>CV + C1 + H1</td>
</tr>
</tbody>
</table>

Table 2.1: Voltage requirements for drift cell power supply.

The power supply was constructed such that these voltages could be supplied in both negative and positive polarity. This was achieved by using two different PCB boards, one for supplying negative voltages, the other for supplying positive voltages.

Circuit diagrams for the power supply can be found in appendix 1.

2.1.5.1 Lens 2 - X-Y Steering

As discussed previously, L2 is split into four segments to allow x-y steering of ions (L2x-right, L2x-left, L2y-top and L2y-bottom). To achieve this, the voltage applied to the lens has to be split between the four segments. This is achieved as follows: the voltage on L2 is set via a resistor pot, this voltage is then applied to two circuits – one for the X segments, one for the Y segments; the voltage is then split on each pair of lenses using a variable resistor; the sum of the voltages on any pair of lens segments is equal to the value of L2 but value on each may be varied by as much as ±50 V e.g. for L2 = 50 V and L2x-right =
30 V, $L_2_{\text{v-len}} = 20$ V. By changing the voltages applied to each of these segments, the centre point of the potential can be shifted thus allowing some steering of the ions.

### 2.1.6 Pulser Design

As described in chapter 1, an ion mobility experiment requires a pulse of ions but as an electrospray source is a continuous ion source, it is necessary to trap ions somewhere in front of the cell and pulse them out of the trapping region into the cell. Returning to the instrument schematic (figure 2.10) it is clear that the ions can only be trapped in the pre-cell transfer hexapole H1. This is achieved by placing a stopping voltage on the top hat lens (TH1). To pulse ions out of the hexapole it is necessary to apply a pulsed voltage to TH1 which will allow the stopping potential to be rapidly lowered for short periods of time at a set frequency.

A pulser unit was therefore designed and built in house to allow the TH1 to be pulsed. The unit requires two inputs: TH1 from the lens power supply described above and a TTL pulse from a signal generator. The signal generator is used to define the pulse frequency and width; the amplitude of the stopping potential is controlled via the pulser unit. A circuit diagram for this unit can be found in appendix 1.

### 2.1.7 Pressure Measurement

The pressure in the drift cell is measured using a MKS Baratron attached to the cell via a $\frac{1}{4}$" Swagelok VCR fitting.

### 2.1.8 Temperature Measurement

The temperature of the cell is monitored using three K-type thermocouples, one on the cell body and two on the cell end cap. The thermocouples are read on a Omega CN1001TC thermocouple controller.
2. The MoQToF – a new ion mobility mass spectrometer

2.1.9 Gas Inlet

Figure 2.17 is a schematic of the gas inlet system used to pressurise the drift cell. The system includes a gas filter used to remove oxygen, water and hydrocarbons from the buffer gas. It has been designed for use with one or two buffer gases (e.g. He or He and H₂O).

![Diagram of Gas Inlet System](image)

**Figure 2.17**: Drift cell gas inlet system. Where T are Swagelok ¼" shut off valve and the arrows are 1/4" needle valves used to fine tune the cell pressure.

By opening and closing the correct combination of valves it is possible to accurately control the pressure in the drift cell using the needle valves. When not in use the inlet system is kept dry and clean by pumping it out using a 70 Ls⁻¹ turbomolecular pump (Pfeiffer Vacuum Ltd., Newport Pagnell, UK).

2.1.10 Cell Hexapole

The drift cell often requires some maintenance (e.g. cleaning) for which it needs to be removed from the instrument. Without the cell in position the instrument can no longer function (or even be pumped down) and lengthy maintenance will therefore leave the lab
down one instrument. To overcome this, Micromass provided a short hexapole to replace the cell. The hexapole mounts off a top vacuum flange and is supplied the same DC and RF as H2.

Figure 2.18 shows the hexapole (10 cm) and vacuum flange.

2.1.11 Quadrupole Upgrade

On a standard Q-ToF I the quadrupole has a maximum isolation m/z of 4000. While this is sufficient for most applications, it will not be suitable for large biomolecular complexes - such as those studied by Robinson and co workers\textsuperscript{11} - which can often appear at above 4000 m/z on a mass spectrum. With this in mind, the quadrupole has been modified to have a mass range of up to 32,000 m/z. This was achieved by replacing the quadrupole RF driver with one capable of lower frequency operation.
2.2 Experiment Set-up

In order to take mobility data (i.e. measure arrival times) on the MoQToF, the Masslynx software was redesigned by Waters Micromass Technologies (Manchester, UK). The new software allows for two modes of operation: (i) MS/(MS) mode - collection of mass spectra only (on point detector or MCPs); (ii) IMS mode - collection of mass spectra with mobility data (MCPs only).

2.2.1 MS Mode

The basic operation for MS mode has not changed greatly, the only significant software change has been to two of the source voltages, those applied to the extractor cone and the sample cone.

The extractor cone voltage is referenced to the collision voltage and prior to the instrument modifications it was typically held at 0 V to 5 V above the collision voltage and crucially at a higher DC voltage than H1. In the modified instrument H1 is held at several tens of volts higher than the collision voltage (typically ~110 V), the extractor must therefore be held at 0 V to 5 V higher than this value, to achieve this the software was altered allowing a greater range of voltages to be applied.

The sample cone is referenced to the extractor cone and therefore the software also required some modification to allow the desired voltage to be applied to it. The maximum voltage that can be applied to the sample cone (sample cone voltage + extractor cone voltage) is 204 V + CV, this can be prove to be a limiting factor in examining high m/z species and some further modification is still required.

MS mode is typically used when tuning for signal; the drift cell is filled with buffer gas to the desired pressure, the drift voltage and injection energy (defined as the difference
between the voltages applied to CI and HI) are set and the source voltages are tuned until the best possible signal is obtained.

**2.2.2 IMS Mode**

For mobility operation significant changes have been made to the software so it can record mobility data (arrival times) along with mass data. In ToF-MS mode the instrument measures the time taken for ions to pass from the pusher to the detector. These times are then converted to m/z values as described in chapter 1. In IMS mode each ion has an associated arrival time (the time it takes to pass through the drift cell + dead time) that must also be recorded.

In the MoQToF software the ion arrival time is the time taken to pass from THI to the pusher. This is measured as follows:

i. THI pulsed to allow packet of ions into drift cell (10 to 40 μs wide, 50 to 100 Hz). The signal generator sends a simultaneous pulse to the instrument TDC card which signals the software to start the IMS experiment.

ii. The first pulse of the pusher (push) after this start signal is scan 1, the second push is scan 2 and so on up to 200 scans. The width of each scan is equivalent to the pusher period being used.

iii. The software records a mass spectrum for each scan to build up a 200 scan total ion count (TIC) chromatogram. The 200 scans can then be summed to give a mass spectrum for the experiment.

iv. The software allows any single m/z or m/z rang to be selected. It then builds up a chromatogram for the selected m/z with a peak after n scans corresponding to that ion's arrival at the pusher.

v. Multiplying the scan number of the peak by the pusher frequency gives the arrival time.

In practice a single set of 200 scans provides insufficient data and the software is set to sum over a number of seconds (10 to 25 s) to obtain a single set of 200 scans. Further to
this it necessary to sum the data over a number of scan sets to obtain good arrival time data. For every set of 200 scans there can be only one pulse of ions into the drift cell, the frequency of the period of this pulse must therefore be greater than 200 x pusher period.

Figure 2.19 shows the pulse sequences of the pusher and IMS pulse. The upper trace shows the pulse sequence used to control the ion mobility pulse while the lower trace shows the ToF pusher pulse. The pulses in red indicate the first push after the IMS pulse i.e. the start of the experiment.

Although the figure indicates the IMS pulse and pusher pulse are synchronised, they are in fact asynchronous (the consequences of this on the measurements will be discussed later). In order for the software to start an IMS experiment, it must see an input pulse whilst it is pushing, for this reason the input pulse to the TDC card must be at least one pusher period in width (thus ensuring the input pulse and pusher pulse are concurrent).

Figure 2.20 is a schematic representation of how the data are presented in the software and how ATDs for individual ions are obtained.
Figure 2.20: Schematic representation of IMS data obtained from Masslynx. A set of TIC arrival time distributions (ATDs) is obtained; the data are combined to give a mass spectrum; from the mass spectrum individual ions are selected and a corresponding ATD is created.
2.3 From ATDs to Drift Times to Mobilities

The ATDs obtained by following the procedure outlined in section 2.2.2 give the time ions take to pass from TH1 to the pusher i.e. the drift time \( t_d \) (time spent in the cell) + dead time \( t_{\text{dead}} \) (time spent outside the cell). To calculate the drift time accurately, it is necessary to determine the dead time or manipulate the data so that the dead time is not needed to calculate the mobility. In reality, both of these conditions can be satisfied by plotting the arrival time \( t_a \) of an ion against \( P/V \) as shown by equations 2.1 and 2.2 where \( L = \) cell length, \( T_0 = 273.15 \) K, \( K_0 \) = reduced mobility, \( P_0 = 7.6 \times 10^4 \) Torr, \( T \) = cell temperature in Kelvin, \( P \) = cell pressure in Torr and \( V \) = drift voltage (the difference between \( C1 \) and \( C2 \)).

\[
\begin{align*}
t_a &= t_d + t_{\text{dead}} \\
t_d &= \frac{L^2 T_0}{K_0 P_0 T V} P
\end{align*}
\]

By substituting equation 2.2 into equation it is clear that a plot of arrival time as a function of \( P/V \) for any one ion should give a straight line of gradient inversely proportional to the reduced mobility. For any experiment the values of \( T \) and \( L \) are well known so by performing the measurement at several different \( P/V \) values, \( K_0 \) can be easily calculated from the plot. The y-intercept of this plot will give the dead time for the ion of interest.

To obtain different values of \( P/V \) it is possible to change one or both of the parameters, however, the cell pressure can take several seconds to stabilise when it is changed while the drift voltage stabilises almost instantly. It is therefore more practical to perform the measurements at a range of drift voltages with constant cell pressure. Figure 2.20 shows a plot obtained for +14 cytochrome C.
2. The MoQToF – a new ion mobility mass spectrometer

Figure 2.20: plot of arrival time vs. P/V + 14 for Cytochrome C.

2.3.1 Obtaining Good ATDs

As the software only allows the ions to be sampled once every pusher cycle the resolution of the data is relatively poor e.g. for data where the maximum m/z = 1000, the pusher period is set to 63 μs; the ions are therefore only sampled once every 63 μs. To try and counteract this low resolution, it is necessary to sum a number of distributions (n ≥ 10) and fit a Gaussian distribution to the peak in order to obtain an accurate arrival time. Summing the data in this manner also allows low abundance peaks to be better sampled.
2. The MoQToF – a new ion mobility mass spectrometer

2.4 Initial Testing

In order to make tuning the instrument as easy as possible, the cell orifices were added in stages. Initial tuning was carried out with no orifices present – using the drift cell as an ion guide only, testing was then carried out with only a 2 mm aperture diameter front orifice in place; a rear 1.67 mm aperture diameter orifice was then added and eventually both orifices were swapped for 1 mm diameter aperture orifices.

2.4.1 No Orifices

SIMION modelling of the einzel lens suggests TH1 and L1 should be held at approximately the same voltage with L2 held 50 to 90 V lower and L3 held 5 to 20 V lower; TH1, L1 and L3 are all required to be at the same or greater potential as CI. However, the construction of the power supply is such that L1 and L2 must be biased in the same polarity i.e. both positive wrt C1 or both negative wrt C1; this clearly changes the capabilities of the lens stack.

Returning to the SIMION modelling it was obvious that the best focussing was achieved with L2 biased negative wrt C1, therefore forcing L1 to be biased –ve wrt C1. In the modelling discussed above, L2 was required to be the minimum of the lens potential, however, as TH1 is required to be positive wrt C1, it seemed obvious to set L1 as the minimum with TH1 and L2/L3 as the high potential points on the lens.

A solution of NaCsI in 50/50 IPA/H2O was used to tune for ion signal on the ToF with no orifices in place. The following optimum values were obtained for each lens: H1 = 35 (75) V; TH1 = 25 (65) V; L1 = -40 (0) V; L2 = -28 (12) V; L3 = 28 (68) V; C1 = 40 (40) V; C2 = 20 (20) V; L4 = 0 (20) V; H2 = TH2 = CV = 20 (18.9) V. Where the values in parentheses indicate the applied voltage relative to ground.
The values applied to H1, L2, C1, C2 and L4 are less crucial those applied on TH1, L1 and L2 but they must meet the following criteria: H1 > TH1; H1 > L3; C1 > L2 > L1; C1 > C2; C2 > L4 > H2.

With the values indicated above, the ion transmission efficiency was approximately 75% (as compared with ion transmission with the cell hexapole in place).

2.4.2 Front Orifice 2 mm Aperture

The tuning experiment was repeated with the front molybdenum orifice in place with a 2 mm aperture.

The presence of this orifice requires a much tighter focus for the ions to enter the cell. This is achieved by varying the potentials applied to the einzel lens. Figure 2.21 shows a plot of (normalised) ion intensity at different values of L1, L2 and L3 with all other voltages held constant (H1 = 37 V, TH1 = 30 V, C1 = 35 V, C2 = 20 V, L4 = 0 V, H2 = TH2 = CV = 20 V).

From the figure it is immediately apparent that there a number of peaks in the ion intensity, the maxima generally occur for L2 = L1 + 20V. Looking left to right along the graph, the amplitude of the peaks increases up to L1 = -50 V then decreases (marginally) again. Looking in the area of experiments 40 to 60, the value of L3 is altered significantly here. The ion intensity is seen to be fairly insensitive to this lens until L3 > H1 where the ion intensity drops off entirely only returning at L3 = 30 V = TH1. The greatest ion intensity is achieved for 30 V ≥ L3 ≥ -40 V (for TH1 = 30V).

This work therefore provides two more tuning criteria, namely L2 ≈ L1 + 20V and L3 ≤ TH1.

The transmission efficiency in this configuration was approximately 60%.
2.4.3 Front Orifice 2 mm Aperture, Rear Orifice 1.67 mm Aperture

The addition of a rear orifice allows gas to be added to the cell; with these apertures in place the maximum achievable cell pressure (i.e. the highest pressure possible without straining the pumps) was 1.75 Torr. The tuning experiment was therefore repeated with 0.5 Torr He in the drift cell.

To obtain good ion transmission, a drift voltage \((C1 - C2)\) of 40 V was required with \(H1 \geq 15\) V. Tuning the einzel lens, maximum signal was achieved at \(L1 = -70\) V, \(L2 = -55\) V, \(L3 = 15\) V.
The presence of two orifices and gas in the cell greatly reduces the transmission efficiency of the instrument to approximately ~10% at 40 V across the cell.

### 2.4.4 Front and Rear Orifices 1mm Apertures

The two larger orifices were then replaced with two 1mm aperture orifices. In this configuration it is possible to maintain good pressure in the rest of the instrument (< 10⁻⁴ mBar in the quadrupole chamber, < 10⁻⁶ mBar in ToF chamber) with up to 4.5 Torr of He in the drift cell. In practice, however, good ion transmission was only possible at ≤3.5 Torr.

Very little tuning of the lens voltages was required to obtain good transmission efficiency in this configuration. At 3.5 Torr, the drift voltage was required to be ≥ 20 V with H1 ≥ 20 V. Transmission was greatly increased by setting L4 = -2 to -5 V (ensuring L4 > H2).

<table>
<thead>
<tr>
<th>Element</th>
<th>Optimum Voltage</th>
<th>Typical Voltage @ V₄ = 60 V, IE = 30 V</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1</td>
<td>≥ C1 + 20 V</td>
<td>30 (105) V</td>
</tr>
<tr>
<td>TH1</td>
<td>H1 - 10 V</td>
<td>20 (95) V</td>
</tr>
<tr>
<td>L1</td>
<td>C1 - 75 V</td>
<td>-75 (0) V</td>
</tr>
<tr>
<td>L2</td>
<td>L1 + 20 V</td>
<td>-60 (15) V</td>
</tr>
<tr>
<td>L3</td>
<td>C1 + 15 V</td>
<td>15 (90) V</td>
</tr>
<tr>
<td>C1</td>
<td>≥ C2 + 20 V</td>
<td>65 (75) V</td>
</tr>
<tr>
<td>C2</td>
<td>≤ C1 - 20 V</td>
<td>5 (15) V</td>
</tr>
<tr>
<td>L4</td>
<td>C2 - 4 V</td>
<td>-4 (11) V</td>
</tr>
<tr>
<td>H2</td>
<td>CV</td>
<td>10 (10) V</td>
</tr>
<tr>
<td>TH2</td>
<td>CV</td>
<td>10 (10) V</td>
</tr>
<tr>
<td>CV</td>
<td>CV</td>
<td>10 (10) V</td>
</tr>
</tbody>
</table>

Table 2.2: Optimum voltages for MoQToF with cell at 3.5 Torr
2. The MoQToF – a new ion mobility mass spectrometer

Table 2.2 lists optimum voltages for good transmission efficiency in this configuration with 3.5 Torr of He in the drift cell. Once more, values in parentheses indicate the applied voltage.
2. The MoQToF – a new ion mobility mass spectrometer

2.5 Mobility Experiments on Standard Compounds

With the instrument tuned as described above, a set of test experiments were carried out to check the accuracy and consistency of mobility measurements made using the MoQToF and use for calibration if necessary.

The experimental cross sections of the proteins Cytochrome C\textsuperscript{12}, Ubiquitin\textsuperscript{13} and Lysozyme\textsuperscript{14} have been widely reported in the literature. As these compounds are cheap and easily available they were ideal test/calibration compounds.

2.5.1 Cytochrome C

Cytochrome C is probably the protein most widely studied by IMS (see section 1.3.2.1). It has been widely used as a test compound for new instruments\textsuperscript{15,16} and as a calibrant molecule for some mobility measurements\textsuperscript{17,18}. It is a 104 residue haem-containing protein of mass 12,360 Da.
Figure 2.22: MoQToF ESI mass spectrum of Cytochrome C from 49:50:1 MeOH:H₂O:CH₃COOH

Figure 2.22 shows a mass spectrum of 40 μM Equine Cytochrome C (Sigma Aldrich, Milwaukee, WI) flown from 49:50:1 MeOH:H₂O:CH₃COOH with p = 3.5 Torr, T = 305 K, Injection Voltage = 35.6 V. Measurements were also taken for cytochrome C flown from 30:70 MeOH:H₂O at the same pressure in order to sample higher charge states. Measurements were taken at V_d = 60, 55, 50, 45, 40, 35 and 30 V with 60 μs and 72 μs pusher frequency.

Arrival times for each ion at each V_d were determined and mobilities were calculated as described above. Figure 2.23 shows a plot of arrival time as a function of P/V for the +7 charge state. Note the excellent R² value for the best fit line. The least squares fit error in the slope of the line is 0.4%
This value gives a mobility of 2.926 cm$^2$ V$^{-1}$s$^{-1}$ which translates to a cross section of 1268 Å$^2$. The cross section of this ion reported by Shelimov et al.$^{12}$ is 1247 Å$^2$ in good agreement with the value measured here. Table 2.3 lists measured cross sections of Cytochrome C versus literature values from the same work.
Table 2.3: Table of measured cross sections and literature values from Shelimov et al for equine Cytochrome C. Reported errors are based on a 3% standard error.

<table>
<thead>
<tr>
<th>Charge State</th>
<th>Measured Cross Section (Å²)</th>
<th>Literature Cross Section (Å²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>1268 ± 38</td>
<td>1247</td>
</tr>
<tr>
<td>8</td>
<td>1855 ± 56</td>
<td>1845</td>
</tr>
<tr>
<td>9</td>
<td>1927 ± 58</td>
<td>1964</td>
</tr>
<tr>
<td>10</td>
<td>2223 ± 67</td>
<td>2226</td>
</tr>
<tr>
<td>11</td>
<td>2182 ± 65</td>
<td>2303</td>
</tr>
<tr>
<td>12</td>
<td>2298 ± 69</td>
<td>2335</td>
</tr>
<tr>
<td>13</td>
<td>2350 ± 70</td>
<td>2391</td>
</tr>
<tr>
<td>14</td>
<td>2486 ± 75</td>
<td>2473</td>
</tr>
<tr>
<td>15</td>
<td>2613 ± 78</td>
<td>2579</td>
</tr>
<tr>
<td>16</td>
<td>2647 ± 79</td>
<td>2679</td>
</tr>
<tr>
<td>17</td>
<td>2782 ± 83</td>
<td>2723</td>
</tr>
</tbody>
</table>

It can be seen that there is excellent agreement between the cross sections measured here; all measure values are within 6% of those reported in the literature and most are within 2%. The large change in cross section between +7 and +8 indicates an unfolding event brought about by coulombic repulsion, the position of this is consistent with studies of Cyt C reported elsewhere.

Shelimov et al\textsuperscript{12} and others\textsuperscript{19} report the presence of multiple resolvable conformations for the +5 to +8 ions of Cyt C. The data obtained here shows only single resolvable conformers for each charge state. There was, however, some evidence of a more extended conformation for the +8 charge state of Cyt C.

Figure 2.24 shows the ATD obtained for this charge state at $V_d = 60$ V. The shoulder on the peak in this distribution represents a lower mobility conformation than the main peak. Unfortunately, this more unfolded species was only observable at this drift voltage; it was
of too low intensity at all other voltages to obtain accurate arrival times. The mobility of this species cannot therefore be determined via the previously outlined method. However, as the more compact species was observable at all drift voltages, the experiment dead time for this charge state was able to be determined accurately. It is reasonable to assume that the dead time for any one charge state should be independent of collision cross section and therefore the drift time for the more extended species at $V_d = 60$ V could be determined. The mobility – and hence collision cross section - of this species can therefore be determined without the need for measurement at multiple drift voltages.

![ATD of +8 Cyt C](image)

**Figure 2.24:** Arrival time distribution of Cyt C +8 at $V_d = 60$ V. The arrival times of the two species (obtained from Gaussian fitting in Origin) are indicated.
2. The MoQToF – a new ion mobility mass spectrometer

The arrival time of the lower mobility species is 1504 μs and the dead time for the +8 species was determined to be 654 μs. This gives a drift time of 850 μs for this species. Using equation 1.8 the mobility was therefore determined to be 1.977 cm²V⁻¹s⁻¹ (c.f. 2.285 cm²V⁻¹s⁻¹ for the more abundant conformer) corresponding to a collision cross section of 2144 Å². This compares well – within 5% - with the cross section of 2061 Å² reported by Shelimov et al. This agreement is especially pleasing as the mobility was only obtained using one arrival time, this suggests the assumption made about the dead time is indeed a valid one.

2.5.2 Ubiquitin

Ubiquitin is a small protein (76 residues, 8564 Da) found in all eukaryotic cells. It has been widely studied by IM-MS.

Mobility measurements were taken on 20 μM Ubiquitin (Sigma Aldrich, Milwaukee, WI) flown from 49:50:1 H₂O:MeOH:CH₃COOH with 3.5 Torr He in the cell at 305 K. Measurements were taken at V_d = 60 V, 50 V, 40 V, 35 V and 30 V with 30 V injection voltage and 65 μs pusher frequency. Figure 2.25 is a plot of measured cross section as a function of charge state for Ubiquitin (plotted in black) along with literature values for Ubiquitin from Valentine et al.¹³ and Li et al.²⁰.
Once more there is excellent agreement between the cross sections measured on the MoQToF and those measured by others (all values are within 3% of the literature values).

### 2.5.3 Lysozyme

Lysozyme is a 129 amino acid protein (~14 kDa) which contains 4 intermolecular disulphide linkages. The presence of these disulphide bonds has been shown to have a stabilising effect on the gas phase conformations adopted by the protein. Here it will be studied in its fully disulphide intact (oxidised) form.

Figure 2.26 shows a mass spectrum of chicken egg white lysozyme (Sigma Aldrich, Milwaukee, WI) flown from 49:59:1 H₂O:MeOH:CH₃COOH with the 3.5 T He in the
drift cell at 305 K. Mobility measurements were taken at $V_d = 60$ V, 50 V, 40 V and 30 V with 30 V injection energy with 87 μs pusher period.

Collision cross sections were determined as described previously. Figure 2.27 shows a plot of cross sections measured in this work for the observed charge states (+5 to +10). Once more only single conformations were observed for each charge state.

Valentine et al.\textsuperscript{14}, however, report the presence of a number of conformations for +7 to +11. They categorise these conformations as either highly-folded, partially-unfolded or unfolded (with the latter category only evident for fully disulphide reduced lysozyme); the +5 and +6 charge states of lysozyme exist as only single conformers categorised as highly-folded. The cross-sections observed in this work for the three lowest charged species (+5, +6 and +7) agree well with the values from Valentine et al. assigned as highly folded. The cross sections measured here for +8, +9 and +10 charge states agree
2. The MoQToF – a new ion mobility mass spectrometer

well with the reported values for partially-folded lysozyme. The conformational change can be seen clearly on figure 2.28.

![Lysozyme Cross Sections](image)

**Figure 2.28:** Collision cross sections of lysozyme at different charge states.

It is worth noting that Valentine *et al.* only report the partially-unfolded species at high injection voltage (120 V) and not at the 30 V used here. The reason the partially-unfolded conformation is sampled here is not clear but it may be due to RF heating of the ions whilst they are being stored in the pre-cell hexapole.
2.6 Issues Affecting Operation of MoQTof

While it is clear that the instrument is performing well and is capable of reproducing measurements made by other groups, there are still a number of issues effecting the operation of the apparatus that need to be overcome. In this section these problems will be discussed and some solutions suggested.

2.6.1 Source Issues

During the course of making the initial measurements it became clear that the characteristics of the source had changed somewhat since with the addition of the drift cell. Prior to the modification it was relatively simple to obtain mass spectra containing high m/z species flown from ‘native’ conditions (e.g. 10 mM ammonium acetate) and non-native conditions (methanol/water/acid). Since the upgrade, however, observing high m/z species has proved to be extremely difficult and further to this it has been extremely difficult to observe any signal for proteins flown from ‘native’ conditions.

While there have been no physical changes to the source itself, there has been a change to the voltages applied in the source area. The total voltage that may be applied to the sample cone (i.e. collision voltage + extractor voltage + cone voltage) is 204 V + collision voltage. In the original configuration the extractor was typically set to 0 V giving a maximum cone voltage of 204 V. For most experiments in the new configuration the extractor is set to 117 V, this gives a maximum cone voltage of 87 V.

The cone voltage effectively determines the kinetic energy given to the ions as they are transferred from the source to the hexapole. High m/z ions require more kinetic energy than low m/z ions in order to be transmitted efficiently from the source to the hexapole. High cone voltage therefore aids the transmission of high m/z ions. However, high cone voltage tends to bring about in-source fragmentation of ions and can therefore lead to a
2. The MoQToF – a new ion mobility mass spectrometer

drop in overall ion intensity, especially for higher charged, low m/z ions. The cone voltage can therefore be viewed as an m/z filter that can be tuned to give the desired ion distribution.

It is clear therefore that the limiting of the cone voltage brought about by the modifications to the apparatus, has led to reduced transmission of high m/z ions by reducing the tuning range of the instrument. This will be overcome by providing the cone voltage externally using a stabilised power supply.

The poor performance of the ion source with regard to proteins analysed from ‘native’ solutions is less easy to explain. The cone voltage limiting may have some effect this - native ESI tends to produce lower charged species than non-native ESI it therefore follows that the ion intensity will be lower for some ‘native’ experiments - but the source conditions are thought to be more relevant.

The presence of gas in the drift cell causes the pressure in the instrument to increase. With 3.5 Torr He in the cell, the pressure in the cell chamber typically increases by 3 orders of magnitude (10^{-6} mBar to 10^{-3} mBar), the quadrupole chamber pressure increases by an order of magnitude (10^{-6} to 10^{-5}) and the temperature in the ToF chamber increases by approximately three times (2 x 10^{-7} to 6 x 10^{-7}). While there is no simple way of measuring the pressure in the source hexapole chamber and source it follows that there will be an increase in the pressure in these regions when gas is introduced to the drift cell.

It has been shown that having increased pressure in a hexapole can improve ion transmission. Indeed the groups of Robinson^{21} and Heck^{22} have shown that introducing argon to the source hexapole can greatly improve the performance of a Q-ToF with regard to the transmission of large, high m/z biomolecules and complexes. They do, however, have to carefully control the pressure in the source chamber and source block (containing the source and sample cones) to achieve good ion signal. It is our belief that the increased pressure in these regions is suppressing the ion signal and that this is
2. The MoQToF – a new ion mobility mass spectrometer

particularly apparent for ‘native’ ESI due to desolvation effects - aqueous solvent being less volatile than organic solvent.

To overcome this it is proposed that additional pumping be provided to the source in the form of a higher capacity rotary pump and that the source pressure should be monitored and optimised for different solvents.

It is hoped that by making these changes, the instrument will be capable of analysing higher m/z ions and molecules flown from more native conditions.

2.6.2 Software Issues

As stated previously, the software used for data collection and analysis is a modified version of Masslynx. While the software has some unique elements, it was originally designed to operate an instrument fitted with a travelling wave ion mobility (t-wave) device and is therefore optimised for this type of system. Some modifications to the software and/or electronics are required in order for to optimise it for the MoQToF.

2.6.2.1 Data Resolution

Typical drift times for a t-wave experiment are of the order of 3 to 10 ms whereas drift times for a MoQToF experiment are of the order of 1 to 3 ms. Additionally, ATDs obtained from the MoQToF are far narrower than those obtained from a t-wave device. Figure 2.29 shows typical ATDs for +8 Ubiquitin obtained from the MoQToF (blue trace) and a t-wave instrument (red line).

The trace from the MoQToF has a centre at ~1240 μs with a full width of ~235 μs while the trace from the t-wave has a centre at ~2735 μs with a width of ~2050 μs - nearly nine times as wide. Both measurements were taken using a pusher period of 65 μs thus the MoQToF peak is approx 4 scans wide while the t-wave peak is approx. 31 scans wide.
2. The MoQToF – a new ion mobility mass spectrometer

Essentially this means ATDs from the MoQToF are sampled far less than t-wave ATDs and are therefore the MoQToF data are of lower resolution (in a data sense – from here on the number of times a peak is sampled will be referred to as data resolution).

ATDs of +8 Ubiquitin on MoQToF and T-Wave

![Graph showing ATDs for +8 ubiquitin from the MoQToF (blue line) and a t-wave instrument (red line).](image)

Figure 2.29: ATDs for +8 ubiquitin from the MoQToF (blue line) and a t-wave instrument (red line). Ion intensities were normalised to the top of each peak.

For species of containing a single conformation the lack of data resolution has not proved to be an issue in assigning cross sections. However, for species with multiple conformations – especially those with similar cross sections – the lack of data resolution may prove problematic. Returning to figure 2.24, the two peaks are not completely resolved and accurate assignment of the arrival time of the second peak proved difficult. If the data resolution had been higher (i.e. the peak was sampled more often) it may have been possible to fully resolve this peak.
To improve the data resolution, there must be a change to the way it is acquired. In the current set up the ion distribution is sampled once every time the pusher is pulsed. The frequency of the push is determined by mass range being studied – the pusher period is set to be slightly longer than the flight time of the highest m/z being studied. If the pusher period is set to a shorter time than this the pusher pulse appears as a set of noise spikes in the mass spectrum that swamp the ion signal. Changing the pusher frequency is therefore not an option for improving data resolution.

The solution to improving the data requires the ability to delay the ion mobility pulse relative to the pusher pulse or vice versa. This can be achieved in two ways:

i. **Delaying the pusher pulse relative to the ion mobility pulse:** this requires taking control of the pusher away from the instrument software and synchronising it with the IMS pulse. The leading edge of the IMS pulse will feed a simultaneous trigger pulse to the pusher circuit triggering the first pulse in the set of 200 required to make up a mobility experiment. The circuit will be set up such that it the trigger pulse can be delayed by some time (e.g. 5 μs) thus effectively sampling the mobility separated peaks that time later. The entire mobility peak can then be sampled by sequentially increasing the delay. The complete ATD can then be formed by summing all the data collected (including the relevant delays).

ii. **Delaying the mobility pulse relative to the pusher:** this involves triggering the mobility pulse with the pusher pulse. A circuit will be set up that includes a digital counter to count the pusher pulses, after a set number of pulses (>200) the circuit will send a trigger pulse to the IMS pulse signal generator, triggering the mobility pulse. The circuit will be set such that the IMS pulse can be delayed relative to the trigger pulse allowing the ions to be sampled in much the same way as described above.
Of these two possibilities the latter solution is the simplest and safest to implement as it does not require major changes to the instrument circuitry. Appendix 1 shows a circuit diagram for the proposed new pulse set-up based on scheme ii.

2.6.2.2 Pulse Synchronisation

In section 2.2.2, the pulsing sequence used in a typical IMS experiment is outlined. Within this it is made clear that the pusher pulse and the IMS pulse are asynchronous.

The pusher pulse is controlled by the instrument and when an acquisition begins, the ToF pushes at the defined frequency. While the frequency of the IMS pulse is determined by the pusher frequency (i.e. \( T_{\text{IMS}} = T_{\text{push}} \times 200 \)) it is not triggered by it and therefore is likely to be offset relative to the pusher pulse. This means the start time of the ion mobility pulse is unlikely to coincide with a pusher pulse making the start time ambiguous (but known within one push).

In order to average out this error, all ATDs in this work are created by summing a minimum of ten individual ion mobility experiments. As can be seen above, this approach has been shown to be a valid one. It is, however, not an ideal situation and it would obviously be better to know the start time of the ions accurately.

Fortunately, the circuit described above to allow increased data resolution uses the pusher pulse to trigger the ion mobility pulse. Using this circuit will therefore allow us to accurately determine the start time of the ions.

2.6.2.3 Data Acquisition

The data sets created by Masslynx operating in IMS mode are extremely large, often as much as ten times larger than an equivalent ToF MS only data set. This is because each IMS file contains an extra dimension of data – namely arrival time – for each scan.
These large data sets require a lot of processing power when they are transferred to the acquisition computer from the TDC on the embedded PC. The computer has 1 Gb of RAM but even this relatively large processor is slowed considerably by an long IMS acquisition.

Currently experiments are limited to twenty minutes in duration with a maximum m/z range of 1000. For experiments requiring longer acquisitions or a wider m/z range it is necessary to create a number of files. While this is not a major problem when running standard IMS experiments, it does present a problem for planned experiments using LC-IMS-MS (c.f. Clemmer) where long run times will be required.

One possible solution to this problem is reducing the size of the data sets created. In the current set-up an IMS experiment is 200 scans. Typically all ions of interest have reached the detector within 40 scans, there are therefore 160 scans per experiment that are not needed. The software will therefore be modified to allow the number of scans per experiment to be reduced thus creating smaller data sets.

An obvious practical solution to this problem in the interim is the addition of extra RAM to the acquisition computer. To this end it will be upgraded to 2 Gb RAM.

### 2.6.2.4 Data Processing

An obvious bottle neck in the experimental method is the transferring of data from the Masslynx files into a format which allows the extraction of arrival times. Currently this involves the creation of several ASCII files of ATD data (one per ion of interest, per data file) followed by extraction of data taken under the same conditions. This data are then summed, plotted and fitted to obtain arrival times. The arrival times are then plotted and a mobility is calculated. This process takes around twenty minutes per ion (longer when multiple raw data files are required).
To attempt to speed this process up some Excel data templates have been created, these, however, require a degree of editing for every data set and do not save much time.

Ultimately it is hoped that Waters will make their ion mobility viewer software available. This software is designed to work with Masslynx files and creates multi-dimensional plots of arrival time, mass, ion intensity and retention time. This software should make data visualisation and processing much easier.
2.7 Conclusions

The MoQToF is a unique instrument that represents the first reported integration of a linear ion mobility device into a commercial mass spectrometer with no loss in the original functionality. As an IMS-MS-MS instrument it has the capability of gaining complimentary structural information from ion mobility, mass spectrometry and tandem mass spectrometry.

Through experiments on a number of test compounds – ubiquittin, cytochrome C and lysozyme – the instrument has been shown to produce ion mobility measurements (i.e. cross sections) comparable with those published by other groups.

Among the more appealing aspects of modifying a commercial instrument is the prior knowledge of its performance one has. The Micromass Q ToF I mass spectrometer has been shown previously by us and others to be extremely useful for the study of proteins under ‘native’ conditions. Whilst this has not proved an easy task to date the potential exists – thanks in part to the modification of the quadrupole – to study some very large complexes using this instrument.

The change from the commercial instrument to the modified ion mobility instrument has brought about many changes not only in the physical instrument but in its performance as a mass spectrometer. Table 2.4 below summarises the performance of the instrument before and after the modifications.
Table 2.4: Instrument specifications before and after modifications. *as compared to ion transmission prior to upgrade; **calculated for +8 cytochrome C at $V_d = 60$ V, $P = 3.5$ T, $T = 305$ K; ***cooling lines require repair before low temperature experiments can be carried out.
References:


3. β-Defensins: Insight from Mass Spectrometry

This chapter will focus on the use of mass spectrometry based techniques to study a category of anti-microbial peptides known as β-defensins.

To protect themselves from infection by micro-organisms, mammals have developed an extremely complex defence mechanism with several levels of defence. The first mechanism of defence against infection is the innate immune response – a non-specific response to infection. An important part of innate immunity is represented by a broad category of peptides known as anti-microbial peptides (AMPs) and an important sub-category of AMPs are defensins.

Defensins are found in a wide range of organisms including humans, mice, plants and penguins. They are small (~30-45 amino acids), cationic and cysteine rich. Broadly speaking, they are active against a wide variety of pathogens: gram-negative and gram-positive bacteria as well as fungi. Defensins are also reported to exhibit chemotactic activity i.e. they can attract or activate specific blood cells such as T-cells across a concentration gradient by acting on specific receptors. Human β-defensin 3 (HBD-3) for example has been shown to attract immature dendritic cells by acting through the CCR6 chemokine receptor.

The genes that express defensins are largely found on chromosome 8. In mammals, they are expressed in a number of tissues but are most abundant in the epithelial tissues of various organs.

One of the more interesting features of defensins is their lack of sequence homology across the series apart from the presence of (typically) six cysteine residues. This variability of sequence is thought to be due to the huge evolutionary pressure applied to the peptides by the constantly changing nature of the pathogens from which the organisms must be protected.
Mammalian defensins are divided into two categories, \( \alpha \)-defensins and \( \beta \)-defensins, based on their gene location, sites of expression and the connectivities of the intra-molecular disulphide bonds formed between the six, conserved cysteine residues. These ‘canonical’ disulphide connectivities are shown in figure 3.1. It is worth noting that these connectivities hold for all defensins that have been isolated from tissue thus far but it has not been proved that all defensins conform to this pattern.

\[
\begin{align*}
\text{\( \alpha \)-defensins:} & \quad \cdots \text{Cys}_1 \cdots \text{Cys}_2 \cdots \text{Cys}_3 \cdots \text{Cys}_4 \cdots \text{Cys}_5 \cdots \text{Cys}_6 \cdots \\
\text{\( \beta \)-defensins:} & \quad \cdots \text{Cys}_1 \cdots \text{Cys}_2 \cdots \text{Cys}_3 \cdots \text{Cys}_4 \cdots \text{Cys}_5 \cdots \text{Cys}_6 \cdots 
\end{align*}
\]

**Figure 3.1:** Canonical disulphide connectivity of \( \alpha \) and \( \beta \)-defensins.

The exact purpose of these disulphide bonds and their topology is not clear. Several groups have shown that removing the disulphide bonds by reduction has little or no effect on activity.\(^6\,8\,9\) Lu has shown that changing the topology of the disulphides in HBD3 has no effect on anti-microbial activity but does have an effect on the ability of the peptide to chemoattract in that only canonically bonded HBD-3 exhibits chemotactic properties.\(^8\)

Klüver *et al.*\(^6\) conducted a systematic study of HBD-3 by synthesising a number of variants of the peptide with different disulphide connectivities, sequence length, net charge and hydrophobicity. They showed that varying the disulphide topology of the full length peptide or removing the disulphides altogether had little effect on anti-microbial activity. It was also demonstrated that small fragments of HBD-3 can exhibit antimicrobial activity.

From the work on varying sequence length, hydrophobicity and charge, the authors demonstrate a qualitative relationship between hydrophobicity, charge and anti-microbial activity from which they were able to categorise the peptides into three groups: (i) peptides with low net-charge and moderate hydrophobicity are active anti-microbial agents; (ii) peptides with low hydrophobicity and positive net-charge are anti-microbially
active and cytotoxic to eukaryotic cells; (iii) peptides with high charge and low hydrophobicity are anti-microbial agents that exhibit no cytotoxic effects on eukaryotic cells.

The mechanism of anti-microbial activity of defensins is not well understood but several studies indicate that the defensins molecules bind to negatively charged cytoplasmic membranes and disrupt their integrity somehow bringing about leakage of intercellular components and inhibition of DNA, RNA and protein synthesis\textsuperscript{2,10,11,12}.

The interaction between the peptides and membranes is not clear; two models have been suggested\textsuperscript{10}:

i. Pore (Barrel Stave) Model – in this model, the peptides form oligomeric pores that span the membrane allowing leakage. This model is supported by the structural studies of defensin which show the presence of oligomers.

ii. Carpet Model – in this model, the defensins bind to negative components on the cell membrane neutralising them. This neutralisation disrupts the integrity of the membrane leading and allow it to be permeabilised.

Figure 3.2 is a pictorial representation of the two models.
Figure 3.2: The carpet and barrel stave (pore) models of membrane permeation. A – peptide accumulates at the cell surface and binds to negative membrane components; B – the peptide neutralises the areas of the membrane destabilising it; C – the membrane disintegrates; A’ – peptide oligomers form on the membrane surface; B’ – the oligomer(s) insert into the membrane forming a pore.

High resolution structural characterisation of defensins has been performed using both XRC\textsuperscript{13,14} and NMR\textsuperscript{15,16,17}. These studies have identified a common ‘defensin-fold’ based on the structure of human α-defensin-3\textsuperscript{18}. The fold consists of a core of three anti-parallel β-sheets constrained by the intra-molecular disulphide bonds. β-defensins additionally exhibit an α-helix region towards the N-terminus. Figure 3.3\textsuperscript{15} shows the NMR structure of HBD-2 with the β-sheet core (blue arrows) and N-terminal alpha-helix (red helix); disulphide bonds are shown in yellow.
Recent work at Edinburgh has focussed on the characterisation of a number of \( \beta \)-defensins from both humans and mice\(^6,19,20\). This work takes many forms and is part of a large collaboration between several groups from the School of Chemistry, the Medical School and the Medical Research Council and externally Cambridge University and University College London. Through this work we endeavour to characterise defensins structurally (via NMR, MS and IMMS), learn about their biological activity (by antimicrobial testing and chemotaxis assays), learn how to produce and extract peptides (through various methods) and attempt to discover how they function \textit{in vitro}.

In our previous work\(^{19,20}\), mass spectrometry has been the core technique for structural characterisation of \( \beta \)-defensins. This work aims to extend that work to include mass spectrometry-based mode-of-action studies.
The peptides featured in this work were all produced by solid phase synthesis techniques; the sequences were obtained for genome mining to identify β-defensin sequences\(^7\). Further to this a number of rationally designed peptides have been synthesised in order to aid our understanding of the importance of sequence, disulphide bonding and dimerisation. The sequences and anti-microbial activities of the peptides studied here can be found in appendix 3.
3.1 Accurate Mass of β-defensins

Upon obtaining a new defensin, it is important to confirm the peptide has been produced correctly and contains the correct number of disulphide bonds. To do this high-resolution, accurate mass measurements have been performed on all peptides using a 9.4T ESI-FTICR-MS (Bruker Daltonics, Billerica). Combining these measurements with isotope modelling allows for precise determination of the oxidation state of each cysteine residue (and hence determination of the number of disulphide bonds present). Modelling individual charge state isotope from raw data or uncharged isotope distributions from deconvoluted data can yield the same information – both are shown here.

3.1.1 Experimental

Peptides are obtained from two sources: (i) synthetic peptides from Albachem Ltd. (Glandsmuir, UK) produced by solid phase methodologies; (ii) synthetic peptides from Dr. Derek MacMillan (UCL, London). Ubiquitin (a calibrant peptide) was obtained from Sigma Aldrich Ltd. (Milwaukee, WI, USA).

For mass spectrometry measurements the peptides were dissolved in 49.5:49.5:1 MeOH:H₂O:CH₃COOH at 20-50μM and ionised by ESI (250μl min⁻¹, 3-4kV) or nano-ESI from gold/palladium coated tips (Proxeon Biosystems) or via a nano-mate (Advion Ltd.).

3.1.2 Defr1

The peptide Defr1 (defensin related peptide 1) is a variant of MBD8 found in black 6 mice. In Defr1, the first cysteine residue has been replaced by a tyrosine leaving only five cysteines and hence opening the possibility of the formation of an intermolecular disulphide bond. Previous work on this peptide has shown it to exist as a covalently bound dimer of mixed disulphide topology. The peptide was shown to be extremely
active against a wide variety of micro-organisms although this activity fell greatly on reduction of the intermolecular disulphide bond.

Work on defensins by other groups has shown that different disulphide connectivities can be separated by a long HPLC gradient. In order to test if this was possible for DefrI it was synthesised again and separated on an extremely long gradient (20% organic to 30% organic over 60 min) collecting 16 fragments across the peak in the hope each fragment would contain, predominantly, one connectivity. In order to confirm the oxidation state of the disulphides in the fractions a representative selection of the more abundant fractions was analysed by FTICR-MS. These were fractions 13, 14, 15, 23, 25 and 26. The spectra were calibrated externally using ubiquitin.

**Figure 3.4:** Monomer sequence of DefrI. Cysteine residues are highlighted in red, the Cys to Tyr mutation is highlighted in blue.

The sequence of the monomer of DefrI is shown in figure 3.4 with the point of the cysteine to tyrosine mutation highlighted in blue.

### 3.1.2.1 Results

By modelling the predicted isotope distribution for DefrI dimer with four intramolecular and one intermolecular disulphide bonds using the X-Mass software (Bruker Daltonics, Billerica), it was possible to confirm that each of the peptides tested was fully oxidised (all five disulphide bonds in tact) as expected. Figure 3.3 shows the +7 charge state of DefrI with the predicted isotope distribution overlain (red triangles).
Figure 3.5: Isotope distribution of +7 Defr1 fraction 23.

There is good mass agreement between the theoretical spectrum and the experimental spectrum but the intensities match poorly. This mismatch is most likely due to space-charge effects which have been reported to affect ion intensities and mass accuracy in FTMS measurements. The deconvoluted mass measured for this peptide was 7566.91 Da as compared to a theoretical mass of 7566.98 Da for Defr1 dimer with all disulphide bonds intact—a mass accuracy of 9ppm. The same result was obtained for each fraction studied and it was therefore concluded that each fraction obtained was fully oxidised.
3. β-Defensins: Insight from Mass Spectrometry

3.1.2 DEFB107

DEFB107 is a five cysteine containing human β-defensin (figure A3.1, appendix 3) obtained from genome database mining; it is the orthologue of the murine β-defensin, defb13. Unlike Defr1, DEFB107 is found to be completely inactive against all pathogens tested.

Low resolution mass spectrometry of DEFB107 showed it to have a mass 304 Da higher than expected. This mass increase corresponds to the addition of a glutathione (a tripeptide of sequence ECG) capping group on present on one of the cysteines. To confirm this accurate mass measurement was performed as described previously.

3.1.2.1 Results

Figure 3.6 shows the deconvoluted FTICR mass spectrum of DEFB107 with the predicted isotope distribution for DEFB107 with two intramolecular disulphide bonds and a disulphide bound glutathione group. Again there is excellent agreement between the theoretical mass and the measured mass; for this spectrum there is also excellent agreement between the predicted abundance and the measured abundance.

3.1.3 Other Defensins studied

A number of other β-defensins have been studied by FTICR-MS during the course of the work. Table 3.1 shows the measured mass for each peptide, the number of disulphides present and the corresponding theoretical mass for a fully oxidised peptide (all disulphides formed).
Table 3.1: Theoretical and measured masses of β-defensins. The masses shown are of the most intense peak in the spectrum.

There is good agreement between measured mass and theoretical mass demonstrating the strength of FTICR-MS as an accurate mass technique for peptides of this size. Additionally, isotope modelling was performed for each of the spectra generated for these peptides allowing confirmation of the assigned cysteine oxidation states.
3. 3-Defensins: Insight from Mass Spectrometry

3.2 Mapping Disulphide Connectivity of β-Defensins

As discussed in the introduction to this section, the topology of the disulphide bonds in β-defensins appears to have no effect on anti-microbial activity but can be important for chemotactic activity. It is therefore important to fully characterise the disulphide connectivities of the peptides studied.

Digestion of peptides by proteolytic enzymes followed by MS analysis is a widely used technique in the identification of peptides. In the case of this work it is important to cleave the peptide between the cysteines while leaving the disulphide bonds in tact. It has been shown previously that using trypsin or a combination of trypsin and chymotrypsin can be used to successfully mass map defensins\textsuperscript{19}. Trypsin cleaves after basic residue (i.e. K and R) and chymotrypsin cleaves after aromatic residues (F, W and Y). After digestion the peptides obtained can be analysed by MS and the masses obtained matched with theoretical peptide masses.

3.2.1 Experimental

Peptides were obtained as previously described. All other chemicals were obtained from Sigma-Aldrich (Milwaukee, WI).

Enzymatic digestion of the peptides (500 μg/ml) was performed with trypsin (25 μg/ml) in 50mM TRIS-HCl buffer pH 5. The reaction was allowed to proceed at 37°C for 4hrs before termination with 0.01% TFA.

The resulting peptides were purified and concentrated using C18 Zip Tips (Millipore) eluting into 20 μl of CH\textsubscript{3}CN:H\textsubscript{2}O:CH\textsubscript{3}COOH (49.5:49.5:1) prior to analysis by nano-ESI MS on a Q-ToF 1 (Waters Micromass Technologies, Manchester, UK) using gold/palladium coated tips (Proxeon Biosystems, Denmark).
3. β-Defensins: Insight from Mass Spectrometry

3.2.2 Results: DEFB107

DEFB107 presents an interesting challenge as the position of the capping group must be determined along with the topology of the two disulphide bonds. This was achieved using trypsin digestion with MS and MS/MS analysis. Figure 3.6 shows the sequence of defb107 with the sites of tryptic cleavage highlighted in red.

![Defb107 sequence](image)

Figure 3.6: Defb107 sequence

Table 3.2 shows the observed peptides obtained from this procedure (the masses indicated are for oxidised disulphide bonds, Glt = glutathione).

<table>
<thead>
<tr>
<th>Fragment Number</th>
<th>Sequence</th>
<th>Theoretical Mass</th>
<th>Measured Mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>R</td>
<td>174.11</td>
<td>174.09</td>
</tr>
<tr>
<td>2</td>
<td>ALISK</td>
<td>530.34</td>
<td>530.33</td>
</tr>
<tr>
<td>3</td>
<td>MEGHC_{1}EAEC_{2}LTFEVK</td>
<td>1722.71</td>
<td>1722.74</td>
</tr>
<tr>
<td>4</td>
<td>[IGGC_{3}] [AELAPFC_{4}C_{5}] [Glt]</td>
<td>1787.75</td>
<td>1788.21</td>
</tr>
<tr>
<td>5</td>
<td>NR</td>
<td>288.15</td>
<td>Not observed</td>
</tr>
</tbody>
</table>

Table 3.2: Identified tryptic digest peptides for DEFB107. Square brackets indicate the presence of disulphide bonds.

Clearly fragment 4 could have the disulphide bond between cysteine 3 and cysteine 4 with the capping group on cysteine 5 or the disulphide bond between cysteine 3 and cysteine 5 with the capping group on cysteine 4 – the two corresponding peptides are indistinguishable on mass grounds only. To determine which of these topologies was observed, CID was performed on +4 full length DEFB107 – the obtained dissociation fragment ions are listed in table 3.3.
Table 3.3: Identified CID fragments from +4 DEFB107.

The important fragments here are fragments y4 and y11. y11 confirms the presence of the glutathione group on the final two cysteine residues; y4 has the sequence [C5:KNR][Glt] indicating the presence of the glutathione group on cysteine 5.

By combining the data from peptide mass mapping and CID measurements it is clear that the peptide has the connectivity C1-C2, C3-C4, C5-Glt. This is of course a non-canonical connectivity, the structural consequences of this topology will be discussed in section 4.1.

3.2.3 Results: Defb14

Defb14 is the murine orthologue of the human β-defensin HBD3. It has been shown to be active against a wide variety of pathogens. The peptide studied was produced synthetically by Albachem. The disulphide connectivity of this peptide was determined by digestion with trypsin. The peptide sequence is shown in figure 3.7.
Analysis of the resulting peptides by MS revealed two/three dominant topologies, the peptides identified by each digestion are shown in table 3.4. The data includes several peptides identified with missed cleavages i.e. incomplete digestion of the peptide.

<table>
<thead>
<tr>
<th>Fragment Number</th>
<th>Sequence</th>
<th>Theoretical Mass</th>
<th>Measured Mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>FLPK</td>
<td>503.31</td>
<td>503.32</td>
</tr>
<tr>
<td>2</td>
<td>TLR</td>
<td>388.24</td>
<td>388.34</td>
</tr>
<tr>
<td>3</td>
<td>K</td>
<td>146.11</td>
<td>146.08</td>
</tr>
<tr>
<td>4+9</td>
<td>[FFC_1R][C_4SNSGR]</td>
<td>1191.50</td>
<td>1191.45</td>
</tr>
<tr>
<td>5</td>
<td>IR</td>
<td>287.20</td>
<td>287.21</td>
</tr>
<tr>
<td>6</td>
<td>GGR</td>
<td>288.15</td>
<td>288.18</td>
</tr>
<tr>
<td>7</td>
<td>[C_2AVLNC_3LGK]</td>
<td>917.45</td>
<td>917.47</td>
</tr>
<tr>
<td>7+11</td>
<td>[C_2AVLNC_3LGK][C_5C_6K]</td>
<td>1295.57</td>
<td>1295.86</td>
</tr>
<tr>
<td>8</td>
<td>EEQIGR</td>
<td>730.36</td>
<td>730.29</td>
</tr>
<tr>
<td>10</td>
<td>K</td>
<td>146.11</td>
<td>146.08</td>
</tr>
<tr>
<td>11</td>
<td>[C_5C_6K]</td>
<td>378.12</td>
<td>378.52</td>
</tr>
<tr>
<td>12</td>
<td>K</td>
<td>146.11</td>
<td>146.08</td>
</tr>
<tr>
<td>13</td>
<td>K</td>
<td>146.11</td>
<td>146.08</td>
</tr>
<tr>
<td>14</td>
<td>K</td>
<td>146.11</td>
<td>146.08</td>
</tr>
<tr>
<td>1+2*</td>
<td>FLPKTLR</td>
<td>873.55</td>
<td>873.65</td>
</tr>
<tr>
<td>2+3*</td>
<td>TLRK</td>
<td>516.35</td>
<td>516.97</td>
</tr>
<tr>
<td>3+4+9*</td>
<td>[KFFC_1R][C_4SNSGR]</td>
<td>1319.61</td>
<td>1319.96</td>
</tr>
<tr>
<td>4+5+9*</td>
<td>[FFC_1RIR][C_4SNSGR]</td>
<td>1461.69</td>
<td>1461.33</td>
</tr>
<tr>
<td>7+8*</td>
<td>[C_2AVLNC_3LGKEEQIGR]</td>
<td>1630.91</td>
<td>1630.78</td>
</tr>
</tbody>
</table>

Table 3.4: Trypsin digest masses for Defb14. Square brackets indicate the presence of disulphide bonds. * indicates the presence of a missed cleavage.
3. β-Defensins: Insight from Mass Spectrometry

The data reveals one clearly defined connectivity of C₁-C₄, C₂-C₃, C₅C₆ (indicated by fragments 4+9, 7 and 11 respectively) and at least one other topology of connectivity C₁-C₄, C₂-C₅, C₃-C₆ and/or connectivity C₁-C₄, C₂-C₆, C₃-C₅ (indicated by peptides 4+9 and 7+11). Due to the low abundance of peptide 7+11 further experiments such as CID were unable to be performed in order to fully characterise this peptide.

It is noteworthy that the C₁-C₄ peptide occurs in all three identified topologies suggesting this disulphide bond is favoured in this peptide while the other disulphide bonds are more variable and may perhaps scramble in the folding process. Unfortunately we are unable to obtain information on the synthesis and folding strategies employed by Albachem when preparing this peptide as this information is viewed as being commercially sensitive. Were this information available, it might be possible to explain why these non-canonical connectivities were obtained.

Once again, there is no evidence of a canonically bonded defensin present in the sample; unlike DEFB107, however, Defb14 is extremely active despite the nature of the disulphide bridges – this is consistent with the findings of Lu⁸ and of Klüver⁹ for HBD3.
3.3 Defensin Membrane Interactions

As discussed previously, the mode of action of β-defensins is not well understood; it is known to involve disruption and permeabilisation of the cell membrane of invading pathogens leading to content leakage and eventual necrosis. There are two proposed modes of action for β-defensins (and AMPs in general):

(i) Carpet Model: where many molecules accumulate on the cell surface eventually disturbing the integrity of the membrane.

(ii) Pore/Barrel Model: where the peptide oligomerises and forms at multimeric pore which permeates the cell membrane to allow leakage.

In reality AMPs may work by one, both or neither of these mechanisms and much work is required to fully, or even partially, understand their mode of action.

The peptide DEFB107 presented itself as an interesting choice for the study of mode of action. At first sight it would seem an unlikely candidate for this type of study because it is inactive. However, this inactivity can be exploited for examination of peptide-membrane interaction; the hope being the peptide is capable of interacting with a cell membrane in a similar way to active defensins but is unable to disturb the integrity of the membrane.

An experiment was devised, based on the work of Demmers et al. who elegantly demonstrated how to determine which parts of peptides are incorporated in cell membranes using hydrogen/deuterium exchange mass spectrometry.

The basic experiment is as follows: the peptide of interest is mixed with a solution of lipid at a high lipid to peptide ratio. The resulting solution is used to form peptide containing vesicles known as proteoliposomes. The parts of the peptide which interact favourably with the lipids (e.g. alpha helices) are enclosed by the lipid bilayer and are therefore unavailable to solvent. By performing H/D exchange on these proteoliposomes
it is possible to determine how much and which parts of a peptide are incorporated in the membrane.

A basic schematic of the experiment is shown in figure 3.8.
1. Mix Lipid & Peptide

2. Extrude to make 'proteoliposomes'

3. Dilute in D$_2$O

4. Analyse by ESI-MS
3.3.1 Experimental

DEFB107 was obtained from Albachem Ltd. (Glansmuir, UK). The lipid, 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), trifluoroacetic acid (TFA), 2,2,2-trifluoroethanol (TFE), ethanol, ammonium acetate, deuterium oxide (D₂O) and methanol were all obtained from Sigma Aldrich (Milwaukee, WI).

250 μg of DEFB107 (1mg/ml in water) was freeze dried under vacuum to produce a lyophilised powder. This powder was dissolved in a small amount (250 μl) of TFA which was removed with a stream of nitrogen. The peptide was then redissolved in TFE (250 μl) and dried under nitrogen once more. The dried peptide was taken up once more in TFE (1mg/ml) and mixed with a solution of DMPC in methanol (5mg/ml) to give a peptide to lipid ratio of approximately 1:25. The resulting solution was vortexed vigorously at 40°C (i.e. above the glass transition temperature of DMPC) before drying under nitrogen (again at 40°C). The resulting film was taken up in 1 ml of methanol and the vortexing and drying process was repeated. The mixed film produced was dried under vacuum for 24hrs prior to hydration at 40°C in 250 μl of 10 mM ammonium acetate solution – giving a final peptide concentration of 1mg/ml.

In order to produce vesicles from this solution a process known as extrusion is carried out. This involves passing the peptide/lipid solution through a 0.1 μm membrane (Avanti Polar Lipids Inc., Birmingham, AL) several tens of times forcing the lipids to form vesicles or in this case, proteoliposomes (once more this is carried out at 40°C).

Prior to H/D exchange the solution is incubated at 40°C for 30 min. It is then diluted 50 times in 10 mM deuterated ammonium acetate solution. At selected time points – ranging from 3 min to 2 days - aliquots of this solution were taken and analysed by nano-ESI-MS on a Q-ToF (Micromass, Manchester, UK) using gold/palladium tips (Proxeon Biosystems, Denmark).
As a control solutions of DEFB107 were analysed at the same concentration from 10mM ammonium acetate and from 10mM deuterated ammonium acetate (ensuring the same final concentration of deuterated solvent was present – 98%).
3.3.2 Results

Figure 3.9: Q-ToF mass spectra of DEFB107 obtained from three different solutions: (A) DEFB107 in 10mM AmAc (Average Mass = 4572.05 Da); (B) DEFB107 proteoliposomes in dAmAc 1 hour after incubation (Average Mass = 4638.14 Da, ~66 exchanged); (C) DEFB107 in 10mM dAmAc 10 minutes after incubation (Average Mass = 4649.98 Da. ~78 exchanged)

Figure 3.9 shows mass spectra obtained for DEFB107 flown from 10mM ammonium acetate (AmAc), deuterated 10mM ammonium acetate (dAmAc) ten minutes after initial incubation in deuterated buffer and for DEFB107 proteoliposomes flown from deuterated 10mM ammonium acetate 1 hr after exposure to deuterated solvent.
Figure 3.10 is a plot of mass increase against time incubated in dAmAc for DEFB107 proteoliposomes. The data clearly shows that there is an instant (<3 minutes i.e. the shortest possible analysis time) increase in mass to ~66 hydrogens exchanged; the number of exchanged hydrogens does not increase beyond this number, even after two days incubation time.

![Mass Increase vs. Time for DEFB107 Proteoliposomes](image)

**Figure 3.10:** Graph of mass increase vs. incubation time for DEFB107 flown from dAmAc. The red line indicates the maximum possible mass increase (i.e. 78 Da) for the peptide.

Returning to figure 3.9, spectrum C shows an average of 78 hydrogens exchanged for DEFB107 in dAmAc after 10 min incubation – this corresponds to the exchange of all available hydrogens (backbone and sidechain) indicating the molecules is completely
solvent accessible. It is clear therefore that the lipid is protecting part of the peptide from the solvent indicating part of the peptide is enclosed by the membrane.

In an attempt to ascertain which part(s) of the peptide was protected from the solvent, CID was performed on the 4+ charge state of the peptide flown from proteoliposomes in dAmAc. At fairly low collision energy (~35 V), loss of the fully exchanged glutathione capping group was seen – indicating it was fully solvent exposed [data not shown]. Due to this group’s proximity to the C-terminus of the peptide, this suggests the C-terminus of the peptide is also fully solvent exposed. Further identification of fragment ions in this spectrum proved to be extremely difficult due to the huge number of possible fragment ion masses for a partially exchanged peptide.

Using this evidence, it is postulated that the N-terminus of the peptide is the part that interacts with the membrane. Examining the sequence (figure A3.1, appendix 3) reveals that the first 11 residues of the peptide are unrestrained by disulphide bonding (in comparison to the rest of the peptide). Three of the first four residues (Ala2, Lys3 and Ile4) are aliphatic residues with low hydrophobicities – i.e. residues that should interact favourably with a lipid bilayer. These residues are flanked by basic residues (Arg1 and Lys6/Arg7) which could interact favourably with the polar head groups of the lipids. If we count the number of exchangeable hydrogens in the section of the peptide before Lys5, the total number is 11, this is very close to the number of protected hydrogens seen in the experiment (12).

A plausible explanation for the reduced exchange seen for the peptide in the presence of the artificial vesicles is that the first five or six residues insert into the bilayer.

Structural studies of the peptide using molecular modelling and ion mobility (see section 4.1) indicate the N-terminal section is a potential helix; this is in keeping with structural findings for other β-defensins where the section of the peptide before the first cysteine is invariably shown to be helical13-17. Similarly, the N-termini of other defensins for which the structure has not been solved (including those peptides studied in this work) is almost
3. β-Defensins: Insight from Mass Spectrometry

always aliphatic and hence likely to interact favourably with a membrane. It is therefore reasonable to suggest that the N-terminal section of β-defensins is the section of the peptide involved in membrane disruption.
3.4 Defensin – Glycosaminoglycan Interactions

With such a large array of AMPs combining to fight off bacterial infection it is not surprising to learn that bacteria have developed their own defence mechanisms with which to fend off AMPs. For example, *Burkholderia cenocepacia* has been shown to be extremely resistant to attack by defensins; closer examination of the structure of the bacteria shows a mutation to its lipopolysaccharide (LPS), neutralising its negative charge, eliminating the possibility of an electrostatic interaction between the peptide and LPS.26

Another reported defence mechanism involves the release of glycosaminoglycans (GAGs).27 On entering an organism the pathogens *Pseudomonas aeruginosa*, *Enterococcus faecalis* and *Streptococcus pyogenes* are reported to release enzymes which degrade epithelial proteoglycans to release dermatan sulphate (a GAG). It has been shown that dermatan sulphate binds to and inactivates AMPs and therefore the increase in GAG concentration brought about by the protease, reduces the effectiveness of the host’s AMPs.

One such GAG is heparin, a highly sulphated polysaccharide comprising repeating disaccharide units. The most common disaccharide repeating unit consists of a 2-O-sulphated iduronic acid and a 6-O-sulphated, N-sulphated glucosamine, IdoA(2S)-GlcNS(6S). It has been shown27 that β-sheet containing α-defensins bind to and are inactivated by GAGs and that the sulphate groups typically found on heparin are important factors in the binding.

A number of authors report the presence of heparin binding domains – stretches of amino acid sequence to which heparin can bind. Cardin and Weintraub28 propose XBBBXXBX and XBBXBX (where X is a hydrophobic or uncharged residue and B is a basic residue) while Sobel et al.29 propose XBBBBXXBBXXBBX and TXXBXXTBXXXTBB (where T represents a turn) as additional binding domains. It is worth noting that these consensus sequences are not always found on heparin binding peptides and it may be
more relevant to examine the spacing between amino acids in order to identify a binding domain; Margalit et al. report a distance of 20 Å between basic residues as a prerequisite for heparin binding.

The exact relation between heparin binding and anti-microbial activity is not known, indeed it could merely be coincidence that cationic AMPs such as defensins bind to anionic heparin. However, if heparin binding is indeed related to anti-microbial activity, the nature of the interaction may help in the design of new, novel AMPs.

In order to study the heparin binding propensity of the β-defensins studied in this work a mass spectrometry based binding assay was developed.

### 3.4.1 Materials and Methods

Peptides were obtained as described previously. The heparin chosen (a disaccharide) was obtained from Dextra Laboratories (Reading, UK).

For the binding assay, the peptides and disaccharide were both dissolved in water at 1mg/ml and mixed in various peptide:heparin ratios (2:1, 1:1, 1:2, 1:4) before dilution in 10mM ammonium acetate (Sigma Albrich, Milwaukee, WI) solution to give a final peptide concentration of 20 μM.

The resulting solutions were allowed to equilibrate at room temperature for 20 minutes before analysis by nano-ESI-MS and MS/MS on a Q-ToF I (Waters Micromass Technologies, Manchester, UK).

### 3.4.2 Results
Figure 3.11 shows an example mass spectrum obtained for the peptide Defr1 mixed 1:2 with the saccharide. A gas phase dissociation constant, $K_d$, can be determined by evaluating the amount of free protein ([P]), free ligand ([L]) and complex ([PL]) present in the mass spectrum – these values are determined by the intensities of the relevant species.
Looking at figure 3.11 the amount of complex seen is clearly charge state dependent. There is complex present for the $+5$ to $+7$ charge states but none for the $+8$ charge state and the ratio of complex to free protein varies from charge state to charge state.

There are a number of possible reasons why no complex is seen for the higher charge state, the two most apparent are: (i) the saccharide is interacting with basic groups on the protein therefore preventing them from becoming charged in the electrospray process – the maximum charge state available to the complexed protein is therefore reduced.
relative to the free species; (ii) the complex is unstable at higher charge states and
dissociates. The question then arises: should $K_d$s be determined from deconvoluted data
or from individual charge states?

Deconvoluted data clearly gives a representation of the whole sample rather than any
individual charge state, however, it takes into account protein ions for which there is no
corresponding complex and may therefore skew the data towards a higher $K_d$ value.
Calculating the $K_d$ from one individual charge state will allow for lower $K_d$s to be
assigned – perhaps closer to the solution phase value – by choosing the charge state for
which the complex is most abundant relative to the free protein.

For this work it was decided that all $K_d$ values should be determined based on
deconvoluted data on the assumption that it is better representative of the solution as a
whole.

$K_d$ values are determined using equation 3.1$^{32}$ where $[L_i]$ is the initial ligand
concentration and $(|L_i|-[PL])$ is the free ligand concentration i.e. free ligand
concentration is equal to initial concentration minus the amount of ligand involved in the
complex.

\[ K_d = \frac{[P][L_i]-[PL]}{[PL]} \]

Equation 3.1

$[P]$ and $[PL]$ are calculated by summing the intensities of the complex and the free
protein and setting that equal to the initial protein concentration i.e. 20 µM. $[P]$ is then
equal to $[P]/([P]+[PL])$ * 20 µM and $[PL]$ is $[PL]/([P]+[PL])$ * 20 µM.

While it is possible to determine $K_d$s based on individual measurements, it is better to
base the values on data obtained from several spectra using different protein:ligand ratios.
This allows a plot of $[PL]/[P]$ against $(|L_i|-[PL])$ to be produced. The gradient of the
A straight line fitted to the data will be equal to the inverse of the dissociation constant. This method was used in this work wherever possible. Figure 3.12 shows a plot of [PL]/[P] against [L] for Defr1.

![K_d plot for Defr1](image)

**Figure 3.12**: Plot of the molar ratio of complex and free protein against free ligand concentration for Defr1. The values were obtained at three protein:ligand ratios: 2:1, 1:1 and 1:2.

In the case of figure 3.12, the complex was evaluated at three peptide:heparin ratios: 2 to 1, 1 to 1 and 1 to 2. The values obtained are plotted and a gradient of 0.0433 is obtained (note the excellent $R^2$ value obtained).

Using the gradient of the line, the $K_d$ of heparin and Defr1 is 23 µM.

This procedure was repeated for a number of different β-defensins. Table 3.5 shows the gas phase dissociation constants measured for the various β-defensin-heparin complexes.
along with their anti-microbial activity (minimum inhibitory concentration – MIC) against *P. Aeruginosa*.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>$K_d$ (µM)</th>
<th>MIC (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Defb107</td>
<td>No binding</td>
<td>&gt;22 (no killing)</td>
</tr>
<tr>
<td>Defrl</td>
<td>23</td>
<td>0.79</td>
</tr>
<tr>
<td>Defrl Y5C</td>
<td>180</td>
<td>13.5</td>
</tr>
<tr>
<td>Defrl 1CysAla</td>
<td>40</td>
<td>0.82</td>
</tr>
<tr>
<td>Defrl 1CysSer</td>
<td>37</td>
<td>0.81</td>
</tr>
<tr>
<td>Defbl4</td>
<td>30</td>
<td>0.29</td>
</tr>
<tr>
<td>Defbl4 1Cys*</td>
<td>19</td>
<td>0.15</td>
</tr>
<tr>
<td>Defbl4 DIP1*</td>
<td>23</td>
<td>0.58</td>
</tr>
<tr>
<td>Defbl4 DIP2</td>
<td>No binding</td>
<td>&gt;20 (no killing)</td>
</tr>
<tr>
<td>Defbl4 DIP3</td>
<td>No binding</td>
<td>&gt;20 (no killing)</td>
</tr>
</tbody>
</table>

Table 3.5: Gas phase dissociation constants of selected β-defensins binding to heparin disaccharide along with MIC against *P. Aeruginosa*. Peptides marked with * had their $K_d$s evaluated at only one peptide:ligand ratio.

Upon examination of the data shown above it is immediately apparent that there is a qualitative relationship between heparin binding and anti-microbial activity: highly active peptides bind more tightly (i.e. have lower $K_d$s) than less active and inactive peptides - which do not bind at all. This presents the assay as a potential high throughput activity screen.

To investigate the effect of the heparin binding on anti-microbial activity, the antimicrobial assays were run again in the presence of heparin for a selected group of β-defensins at a 1:2 peptide:ligand ratio. Table 3.6 shows the results against *P. Aeruginosa* (a gram-negative bacteria) and *S. Aureus* (a gram-positive bacteria).
Table 3.6: Anti-microbial activities of selected defensins against *P. Aeruginosa* (PA) and *S. Aureus* (SA) in the presence and absence of heparin disaacharide.

It is interesting to note that the two 'natural' defensins studied (Defr1 and Defb14) show partial or complete loss in activity in the presence of heparin whereas the designed peptides lose little or no activity in the presence of the saccharide.

It is noteworthy that the effect of heparin is much more pronounced for the activity against the gram-positive bacteria studied. This is thought to be due to the different cell membrane structures of each type of bacteria. Figure 3.13 shows a schematic representation of each type of bacteria.
3. 3-Defensins: Insight from Mass Spectrometry

Gram-negative bacterial membranes are comprised of a thick peptidoglycan layer followed by a cell membrane. Gram-negative bacteria have an outer membrane followed by a much thinner peptidoglycan layer in the periplasmic space between the outer membrane and the subsequent inner membrane.

In order for a defensin to reach the cell membrane of a gram-positive bacterium, it first must traverse only the peptidoglycan layer; to reach the cell membrane of a gram-negative bacterium a defensin must also pass through the outer membrane. It is therefore possible that during the more complex path the molecule must take in order to reach a gram-negative cell membrane, the bound heparin can be lost through interaction with the cell membrane. For this reason, in the presence of heparin the peptide can remain active against gram-negative bacteria at lower concentration than against gram-positive bacteria.

If we take this hypothesis to be correct then we must assume that the two 'natural' defensins better protect the heparin from interactions with the membrane components hence heparin has a more pronounced effect on their activity. Further insight into this will be provided by ion mobility in section 4.4.
3.5 Conclusions

Studying β-defensins with MS can yield many different pieces of information from the relatively simple structural data provided by an accurate mass measurement to the more complex and difficult to interpret mode of action data obtained from a binding assay.

From the accurate mass data shown above, it is clear that the synthetic peptides are being produced correctly and have the correct number of disulphide bonds. The formation of these disulphide bonds and the resulting connectivity is, however, a more complex issue.

The two peptides studied in detail above did not possess canonical disulphide connectivity. This suggests a failing in the synthesis/refolding strategies employed in the production of these peptides. Current work within the project is focussing on new synthetic strategies (selective capping of cysteines etc.) to produce canonical disulphide connectivity Defb14. The fact remains, however, Defb14 with 'incorrect' disulphide bonding is a highly active anti-microbial agent - this is of course in agreement with the work of Lu and others. However, it is interesting to note that Defb14 is chemotactically active - this is in opposition to the findings of Lu's work on HBD3 (the human orthologue of Defb14) where he found canonical disulphide bonding was a requirement for chemotaxis.

The work carried out on membrane interaction provides good evidence for the n-terminus of β-defensins being important for antimicrobial activity. Armed with this information it is possible to rationalise the differences in activity of other defensins studied. Defr1 exists as a covalently bound dimer with five disulphide bonds and is a potent AMP; its six cysteine analogue Defr1 Y5C exists as a non-covalent dimer with three intramolecular disulphide bonds and is seen to be nearly twenty times less active. If one examines the n-termini of these two peptides they are obviously globally similar, however, the n-terminus of Defr1 is less restrained by disulphide bonding than that of Defr1 Y5C - the first cysteine in Defr1 is residue 11, in Defr1 Y5C it is residue 5. The less constrained n-
terminus of Defr1 may be able to present a structure favourable for membrane interaction and may also be able to penetrate the membrane more deeply than Y5C.

Clearly the artificial vesicles used in this work represent a vastly simplified model of a bacterium. Bacterial membranes are vastly complex structures comprised of a mixture of lipids, carbohydrates and proteins. The first interactions between these membranes and β-defensins are reported to be between the basic residues of the peptide and anionic structures on the surface of the cell such as lipopolysaccharide (LPS), techoic acid and sialic acid. Indeed it has been shown that *B. cepacia* with mutated LPS is highly resistant to attack by β-defensins (also appendix 3, Table A3.1). It is therefore important to consider the interactions with these membrane components when considering the mode of action of β-defensins.

The heparin binding assay described above has been shown to be a good qualitative assay for ranking anti-microbial activities of peptides. It has also been shown that some β-defensins are inactivated in the presence of heparin. The nature of the interaction may therefore be important in understanding how β-defensins work and will be studied in more detail by ion mobility and molecular modelling.

Ultimately it would be desirable to extend this work on β-defensin binding to include interactions with bacterial membrane components such as LPS and teichoic acid.

References:

3. β-Defensins: Insight from Mass Spectrometry

11 O. Toke, Biopolymers, 2005, 80, 717-735
28 A.D. Cardin and H.J. Weintraub, Arteriosclerosis, 1989, 9, 21-32
32 www.zenobi.ethz.ch/binding.html
33 S. Baron, in Medical microbiology, 3rd ed., The University of Texas Medical Branch at Galveston, 1991
4. Ion Mobility Studies of β-Defensins and Defensin-Related Peptides

As stated in chapter 3, producing correctly folded defensins with the correct disulphide topology is difficult. Understanding this – coupled with a wish to understand the defensin related peptides produced – drives our desire to characterise the peptides studied here with structural techniques.

Most structural characterisation of β-defensins has been carried out using NMR and XRC although some mass spectrometry based studies have been published. Thus far only three human β-defensin structures have been solved: HBD-1 (NMR and XRC); HBD-2 (NMR and XRC); and HBD-3 (NMR only). The three structures were shown to share a common topological fold consisting of a core of three anti-parallel β-sheets with an N-terminal α-helix. Additionally, HBD-2 was reported to exist in two different quaternary structures: a non-covalent dimer and a non-covalent octamer composed of four dimers. This latter form was taken as good supporting evidence for the pore formation model of defensin activity (see section 3).

It is interesting to note, however, that static light scattering studies by Sawai et al. and size exclusion studies by Bauer et al. show HBD-2 to exist as a monomer in solution. The latter authors suggest that the conditions used in the crystallisation process (e.g. high concentration) favour oligomerisation and the XRC studies do not therefore report on the solution behaviour of the peptide.

Given the number of defensins studied here, it is unfeasible to determine the structures of all the peptides studied using NMR or X-ray crystallography. It is, however, possible to study a number of these peptides by ion mobility mass spectrometry using the MoQToF.

This chapter will focus on the structural characterisation of a number of β-defensins, defensin related peptides (DIPs) and of defensin-heparin complexes. Due to the challenges associated with obtaining reliable structures from molecular modelling there
are only a small number of model structures presented here. IMMS measurements will be used here primarily to examine differences in defensin conformations under different conditions such as different disulphide connectivities. This may give some insight into the importance of the disulphide bonds and their connectivity.

The heparin binding experiments will be used to investigate the structural change on binding heparin – can this be related to the degree of inhibition seen?

While most of the work discussed in this chapter has been carried out on the MoQToF, certain experiments have also been carried out in UCSB and therefore are useful for quality control purposes.
4. Ion Mobility Studies of \( \beta \)-Defensins and Defensin-Related Peptides

4.1 DEFBI07

Mobility measurements of the peptide DEFBI07 have previously been performed at UCSB\(^2\), this peptide is therefore an ideal test and calibration compound for our defensin studies.

4.1.1 Experimental: Large Apertures

Initial experiments were carried out with large aperture orifices in place – 2 mm diameter at the front of the cell, 1.67 mm diameter at the rear – allowing experiments to be carried out at \( \leq 1.75 \text{ Torr} \).

The experiments were therefore performed at 1.6 Torr of He at 305 K with injection voltage \((V_{\text{inj}}) = 35.5 \text{ V}\). DEFBI07 was analysed at 40 \( \mu \text{M} \) in 49:50:1 \( \text{H}_2\text{O}:\text{MeOH}:\text{CH}_3\text{COOH} \) using ESI with 3.2 kV capillary voltage and 75 V cone voltage. Measurements were performed at \( V_d = 35 \text{ V}, 30 \text{ V}, 25 \text{ V}, 20 \text{ V}, 15 \text{ V} \) and 10 V with 60 \( \mu \text{s} \) pusher period. Arrival times, mobilities and cross sections were obtained as described in section 2.3.

Table 4.1 shows the cross sections obtained from this method for the three observed charge states (+5, +6 and +7) along with cross-sections measured in previous work.
4. Ion Mobility Studies of β-Defensins and Defensin-Related Peptides

<table>
<thead>
<tr>
<th>Charge State</th>
<th>Cross Section (MoQToF)</th>
<th>Cross Section (UCSB)</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>+5</td>
<td>657 Å²</td>
<td>768 Å²</td>
<td>14%</td>
</tr>
<tr>
<td>+6</td>
<td>694 Å²</td>
<td>819 Å²</td>
<td>15%</td>
</tr>
<tr>
<td>+7</td>
<td>712 Å²</td>
<td>865 Å²</td>
<td>18%</td>
</tr>
</tbody>
</table>

Table 4.1: DEFB107 cross sections from MoQToF vs. UCSB.

Clearly the cross sections are being significantly underestimated under these conditions with a larger underestimation occurring at higher charge.

One possible explanation for this is that the ions are being injected with too high energy and are not reaching a constant drift velocity until a short distance into the cell. Under the conditions described above, the ions have the following kinetic energies: +5 – 177.5 eV; +6 – 213 eV; +7 – 248.5 eV. This means a +7 ion of DEFB107 will have higher velocity upon entering the cell than a +6 ion which will in turn have a greater velocity than a +5 ion. If the hypothesis is correct that the ions are not ‘drifting’ for the entire length of the cell then it follows that the +7 ion will take the longest to reach a constant drift velocity and hence be underestimated by the largest degree.

To test this hypothesis, the experiment was repeated at V_{inj} = 20.5 V. The cross sections obtained were as follows: + 5 ion found to be 689 Å (underestimation by 10%); +6 was found to be 722 Å² (12%) and +7 was found to be 745 Å² (14%). Once more, there is a large underestimation of the cross-section. The MoQToF values are, however, closer to those measured previously suggesting the hypothesis is valid.

Assuming the ions begin to drift within the first few millimetres of the drift cell (i.e. the pressure is sufficient to allow them to reach a constant drift velocity), it should be possible to bring the measured values into calibration by adjusting the drift length used in the mobility calculation. For the data shown in table 4.1, setting the drift length to 4.72
cm for +5, 4.70 cm for +6 and 4.62 cm for +7 gives cross sections of 767 Å, 817 Å² and 868 Å² respectively all within less than 0.5% of the UCSB values².

While it is possible to apply this sort of calibration to any data collected using the MoQToF, it is not ideal to have such an obvious unknown in our experiment, especially given the charge state dependence of the error.

The experiment was therefore repeated at higher pressure.

### 4.1.2 Experimental: Small Apertures

The large aperture orifices were replaced with 1mm aperture orifices thus allowing higher pressures to be reached in the cell.

The above experiment was repeated with 3.4 T He in the drift cell at 305 K with 34.8 V injection voltage. Measurements were taken at $V_d = 60$ V, 50 V, 40 V, 30 V, 25 V and 20 V with 60 µs pusher period.

The experiment was performed three times under these conditions. +5 and +6 charge states were observed for all experiments while +7 and +8 charge states were observed on two occasions with the +4 charge state observed in one repeat. Table 4.2 summarises the results obtained.
4.1.3 Results

<table>
<thead>
<tr>
<th>Charge</th>
<th>Cross Section (MoQToF)</th>
<th>Cross Section (UCSB)</th>
</tr>
</thead>
<tbody>
<tr>
<td>±4</td>
<td>n/a</td>
<td>627 Å²</td>
</tr>
<tr>
<td>±5</td>
<td>742 Å²</td>
<td>743 Å²</td>
</tr>
<tr>
<td>±6</td>
<td>808 Å²</td>
<td>825 Å²</td>
</tr>
<tr>
<td>±7</td>
<td>855 Å²</td>
<td>874 Å²</td>
</tr>
<tr>
<td>±8</td>
<td>920 Å²</td>
<td>955 Å²</td>
</tr>
</tbody>
</table>

Table 4.2: Defb107 cross sections from MoQToF. Measurements were obtained at 3.5 Torr from 49:49:2 HO:MeOH:CH₃COOH solution.

As can be seen, there is good reproducibility across all charge states and good agreement with previous results. Figure 4.1 shows a plot of cross section versus charge for DEFB107 using cross sections obtained by averaging the MoQToF data shown in table 4.2.
4. Ion Mobility Studies of β-Defensins and Defensin-Related Peptides

**Figure 4.2:** Plot of average cross section against charge for Defb107. All values are MoQToF data apart from +3 which is UCSB data. Errors are ± one standard deviation where appropriate and ± 3% otherwise.

It can be seen from figure 3.1 that there is a steady increase in cross section from +4 to +8. The transition from +3 to +4 shows a comparatively small increase in collision cross section, perhaps indicating an unfolding transition between +4 and +5.

### 4.1.4 Molecular Modelling

In order to characterise the types of structures seen in this work, molecular modelling calculations were performed using the AMBER 99 force field within the Amber 8.0 program suite. The peptide with glutathione capping group was built in X-leap with four
different connectivities: the canonical disulphide connectivity and the three possible connectivities with the glutathione group attached to the final cysteine.

All amino acids were held at their physiological charge states apart from the histidine and the glutamic acid on the capping group which were both neutralised. This gave the modelled peptide a net charge of ±2.

The peptide was modelled using a simulated annealing approach to yield minimised gas phase structures: dynamics are performed at 800 K for 30 ns before cooling exponentially to 0 K. The resulting structure is then minimised. This minimised structure is then used as the starting point for the next set of dynamics and so on until 100 structures have been generated.

The collision cross section for these structures was then calculated using the projection approximation. Table 3.3 shows the average cross sections obtained for the 20 lowest energy structures and all structures obtained for each disulphide topology.

<table>
<thead>
<tr>
<th>Connectivity</th>
<th>Average Cross Section (20 lowest energy structures)</th>
<th>Average Cross Section (all structures)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-4 3-6 5-Glt</td>
<td>596 Å²</td>
<td>608 Å²</td>
</tr>
<tr>
<td>2-4 3-5 6-Glt</td>
<td>582 Å²</td>
<td>607 Å²</td>
</tr>
<tr>
<td>2-5 3-4 6-Glt</td>
<td>592 Å²</td>
<td>609 Å²</td>
</tr>
<tr>
<td>2-3 4-5 6-Glt</td>
<td>614 Å²</td>
<td>620 Å²</td>
</tr>
</tbody>
</table>

Table 3.3: Collision cross sections of for different disulphide topologies of ±2 DEFB107 found from low energy calculated geometries.

Figure 4.2 shows two representative low energy structures of DEFB107 obtained from this method. Figure 4.2-A shows a structure obtained for the ‘canonical’ connectivity; figure 4.2-B shows a structure obtained for the observed species.
Figure 4.2-A: Low energy molecular modelling structure of canonical DEFB107

Figure 4.2-B: Low energy molecular modelling structure of DEFB107 with the experimentally observed disulphide topology. In both figures the polypeptide backbone is represented by a light blue ribbon, and the cysteine and glutathione residues are shown as CPK structures.
4.1.5 Discussion

While it has not been possible to obtain experimental data for a +2 charge state of DEFB107, the cross section for such a species may be extrapolated from figure 3.1.

Clearly, given the variability of the measurement and the similarity of the calculated cross sections, the measured geometries could correspond to any of the modelled topologies. However, the large increase in cross section seen from +4 to +8 (~50% increase) may reveal something about the disulphide connectivity.

The increase in collision cross section seen for ubiquitin\(^9\) between the +4 and +8 charge states is approximately 50% i.e. comparable to that seen here for DEFB107. Ubiquitin does not posses any disulphide bonds and is therefore relatively unrestricted in the conformation it can adopt. If we then assume that DEFB107 must be similarly unrestricted it is clear that the disulphide bonding topology present for this peptide cannot greatly restrain it conformationally. Of the four topologies modelled it is clear that the 2-3, 4-5, 6-Glt topology is the most unrestricted by disulphide bonding given that the disulphide bonds present are between adjacent cysteine residues. This is of course the connectivity observed for this peptide by the mass mapping procedure described in section 3.2.2.

While this method does not give absolute confirmation of the disulphide topology seen for this peptide it does represent a potential method for reducing the number of possibilities.
4.2 Defr1

As discussed previously, Defr1 is a five cysteine containing defensin related peptide which forms a covalently bound dimer between the penultimate cysteine of each monomer. In order to fully understand the activity of the peptide a number of variants of this peptide have been created:

i. Defr1 dimer: the original peptide containing four intra-molecular disulphide bonds and one intermolecular disulphide bond.

ii. Defr1 Glt: a partially reduced form of Defr1 wherein the intermolecular disulphide bond has been reduced and the cysteine has been capped with a glutathione group.

iii. Defr1 Red: fully reduced Defr1 i.e. no disulphide bonds present

iv. Defr1 Y5C: Defr1 with residue 5 (tyrosine) replaced by a cysteine with three intra-molecular disulphide bonds.

v. Defr1 1Cys: Defr1 with all bar the penultimate cysteine replaced with alanine. Exist as a covalently bound dimer.

The disulphide connectivity of Defr1 (and by inference Defr1 Glt) has been shown to be a mixture of non-canonical topologies. The connectivity of Defr1 Y5C has been shown to be canonical. There is only one possible connectivity for Defr1 1Cys.

Each of these peptides will be studied by IM-MS in the hope of revealing the influence of the disulphide bonds on the conformations adopted by defensins.

4.2.1 Experimental

All peptides were analysed at 40 µM from 49:50:1 H2O:MeOH:CH3COOH using ESI with typical capillary voltages of 3.25 kV and typical cone voltage of 65 to 80 V. For Defr1 1Cys a pusher period of 63 µs was used; for Defr1 Red and Defr1 Glt a pusher
period of 70 µs was used and for Defr1 Y5C a pusher period of 65 µs was used. As there was insufficient Defr1 to obtain mobility data on the MoQToF, the data reported here are from the analogous Bowers instrument at UCSB.

For the data obtained on the MoQToF, measurements were performed at 3.5 Torr of He at 305 K with an injection voltage of 40 V. Measurements were taken at $V_d = 60$ V, 50 V, 40 V, 30 V and 25 V. For Defr1, data was recorded at 5 Torr of He at 300 K with $V_{\text{inj}} = 42$ V. Measurements were taken at $V_d = 90$ V, 60 V, 45 V, 30 V and 20 V.

### 4.2.2 Results

Table 4.4 shows measured cross sections for all peptides studied.

<table>
<thead>
<tr>
<th>Charge</th>
<th>Defr1</th>
<th>Defr1 Glt</th>
<th>Defr1 Red</th>
<th>Defr1 Y5C</th>
<th>Defr1 1Cys</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>-</td>
<td>513 Å²</td>
<td>551 Å²</td>
<td>523 Å²</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>595 Å²</td>
<td>581 Å²</td>
<td>538 Å²</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>642 Å²</td>
<td>652 Å²</td>
<td>605 Å²</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>1071 Å²</td>
<td>-</td>
<td>735 Å²</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>1242 Å²</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1332 Å²</td>
</tr>
<tr>
<td>8</td>
<td>1342 Å²</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1401 Å²</td>
</tr>
<tr>
<td>9</td>
<td>1430 Å²</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1520 Å²</td>
</tr>
<tr>
<td>10</td>
<td>1406 Å²</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1566 Å²</td>
</tr>
<tr>
<td>11</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1620 Å²</td>
</tr>
</tbody>
</table>

Table 4.4: Experimental cross sections of Defr1 and related peptides.

### 4.2.3 Discussion: Defr1 Dimers

As no reliable computational structures are available for these peptides, we will focus here on differences between the patterns seen for each peptide.

* P.E. Barran, unpublished results
Comparison of Defr1, Defr1 1Cys and Defr1 Y5C will provide insight into the influence of intra-molecular disulphide bonds on conformation. Unfortunately, non-covalent dimers were not observed for any of three monomers studied despite previous evidence that Defr1 Y5C exists as a non-covalent dimer. Cross sections for the Defr1 Y5C dimer were therefore estimated by doubling the cross section seen for the monomer species. While this is clearly not a valid method for calculating the cross section of a non-covalent dimer, it is instructive to illustrate the influence of the inter-molecular disulphide bond. Figure 4.3 shows a plot of cross section vs. charge state for these three peptides.

**Figure 4.3:** Collision cross sections of Defr1 related dimers at different charge states. Defr1 and Defr1 1 Cys are experimentally determined values for covalently bound dimer species while Defr1 Y5C cross sections are extrapolated values for a covalently bound dimer.
Clearly for any one charge state, Defr1 is a more compact ion than Defr1 1Cys and the putative Defr1 Y5C dimer is more compact than both species.

If we first examine the Defr1 and Defr1 1Cys data we can see that for the lower charged species, the two molecules can be seen to behave fairly similarly, showing a similar increase in cross section with charge: for ±7 to +9 Defr1 increases by 15% while Defr1 1Cys increases by 14%. However, at +10 the cross section of Defr1 was seen to flatten while the cross section of Defr1 1Cys was seen to continue to increase fairly linearly with charge.

We can interpret these data in terms of the influence of intra-molecular disulphide bonds which conformationally restrict Defr1 in comparison to Defr1 1Cys. In lower charge species this influence is only apparent in terms of the slightly larger cross section measured for Defr1 1Cys (5% to 8%) as would be expected for a less restrained peptide. This finding is similar to that shown for Lysozyme by Valentine et al\textsuperscript{10} where they showed the disulphide reduced form to present a considerably larger cross section than the disulphide intact form.

The apparent drop in cross section from +9 to +10 for Defr1 is perhaps more interesting, the peptide has reached its most extended form at +9. Up until the +9 charge state, the increase in cross section can be explained in terms of coulombically driven unfolding of the secondary and tertiary structure of the peptide. For Defr1 1Cys, this process is allowed to continue with the addition of a tenth and then eleventh charge; the presence of the intra-molecular disulphide bonds in Defr1 does not allow this unfolding to continue. It is tempting to interpret the slight drop in cross section as a structural rearrangement of the peptide with the addition of the tenth charge. It is also possible that the drop in cross section is merely an experimental error, the cross sections measured at +9 and +10 are within 2% of each other, a reasonable estimate of the experimental error. Without reliable molecular modelling structures it is not possible to confirm which hypothesis is correct.
Turning to the Deflr Y5C data, the cross sections obtained for the putative dimer are smaller than those obtained for Defrl with the difference more pronounced at higher charge (~3% at +6, 20% at +8 and 15% at +10). This difference highlights two important factors: firstly the influence of a third intermolecular disulphide bond; secondly – and more clearly – the difference between a covalently bound dimer and a non-covalently bound dimer.

From +6 to +10 for Defrl there is an increase in cross section of 30%, for Defrl Y5C the increase is only 16% indicating Defrl Y5C is a more restrained peptide than Defrl. Looking at the sequence, this is unsurprising. The first cysteine – and hence disulphide bond - in Defrl Y5C is residue 5 with the final cysteine occurring one residue from the end; this means almost the entire peptide is within the disulphide restrained region. Defrl, however, has no cysteine until residue 12 meaning a far larger proportion of the peptide is unrestrained by disulphide bonds – this is supported by the far greater increase in cross section seen for Defrl.

The large difference in cross section between these two species is not only due to an extra intra-molecular disulphide bond, the inter-molecular disulphide bond in Defrl must also have some influence over this. To explain this fully it is first necessary to examine the data obtained for the three monomer species.

### 4.2.4 Discussion: Defrl Monomers

Figure 4.4 is a plot of cross section against charge for Defrl Git, Defrl Red and Defrl Y5C.
4. Ion Mobility Studies of β-Defensins and Defensin-Related Peptides

**Figure 4.4:** Experimental collision cross sections of Defr1 Glt, Defr1 Red and Defr1 Y5C

At this stage it is worth reiterating the differences between these three peptides: Defr1 Glt is a monomer of Defr1 with two intra molecular disulphide bonds with a mixture of connectivities (two or three) and a glutathione capping group on the penultimate cysteine; Defr1 Red is a monomer of Defr1 with no disulphide bonds i.e. all the cysteines present are reduced; Defr1 Y5C is a mutant of Defr1 in which the fifth residue – a tyrosine – has been replaced with cysteine, it contains three disulphide bonds of canonical connectivity.

It is immediately apparent that Defr1 Red and Defr1 Y5C behave in a very similar way. For Defr1 Red the cross section increases by 5% between +3 and +4 and by 12% between +4 and +5; for Defr1 Y5C the cross section increases by 3% between +3 and +4 and by 12% between +4 and +5. This indicates that intra-molecular disulphide bonds are not influencing the degree of charge driven unfolding seen for Defr1 Y5C at these charge
states. We do see, however, that the cross sections obtained for Defr1 Red are around 8% larger than those measured for Def1 Y5C indicating that the disulphide bonds confer a more compact structure on the peptide. This is of course in good agreement with what was seen for Defr1 and Defr1 1Cys.

The data obtained for Defr1 1Glu show slightly different behaviour for this peptide. It shows a larger increase from +3 to +4 (16%) followed by a slightly smaller increase between +4 and +5 (8%). This is perhaps best explained with reference to DEFB107 which also contains a glutathione capping group. For DEFB107, we saw from modelling that the glutathione group had a great influence on the structures adopted with the peptide folded around the capping group. The large increase in cross-section between +4 and +5 measured for DEFB107 can be attributed to a loss of the interaction between the peptide and capping group. It is therefore tempting to assume Defr1 1Glu behaves similarly: the lowest charged species interacting strongly with the glutathione group and hence giving a smaller cross section than Defr1 Y5C; moving from +3 to +4 the interaction is lost cross section increases, it then unfolds with charge in a similar fashion to the other two species studied.

The presence of the glutathione group makes it difficult to directly compare Defr1 Glt with Defr1 Y5C and Defr1 Red (and hence gain insight into the influence of the 1-5 disulphide bond in Defr1 Y5C) but if the data are normalised to cross section per residue (i.e. the cross section is divided by the number of residues present), Defr1 Y5C and Defr1 Red are seen to give similar values for their +4 and +5 charge states perhaps indicating that the more compact geometry seen for Defr1 Y5C can be as easily inferred by two disulphide bonds as it can by three. Table 4.5 summarises this normalised data for the three monomer peptides.
Table 4.5: Normalised cross sections for Defrl monomers. All values are obtained by dividing measured cross section by number of residues and are in Å² per residue.

<table>
<thead>
<tr>
<th>Charge</th>
<th>Peptide</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>+3</td>
<td>Defrl Glt</td>
<td>13.9 ± 0.4</td>
<td>16.2 ± 0.5</td>
<td>15.4 ± 0.5</td>
</tr>
<tr>
<td>+4</td>
<td>Defrl Red</td>
<td>16.1 ± 0.5</td>
<td>17.1 ± 0.5</td>
<td>15.8 ± 0.5</td>
</tr>
<tr>
<td>+5</td>
<td>Defrl Y5C</td>
<td>17.4 ± 0.5</td>
<td>19.2 ± 0.6</td>
<td>17.8 ± 0.5</td>
</tr>
<tr>
<td>+6</td>
<td></td>
<td></td>
<td>21.6 ± 0.6</td>
<td></td>
</tr>
</tbody>
</table>

From the above work, we would not necessarily expect to see the great difference between the cross sections of Defrl and a Defrl Y5C dimer. The larger cross sections observed for Defrl must therefore be related to the inter-molecular disulphide bond. For a non-covalent dimer, the interactions between the two monomers must be between the peptide backbones and/or the side-chains. In order to maintain a stable dimer there are usually several important interactions and therefore the two monomers are quite restrained. For a covalently bound dimer, the only required interaction between the two monomers is the covalent linker, in this case the disulphide bond. Each Defrl monomer will therefore be able to flex and rotate about the intermolecular disulphide bond allowing it to form a more open structure hence the larger cross section measured.

While comparing cross sections of peptides at different charge states gives a good indication of the over degree of flexibility of each peptide, it is also worth quantifying this for each individual charge state. This can be done by comparing the widths of the ATDs obtained for different peptides under the same conditions – a wider peak indicating a more flexible molecule. Table 4.6 shows the widths of the ATDs obtained for the Defrl monomers. The data were obtained by measuring the full width at half height (FWHH) of each peak obtained at $V_d = 60 \, \text{V}$.
### Table 4.6: FWHH of ATDs of Defr1 monomers at $V_d = 60$ V.

<table>
<thead>
<tr>
<th>Charge</th>
<th>Defr1 Glt</th>
<th>Defr1 Red</th>
<th>Defr1 Y5C</th>
</tr>
</thead>
<tbody>
<tr>
<td>+3</td>
<td>124 ± 4 μs</td>
<td>118 ± 4 μs</td>
<td>75 ± 2 μs</td>
</tr>
<tr>
<td>+4</td>
<td>88 ± 3 μs</td>
<td>106 ± 3 μs</td>
<td>65 ± 2 μs</td>
</tr>
<tr>
<td>+5</td>
<td>90 ± 3 μs</td>
<td>101 ± 3 μs</td>
<td>60 ± 2 μs</td>
</tr>
<tr>
<td>+6</td>
<td>-</td>
<td>77 ± 2 μs</td>
<td>-</td>
</tr>
</tbody>
</table>

For any one peptide, it is expected that the peak width should decrease with increasing charge – more highly charged species spend less time in the drift cell than lower charged species hence there is less peak broadening due to diffusion. This trend holds for all ions studied. It is interesting to note, however, there is a comparatively large drop in peak width from +3 to +4 for Defr1 Glt indicating a change in conformational flexibility or the presence of multiple unresolvable conformations. The +3 to +4 transition has been assigned as a point at which there is a large conformational change for this species, these data would seem to support that finding.

The general trend from these data is that Defr1 Red is more flexible than Defr1 Glt which is in turn more flexible than Defr1 Y5C. This seems logical from what we know about the peptides in that conformational flexibility is related to the number of intra-molecular disulphide bonds present – zero, two and three respectively.

Defr1 is a mutant form of the murine defensin MBD-8 found in black 6 mice\(^1\). MBD-8 has six cysteine residues and is therefore an orthologue of Defr1 Y5C; the sequences of these two peptides are shown below.

**Defr1 Y5C:**
*DPVTClRNGGICQYRCIGLHKGTCGSPFKCCK*

**MBD-8:**
*NEPVSICRNGGICQYRCIGLHKGTCGSPFKCCK*
The sequence alignment between Defr1 Y5C and MBD-8 reveals the presence of three mutations in the N-terminus of Defr1 Y5C as compared to MBD-8: (i) the loss of N-terminal asparagine 1; (ii) glutamate 2 to aspartate; and (iii) serine 5 to threonine.

The NMR structure of MBD-8 has previously been determined by Bauer et al. who demonstrated it to have the 'defensin fold' described previously in this section. Given the large degree of sequence homology between the peptides it is reasonable to assume the Defr1 Y5C and MBD-8 will have very similar solution structures. The gas phase collision cross sections of Defr1 Y5C can therefore be compared to the NMR structure of MBD-8; a collision cross section was calculated for the 20 solution phase structures of this molecule using the Mobcal program.

Using trajectory methods the average collision cross section calculated was 666 Å² (ranging from 625 to 701 Å²) as compared to cross sections of 523 Å², 538 Å² and 605 Å² for the +3, +4 and +5 charge states of Defr1 Y5C i.e. the solution structure of MBD-8 has a larger cross section than one might expect for Defr1 Y5C.

Figure 4.5 shows the NMR structure of MBD-8. The core of the peptide containing the regions of defined secondary structure can be seen to be restricted by the disulphide bonding. The five N-terminal residues, however, are unrestrained by these interactions allowing this section of the peptide a larger degree of conformational freedom than the rest of the peptide – this region of the peptide can be seen to be unrestrained in the NMR structures. As this is the region where the mutations occur in Defr1 Y5C a change in the flexibility of this region may account for the smaller than expected cross section.

It is also worth remembering that MBD-8 contains one more residue than Defr1 Y5C, to better compare the two molecules the cross section calculated for MBD-8 will be mass-normalised to Defr1 Y5C. This gives a normalised collision cross section of 646 Å² for MBD-8.
Figure 4.5: NMR structure of MBD-8. The figure shows the position of the six cysteine residues, the disulphide bonds connecting them and the areas of defined secondary structure: the three anti-parallel β-sheets of the core and the helical N-terminal section.

To test estimate the importance of the unrestrained region of the peptide on the overall cross section, the 5 n-terminal residues were removed from the structures and the cross sections were recalculated giving an average value of 588 Å². This value is comparable to the cross sections measured for Defr1 Y5C suggesting an interaction between the n-terminal section and the core of the peptide in the gas phase structure.
4.3 Defb14

Defb14, the murine orthologue of HBD-3\textsuperscript{14}, was synthesised in a number of different forms in order to fully study its physical, chemical and biological properties. A number of the peptides created were studied by IMMS on the MoQToF:

i. Defb14: full length Defb14, fully oxidised containing 3 disulphide bonds of the topology described in section 3.2.3.

ii. Defb14 1Cys monomer: full length defb14 with all but the penultimate cysteine mutated to alanine.

iii. Defb14 Ala: full length Defb14 with all cysteines mutated to alanine


v. Defb14 DIP2: the final 23 residues of Defb14 Ala


Experiments were performed on each of these peptides.

4.3.1 Experimental

All peptides were analysed at 40 $\mu$M from 49:50:1 H\textsubscript{2}O:MeOH:CH\textsubscript{3}COOH using ESI with typical capillary voltages of 3.25 kV and typical cone voltage of 65 to 80 V. For the full length peptides a pusher period of 70 $\mu$s was used; for the short defensin inspired peptides (DIPs) a pusher period of 60 $\mu$s was used.

Measurements were performed at 3.5 Torr of He at 305 K with an injection voltage of 35 V. Measurements were taken at $V_d = 60$ V, 50 V, 40 V, 30 V and 25 V.
4.3.2 Results

Table 4.7 shows the experimental collision cross sections obtained for the various Defb14 peptides studied here.

<table>
<thead>
<tr>
<th>Charge</th>
<th>Defb14</th>
<th>Defb14 1Cys</th>
<th>Defb14 Ala</th>
<th>DIP1</th>
<th>DIP2</th>
<th>DIP3 monomer</th>
<th>DIP3 dimer</th>
</tr>
</thead>
<tbody>
<tr>
<td>+2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>+3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>465 Å²</td>
<td>444 Å²</td>
<td>434 Å²</td>
<td>-</td>
</tr>
<tr>
<td>+4</td>
<td>709 Å²</td>
<td>-</td>
<td>-</td>
<td>546 Å²</td>
<td>486 Å²</td>
<td>491 Å²</td>
<td>666 Å²</td>
</tr>
<tr>
<td>+5</td>
<td>751 Å²</td>
<td>916 Å²</td>
<td>-</td>
<td>-</td>
<td>585 Å²</td>
<td>580 Å²</td>
<td>758 Å²</td>
</tr>
<tr>
<td>+6</td>
<td>881 Å²</td>
<td>962 Å²</td>
<td>979 Å²</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>827 Å²</td>
</tr>
<tr>
<td>+7</td>
<td>1008 Å²</td>
<td>1018 Å²</td>
<td>1070 Å²</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>971 Å²</td>
</tr>
<tr>
<td>+8</td>
<td>-</td>
<td>1105 Å²</td>
<td>1170 Å²</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 4.7: Experimental cross sections of Defb14 and related peptides.

4.3.3 Discussion: Full Length Peptides

Figure 4.6 is a plot of cross section against charge state for the three full length peptides studied here.
Figure 4.6: Plot of experimental cross sections of full length Defb14 and related peptides analysed from 49:49:2 H$_2$O:MeOH:CH$_3$COOH. The dotted line represents the average cross section of HBD-3 taken from the NMR structures.

The data shown here shows Defb14 behaves in a similar fashion to Defr1 in that the disulphide intact form (Defb14) unfolds with charge in a similar fashion to the no-disulphide form (Defb14 Ala) i.e. fairly linearly with charge. Once more the presence of intra-molecular bonds can be seen to give rise to a more compact form of the peptide.

The behaviour of Defb14 1Cys is perhaps more interesting. In terms of atomic structure it is practically identical to Defb14 Ala, the only difference being the presence substitution an −SH for an H at residue 40. For this reason, it would be expected that the two molecules would behave in a similar fashion. This hypothesis holds at lower charge.
4. Ion Mobility Studies of β-Defensins and Defensin-Related Peptides

states (+5 and +6) but at higher charge Defb14 lCys behaves more like Defb14. This is an interesting finding and suggests the presence of the –SH group allows the molecule to form different intra-molecular interactions allowing it to adopt more compact gas phase structures. This perhaps gives us some insight into the different behaviour of this molecule in vivo; Defb14 lCys was shown to be active chemotactically while Defb14 Ala is significantly less active. It is thought that in order for chemotaxis to occur the peptide must adopt a specific conformation, this does not occur for Defb14 Ala but does for Defb14 lCys – further study of these two peptides by IMS and molecular modelling may allow us to discover the structural importance of the Ala to Cys substitution.

It is interesting to note that despite the presence of two, distinct disulphide bonding topologies for Defb14, there is only one resolvable conformer obtained from the ion mobility measurement. This is of course in agreement with the data obtained for Defr1 where one conformation is observed despite the presence of more than one disulphide bonding topology. Once more, it may be interesting to examine the peak widths of each of the three full length species. Table 4.8 shows the peak widths of these three species at V\textsubscript{d} = 60 V.

<table>
<thead>
<tr>
<th>Charge</th>
<th>Peptide</th>
<th>Defb14</th>
<th>Defb14 lCys</th>
<th>Defb14 Ala</th>
</tr>
</thead>
<tbody>
<tr>
<td>+4</td>
<td></td>
<td>85 ± 3 µs</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>+5</td>
<td></td>
<td>75 ± 2 µs</td>
<td>73 ± 2 µs</td>
<td>-</td>
</tr>
<tr>
<td>+6</td>
<td></td>
<td>72 ± 2 µs</td>
<td>86 ± 3 µs</td>
<td>53 ± 2 µs</td>
</tr>
<tr>
<td>+7</td>
<td></td>
<td>82 ± 3 µs</td>
<td>90 ± 3 µs</td>
<td>56 ± 2 µs</td>
</tr>
<tr>
<td>+8</td>
<td></td>
<td>-</td>
<td>79 ± 3 µs</td>
<td>63 ± 2 µs</td>
</tr>
</tbody>
</table>

**Table 4.8:** ATD peak widths for full length Defb14 peptides.
For Defb14, the ATD peak width decreases with charge from +4 to +6 before increasing again. The ATD widths measured are larger for Defb14 than for Defb14 Ala i.e. Defb14 occupies a greater amount of conformational space than Defb14 Ala. The presence of intra-molecular disulphide bonds in Defb14 confers a more compact geometry on Defb14 in comparison to Defb14 Ala and one might assume the disulphide bonds would restrict the molecule more leading to narrower peaks. If we take this to be the case, then the width of the ATD must be due to the presence of multiple conformers of similar collision cross section. In section 3.2.3, it was shown that Defb14 exists in two different disulphide topologies, these may result in two slightly different gas phase structures giving rise to a more diffuse ATD. The IM resolution of the MoQToF is around 20, this is clearly too low to separate any possible multiple conformers seen here but by studying the same system on a high resolution ion mobility instrument it may be possible to separate two disulphide topoisomers.

For comparison, the average cross section of the 20 NMR cross sections of HBD-3 is included in figure 4.6. This is 883 Å², very close to the value measured for +6 Defb14. Clearly the two species are not directly comparable due to the difference in sequence and disulphide topology but the agreement does suggest the measurement is valid.

4.3.4 Discussion: Defb14 DIPs

Figure 4.7 is a plot of collision cross sections obtained for DIP1, DIP2 and DIP3.
The data shows that the two c-terminal peptides (DIP2 and DIP3) are more compact than DIP1, the n-terminal peptide. The cross sections of all three peptides increase fairly linearly with charge.

For the full length Defb14 peptides, it was shown that Defb14 1Cys behaved differently to Defb14 Ala despite there only being a minor change to the side chain of one amino acid. For DIP2 and DIP3, the same change has been made i.e. Ala to Cys. In contrast to what was shown for the full length peptides, DIP2 and DIP3 give virtually identical cross sections for all coincident charge states (within 2.5%). This suggests that the interaction which causes a change in the behaviour of Defb14 1Cys is between the cysteine residue and the n-terminus of the peptide.
Figure 4.8 shows a mass spectrum obtained for DIP3 from the conditions described in section 4.3.1.

Figure 4.8: Mass spectrum of DIP3 from 49:49:2 H$_2$O:MeOH:CH$_3$COOH. The poor signal-to-noise seen for the spectrum illustrates the loss of transmission seen for the IMMS configuration.
There is clear evidence for the presence of both monomer and dimer species in this mass spectrum. As DIP3 has a reduced cysteine residue it is tempting to assume the dimer present is covalently linked, however, if harsher source conditions are used (higher cone voltage etc.) the dimer dissociates easily suggesting a non-covalent dimer; the mass data - although not high enough resolution or mass accuracy for absolute confirmation – would seem to support this assertion.

Figure 4.9 shows a plot of the cross sections obtained for the DIP3 monomer, dimer and, for clarity, the cross sections for a putative dimer of DIP3 obtained by doubling the cross sections obtained for the +2 and +6 monomers.

![DIP3 Cross Sections](image)

**Figure 4.9**: Cross sections of DIP3 monomer and dimer from 49:49:2 H₂O:MeOH:CH₃COOH.
We can see that, in contrast with the Defrl dimer data shown previously, the dimer cross sections obtained for DIP3 are more compact than twice the monomer cross section. This supports the assertion that the DIP3 dimer is non-covalent as oppose to covalent; the apparent drop in cross section being due to multiple interactions between each monomer as oppose to the single, covalent interaction required to keep Defrl in dimer form. This is analogous to the drop cross section seen by Wyttenbach et al$^{15}$ for the Bradykinin dimer, which can only be held together by non-covalent interactions.
4. Conclusions

Ion mobility studies of a number of different defensin molecules and related peptides have been presented. This represents the first concerted effort at studying the structures of these species in the gas phase.

Thanks to previous studies at UCSB\textsuperscript{2}, DEFB107 was an ideal test molecule for these studies. Initial experiments at lower buffer gas pressures demonstrated the importance of reaching a constant drift velocity on entering the cell. At these lower pressure the ions do not start to ‘drift’ until they are several millimetres into the cell; this causes the drift time to be reduced and hence the measured cross-section is underestimated. Repeating these experiments at higher pressure gave good agreement with previous results indicating the ions drift for the entire length of the cell and hence validating the MoQToF as a tool for these studies.

A number of DefrI related peptides were studied yielding important information about the importance of disulphide bonds on conformational flexibility. From the dimer studies we see that the presence of an intermolecular disulphide bond in DefrI and DefrI 1Cys (forming a covalent dimer) allows a more extended conformation to be adopted than might be expected for a non-covalent dimer. This indicates that at the charge states studied the interactions between the monomers are dominated by the covalent intermolecular bond; it is only at the lowest charge state (+6) that non-covalent interactions can be seen to have an influence on the conformation.

The influence of intramolecular disulphide bonds is illuminated by all the DefrI species studied. DefrI Y5C has three intramolecular disulphide bonds – it forms the most compact monomer gas-phase ions. DefrI Red has no disulphide bonds – it forms the most extended monomer gas phase ions. Similarly, DefrI (two intramolecular disulphide bonds per monomer) forms more compact ions that DefrI 1Cys (no intramolecular disulphide bonds). The presence of intramolecular disulphide bonds constrains the molecules.
Examining these findings in the wider context of the biological activity of these molecules is important. From previous studies\(^1\) we know the intermolecular disulphide bond is vital for the anti-microbial activity of Defr1 (reduced Defr1 is virtually inactive); in contrast the data shown in Appendix 3 (table A3.1) shows that the intramolecular disulphide bonds are not necessary necessary for its anti-microbial activity (Defr1 1Cys is as active as Defr1). It is postulated therefore that the flexibility allowed by the intermolecular disulphide bridge is an important feature of the biological activity of the molecule.

The studies of Defb14 and related peptides reveal the influence of very minor changes in sequence on gas phase structure. Once more the influence of intramolecular disulphide bonds was demonstrated – Defb14 being more compact than the mutants Defb14 1Cys and Defb14 Ala. However, it is change imparted by a cysteine to alanine mutation that is most intriguing. Defr1 1Cys has a cysteine residue at position 40, in Defb14 Ala this residue is replaced by alanine. For the lower charge states studied this had little or no influence on the structure, however, for higher charge states (+7 and +8) it was shown that the presence of the cysteine allows a more compact conformation to be adopted.

Further study of this using only the c-terminal half of Defb14 Ala and Defb14 1Cys (Defb14 DIP2 and Defb14 DIP3 respectively) showed the cysteine residue to have to influence on the cross-section of these species (i.e. DIPs 2 and 3 have essentially the same cross-sections at all charge states studied). This indicates that there must be an important interaction between the c-terminal cysteine and the n-terminal half of Defb14 which allows a more compact geometry to be maintained for Defb14 1Cys compared with Defb14 Ala. Once more this is echoed in a biological context where Defb14 1Cys has been shown to be chemotactically active while Defb14 Ala is not\(^1\).

References:

4. Ion Mobility Studies of β-Defensins and Defensin-Related Peptides

11 http://nano.chem.indiana.edu/Software/mobca1.txt
5. Defensin – Glycosaminoglycan Interactions by IMMS

In section 3.4, a method was presented in which the binding of a GAG, heparin, to β-defensins was studied using MS. For a number of the Defb14 related peptides the study has been extended to also include ion mobility data. The use of IMMS to study complexes has been discussed previously in section 1.3.2.5.

These studies were performed to gain insight into the conformational effect of heparin binding to a defensin or DIP. For clarity this study has been restricted to Defb14 and some of the Defb14 related peptides studied in section 4.3. The peptides chosen were:

i. Defb14
ii. Defb14 1Cys
iii. Defb14 DIP1

The experiment was also carried out with Defb14 Ala and Defb14 DIP2; no binding was observed for these peptides and the results are therefore omitted from this section.

5.1 Experimental

Samples were prepared as described in section 3.4.1 using a 1:2 peptide:heparin ratio. IM-MS spectra were obtained using ESI with a typical capillary voltage of 3.25 kV and cone voltages of 65 to 85 V.

For Defb14 a pusher period of 67 μs was used, for Defb14 1Cys a pusher period of 60 μs was used and for Defb14 DIP1 a pusher period of 65 μs was used.

Measurements were performed at 3.5 Torr of He at 305 K with an injection voltage of 35 V. Measurements were taken at \( V_d = 60 \text{ V}, 50 \text{ V}, 40 \text{ V}, 30 \text{ V} \) and 25 V.
The experiment was also performed using Defr1 Y5C, DIP2 and Defb14 Ala, however, no binding was seen to occur for these species.

Molecular modelling of the Defb14 DIP1 – heparin complex was carried out in the Amber Suite of programs. The two molecules were built in X-Leap and minimised via a short simulated annealing run generating ten low energy structures. The two molecules were then placed together and 100 low energy structures were generated using the simulated annealing protocol outlined in section 4.1.4.

5.2 Results

Table 5.1 shows cross sections obtained for the complexes of the three peptides studied with heparin.

<table>
<thead>
<tr>
<th>Charge</th>
<th>Peptide</th>
<th>Defb14 + 1 Hep</th>
<th>Defb14 + 2 Hep</th>
<th>Defb14 1Cys + 1Hep</th>
<th>Defb14 DIP1 + 1 Hep</th>
</tr>
</thead>
<tbody>
<tr>
<td>+3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>511 Å²</td>
</tr>
<tr>
<td>+4</td>
<td>726 Å²</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>+5</td>
<td>757 Å²</td>
<td>762 Å²</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>+6</td>
<td>-</td>
<td>-</td>
<td>1006 Å²</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 5.1: Experimental collision cross sections of heparin complexed with Defb14 and related peptides.

5.3 Discussion

The data obtained are best viewed in terms of the differences in cross section between the uncomplexed peptide and the complex.
Figure 5.1: Plot of cross section against charge for Defb14 and Defb14 heparin complexes.

Figure 5.1 shows the cross sections obtained for Defb14 and the observed Defb14 complexes. The complex was observed in +4 and +5 charge states with evidence of one and two heparin molecules binding for the +5 charge state.

For the +4 charge state, the complex gives a collision cross section of 726 Å² around a 2.5% increase in cross section in comparison to the free peptide. This increase in cross section is to be expected as the complex will contain 55 more atoms than the free peptides although a larger increase in cross section may have been expected as previous work on a similar heparin derived disaccharide showed it to have a cross section of ~630 Å². This suggests that there is some conformational tightening of the peptide structure on binding the heparin.
5. Defensin – Glycosaminoglycan Interactions by IMMS

This hypothesis is lent more weight by the cross sections measured for the +5 complex: the free peptide has a cross section of $751 \text{Å}^2$ while the peptide with one heparin bound has a cross section of $757 \text{Å}^2$ and the peptide with two heparin bound has a cross section of $762 \text{Å}^2$ – increases of 0.8% and 1.5% respectively. These are extremely small changes in cross section for such a large change in the make up of the species studied (for the Defb14 + 1 heparin species, the saccharide represents 18% of the mass of molecule). This strongly suggests the peptide is highly conformationally restricted by the heparin. Another explanation is that the presence of heparin brings about a significant rearrangement of the molecule resulting in a similar cross section.

If we again examine the peak widths of the ATDs of each ion studied here we see the conformational space occupied by the ion is reduced for the defensin-heparin complexes (i.e. the ATDs for the complex ions are sharper than those seen for the free peptide). At $V_d = 60 \text{V}$: +4 Defb14 ATD has a FWHH of 98 μs, the complex ATD has a FWHH of 76 μs; +5 Defb14 ATD has a FWHH of 82 μs, the +1 heparin and +2 heparin ATDs have FWHH of 68 μs and 72 μs respectively.

This conformational tightening and stabilisation of the peptide by heparin is analogous to the work of Guo et al. on metallothionein complexes whereby they observed a decrease in cross section of metallothionein with increasing numbers of ligand bound. This was interpreted in terms of peptide stabilisation by the ligand.

Figure 5.2 shows a plot of collision cross section versus charge for Defb14 ICys and its complex with heparin.
The cross section measured for the +6 complex was 1006 Å², an increase of ~5% compared to the cross section measured for the free peptide. The peak widths of these species at $V_d = 60$ V were 103 μs (free peptide) and 92 μs (complex), once more indicating conformational restriction of the peptide by the saccharide. Figure 5.3 shows the ATDs obtained for these two species at $V_d = 60$ V.
Figure 5.3: ATDs of Defb14 1Cys and Defb14 1Cys Complex
at $V_d = 60$ V

Figure 5.4 shows a plot of collision cross section as a function of charge state for DIP1 and the DIP1-heparin complex. The complex was only observed in the +3 charge state, this – perhaps coincidentally – corresponds to the net charge of the DIP3-heparin complex in solution with the peptide having seven positive charges and the saacharide four negative charges. The complex cross section is 511 Å$^2$, approximately 10% larger than the cross section of the free peptide. While this is the largest relative increase in cross section seen for the three peptides studied it is still a remarkably small increase in cross section, especially when considering the disaccharide constitutes 19% of the complex by mass. It is noteworthy that the increase in cross section on binding heparin of 46 Å$^2$ seen here is extremely close to that seen for Defb14 1Cys where an increase of 44 Å$^2$ was observed.
Figure 5.4: Collision Cross Sections of DIP1 and DIP1-heparin complex.

Once more we see a reduction in the ATD width for the complex suggesting a conformational stabilisation of the peptide by the heparin: for the free peptide at $V_d = 60$ V the ATD FWHH was 92 $\mu$s; for the complex under the same conditions the ATD FWHH was 73 $\mu$s.

In section 3.4.2 it was shown that the presence of heparin greatly reduced the activity of Defb14 against both gram-negative and gram-positive bacteria but had a far less pronounced effect on the activity of the other two peptides. It was proposed that the difference seen here was due to a different degree of protection of the heparin from being lost to the cell wall.
Kₐ values measured for each of the three peptides differ by less than a factor of two and are therefore essentially identical i.e. the peptide-heparin interaction is equally as strong for each peptide studied. If the difference in activity in the presence of heparin is indeed related to how easily the heparin can be lost – hence returning the peptide to its original, active form – then the difference between each species is not governed by thermodynamic control.

In terms of increase in cross section on binding heparin it was shown that DIP1 increased the most, followed by Defb14 1Cys with Defb14 showing the least increase in cross section, even with two heparin molecules bound. The relatively small increase in cross section seen for each species suggests a conformational tightening of the peptide on binding heparin. This conformational tightening presumably better protects the peptide-saccharide interaction from disruption by cell membrane components.

As the largest degree of conformational tightening is seen for Defb14 it is not surprising that the presence of heparin has the greatest effect on the activity of this peptide against both gram-negative and gram positive bacteria. The similar degree of conformational tightening seen for DIP1 and Defb14 1Cys suggests both peptides protect the heparin-defensin interaction to a similar degree – this is broadly supported by the similar trends in activity of these peptides in the presence of heparin.

In order to gain further insight into the interaction it was clear that molecular modelling should be carried out. The complexes of the full length Defb14 peptides and heparin are very large systems and the position of the charges on each peptide is non-trivial (Defb14 has 14 basic residues where a charge can be carried; data were obtained for +4 to +7 charge states). Modelling of these systems will therefore require a great deal of work both experimentally and computationally before reliable structures can be generated. DIP1, however, is a far smaller system and the observed complex charge state corresponds to the net charge of the complex in solution thus allowing the complex to be modelled using the solution charges on the peptide and saccharide.
Figure 5.5 shows a gas-phase simulated annealing structure obtained for the free peptide in its solution charge state i.e. +7.

While it is worth noting that this species is not observed experimentally, DIP\(1\) is clearly an unstructured peptide at this charge state. The theoretical cross section for this structure is \(677 \text{ Å}^2\).

Figure 5.6 shows a low energy gas phase structure obtained for the +3 charge state of the DIP1-heparin complex. This theoretical cross section of this system obtained using the exact hard sphere scattering\(^2\) algorithm in Mobcal\(^3\), is \(524 \text{ Å}^2\). This agrees very well with the experimental value of \(511 \text{ Å}^2\).
As suggested by the mobility data, the addition of the heparin to the defensin brings about a conformational tightening of the peptide. The peptide wraps around the heparin enclosing it. The interaction causes the formation of a helical region towards the N-terminus of the peptide between residues 9 and 17. The primary interactions between the peptide and saacharide are ionic interactions between the protonated basic groups on the peptide and the deprotonated acidic groups on the saccharide along with hydrogen bonding interactions between the peptide and saccharide.

Comparing this structure to the solution structure of HBD-3 (the human orthologue of Defb14) reveals a remarkable similarity between the two structures. Figure 5.7 shows an NMR structure of HBD-3 (Schibli et al\textsuperscript{4}).
Figure 5.7: Solution phase structure of HBD-3 solved by NMR spectroscopy. The peptide shows the characteristic ‘defensin fold’ consisting of a core of three β-sheets and an N-terminal helical section.

It can be seen that the key structural motifs of the N-terminal section of HBD-3 are mirrored by the DIP1 – heparin complex. Both structures contain a helix towards the N-terminus followed by a turn; the C-terminus of DIP3 maps quite closely onto the first β-sheet of HBD-3. The heparin molecule takes the place of the second and third β-sheets of HBD-3. Clearly, the presence of the heparin induces a ‘defensin fold’ in DIP1 – a remarkable discovery.
5.4 Conclusions

In chapter 3 there was shown to be a link between heparin binding and defensin activity, further to this it was demonstrated that the presence of heparin has a profound effect on the anti-microbial activity of a number defensins. In this chapter, ion mobility studies of heparin binding to Defb14, Defb14 ICys and Defb14 DIP1 were presented.

Upon addition of a heparin molecule to a peptide, the collision cross-section was seen to increase. The absolute increase in cross-section was seen to be comparable for Defb14 ICys and Defb14 DIP1 (~45 Å²) while it was seen to be much smaller for Defb14, even for the complex between Defb14 and two heparin molecules. The increases in cross section seen for all species observed was much less than might be expected, this is indicative of more compact gas phase conformations being adopted on heparin binding – this is especially pronounced for Defb14.

Relating this to anti-microbial activity in the presence of heparin (table 3.6) we see that Defb14 ICys and Defb14 DIP1 behave similarly showing no change in activity against gram-positive bacteria and a small decrease in activity against gram-negative bacteria; Defb14, however, shows a marked decrease in activity against both gram-negative and gram-positive bacteria in the presence of heparin. In other words the peptide which is most restrained by the heparin is most affected by its presence.

References:

6. Ion Mobility of Other Systems

Work in the group extends beyond the study of β-defensins to include a number of different molecules from small peptides such as LHRH\(^1\) to large protein complexes such as cyclophilin-cyclosporin. Past work in the group has primarily involved the use of mass spectrometry as a probe of these molecules\(^2,3\). In the future further insight into these systems will be provided by performing IM-MS experiments on the MoQToF.

This chapter will focus on some initial work carried out on three different systems: Trp-Cage, a ‘mini-protein’ with a well known structure; Melittin a small peptide present in honey bee venom and Calmodulin, a small calcium binding protein.
6.1 Trp-Cage

Trp-cage\textsuperscript{4,5} is the smallest peptide known to have a defined fold. It has 20 residues and has been shown to fold spontaneously into its native structure\textsuperscript{4}. In the native structure, the Trp6 residue is 'caged' by Gly11, Pro12, Pro18 and Pro 19 with a stabilising salt bridge between Lys8, Asp9 and Arg16. It is a designed peptide based on extendin-4 a 39 residue peptide originally isolated from the Gila Monster lizard.

It has been shown using a fluorescence based technique\textsuperscript{6} that the structure of the +2 ion of this peptide is stable up to $\sim$420 K and the structure of the +3 ion is stable up to $\sim$360 K. In this section, four different mutants of Trp-cage will be studied using IMMS with temperature dependent measurements performed on one of these mutants.

The solution phase structure of wild type Trp-cage is shown in figure 6.1\textsuperscript{4}.
6.1.1 Experimental

Four different Trp-cage mutants were synthesised using solid phase synthesis techniques, the sequences are shown in figure 6.2.
6. Ion Mobility of Other Systems

<table>
<thead>
<tr>
<th>Variant</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>NLYIQWLKDGGPSSGRPPPS</td>
</tr>
<tr>
<td>K8R</td>
<td>NLYIQWLRDGGPSSGRPPPS</td>
</tr>
<tr>
<td>R16K</td>
<td>NLYIQWLKDGGPSSGKPPPS</td>
</tr>
<tr>
<td>DM</td>
<td>NLYIQWLRDGGPSSGKPPPS</td>
</tr>
</tbody>
</table>

**Figure 6.2:** Sequences of Trp-cage wild type and mutants. Mutations are labelled in blue.

The four variants studied are the wild type (WT), a Lys to Arg mutant (K8R), an Arg to Lys mutant (R16K) and a Arg to Lys, Lys to Arg mutant (double mutant – DM).

The four species were studied on the MoQT0F under the same conditions: 3.5 Torr He, 305 K, $V_{inj} = 40$ V with 63 μs pusher period. Solutions were prepared at 40 μM in 50:50 MeOH:H₂O and ionised using ESI with 3.5 kV capillary voltage and 70 V cone voltage. Measurements were taken at $V_d = 60$ V, 55 V, 50 V, 45 V, 40 V, 35 V and 30 V.

Further experiments were performed on DM at 340 K, 375 K and 395 K.

### 6.1.2 Results

Figure 6.3 shows a typical mass spectrum obtained for WT under these conditions. Note the dominance of the single sodium and single potassium adducted peaks for the 3+ species.
Figure 6.3: ESI mass spectrum of Trp-cage WT.

Table 6.1 shows the measured cross sections for each species at 305 K.

<table>
<thead>
<tr>
<th>Charge</th>
<th>WT</th>
<th>K8R</th>
<th>R16K</th>
<th>DM</th>
</tr>
</thead>
<tbody>
<tr>
<td>[M+2H]$^2+$</td>
<td>378 Å$^2$</td>
<td>386 Å$^2$</td>
<td>380 Å$^2$</td>
<td>382 Å$^2$</td>
</tr>
<tr>
<td>[M+3H]$^3+$</td>
<td>401 Å$^2$</td>
<td>412 Å$^2$</td>
<td>394 Å$^2$</td>
<td>-</td>
</tr>
<tr>
<td>[M+2H+Na]$^3+$</td>
<td>403 Å$^2$</td>
<td>406 Å$^2$</td>
<td>392 Å$^2$</td>
<td>393 Å$^2$</td>
</tr>
<tr>
<td>[M+2H+K]$^3+$</td>
<td>400 Å$^2$</td>
<td>401 Å$^2$</td>
<td>394 Å$^2$</td>
<td>390 Å$^2$</td>
</tr>
</tbody>
</table>

Table 6.1: Collision cross sections of four different Trp-cage mutants.

For the high temperature studies of DM, only the +2 species was obtained with sufficient intensity to accurately assign arrival times. Table 5.2 shows the cross sections obtained for +2 DM at the four temperatures studied.
6. Ion Mobility of Other Systems

<table>
<thead>
<tr>
<th>Temperature</th>
<th>305 K</th>
<th>340 K</th>
<th>375 K</th>
<th>395 K</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cross Section</td>
<td>382 Å²</td>
<td>380 Å²</td>
<td>378 Å²</td>
<td>378 Å²</td>
</tr>
</tbody>
</table>

Table 6.2: Collision cross sections of +2 Trp-cage DM at 4 different temperatures.

6.1.3 Discussion

The mass spectrum shown in figure 6.2 is a fairly typical mass spectrum for any of the four species studied in that the dominant species of the spectrum is the +2 and the +3 species ions are dominated by the singly sodiated peak with the fully protonated being the weakest of the three seen (i.e. triply protonated; doubly protonated and single sodiated; and doubly protonated and singly potassiated).

While this is unusual for larger peptides and proteins it is not unexpected for smaller peptides which tend to have less basic groups and therefore less sites for protonation. All four mutants have two basic groups (K and R, K and K or R and R) each and these are assumed to be the sites of protonation for the +2 species. For the +3 species the final protonation site is not clear, however, it is clear that this site must interact more favourably with the alkali metal cations than with a proton hence the adduct species dominate.

Turning to the mobility data, it can be seen that for all four mutants the +2 species is smaller than the +3 species.

Generally the cross sections measured for the three different +3 species (fully protonated; one sodium and one potassium) for each mutant are the same regardless of the third charging group. K8R is a slight anomaly here as the cross section decreases marginally from protonated to sodiated to potassiated.

Looking across the different peptides we see that for the +2 ion, the cross sections are essentially identical – within 2% - for all four mutants. This trend holds less well for the
6. Ion Mobility of Other Systems

+3 ions where the cross sections generally go as: K8R > WT > R16K ~ DM. The variation is small – less than 5% across the series – but consistent.

For the first three mutants the trend would seem to go with the number of arginine residues: arginine has a bulkier side chain than lysine; the largest cross section was measured for the species with the most arginine residues and the smallest for the one with the least. Going by this trend, however, one might have expected Trp-cage DM to have the same cross section as Trp-Cage WT as they both contain one arginine - this does not happen. This then suggests that the presence or absence of an arginine at residue 8 is a more important factor for the structures measured.

To better understand the results seen here, extensive computational studies are under way, examining the gas phase structures and stability of the different Trp-cage mutants. Here we will show a computed structure for +2 Trp-cage WT.

The process used to produce the computational structures is as follows: the +2 peptide was built using the AMBER8 molecular mechanics package with the parm99 force field using the co-ordinates obtained from the NMR structure. The structure was then heated sequentially to 350 K (50 K steps, 20 ps duration). Molecular dynamics of the resulting structures were then run for 10 ns at 350 K. Figure 6.4 shows a computed structure of +2 Trp-cage WT with charges located on the two basic residues.
6. Ion Mobility of Other Systems

Figure 6.4: Molecular mechanics structure of +2 Trp-cage WT at 350 K. The N-terminus of the peptide is to the left with the c-terminus to the right of the figure. Note the retention of the N-terminal helix.

The calculated cross section for this peptide at the end of the molecular dynamics run was 387 Å² in excellent agreement with the measured cross section of 378 Å² for this structure.

For Trp-cage DM, the results at each of the four temperatures studied were essentially identical suggesting that, as suspected, the +2 charge state of this mutant is thermostable up to 395 K. Ideally the experiment would have been performed at a number of higher temperatures in order to study the thermal unfolding of the peptide. This was not possible due to the failure of an internal solder at higher temperature.
6.2 Melittin

Melittin is a small, cationic peptide that is the major component of honey bee (Apis mellifera) venom\textsuperscript{8}. It shows a number of biological activities including increasing cell permeability and haemolysis\textsuperscript{9}; the activity it shows is thought to be directly related to its interaction with and perturbation of lipid bilayers\textsuperscript{10}.

Previous work by a number of groups has shown melittin to exist in two forms: in aqueous solution it adopts a random coil structure; in a membrane environment it adopts a helical structure\textsuperscript{11} thought to involve the formation of a tetramer\textsuperscript{12}. The helical structure has also been shown to form in methanol\textsuperscript{10} and high salt concentration\textsuperscript{13}. The methanol monomer structure\textsuperscript{10} shows melittin to exist as two \( \alpha \)-helices with a ‘kink’ between them at Pro14. The sequence of melittin is shown in figure 6.5.

\begin{center}
\textbf{Figure 6.5: Melittin Sequence}
\end{center}

\begin{center}
\begin{tabular}{c}
10 & 20 \\
GIGAVLKVL TGLPALISWI KRKQQ-CONH\textsubscript{2}
\end{tabular}
\end{center}

Current work at Edinburgh is focussed on trying to understand the influence of the environment on the structure adopted by melittin. The peptide is being studied at various concentrations and pHs, in different buffers of varying concentration and in different solvents. These experiments are primarily being carried out using H/D exchange mass spectrometry in both the solution and gas phases.

The molecule has also been studied by CD spectroscopy\textsuperscript{13,14,15,16}, a technique by which the degree of helicity of a peptide or protein can be determined. Figure 6.6\textsuperscript{*} shows CD spectra of melittin under different solvent conditions from 100\% MeOH to 2\% MeOH.

\begin{center}
\textsuperscript{*} H.V. Florance, unpublished results
\end{center}
It can be seen that the helical content of the solution increases with increasing methanol concentration. From 2% to 27% methanol, the peptide exists as primarily random coil; at 52% there is evidence of some alpha-helical structure; the degree of helicity then increases with methanol content to a maximum at 100% methanol. An increase in helicity has been similarly demonstrated for increasing phospholipid concentration.

Clearly solvent composition has a strong influence on the solution phase structures adopted by melittin. The question then arises: can the solution phase structure be preserved into the gas phase? Ion mobility mass spectrometry is clearly the ideal way to answer this question.
6. Ion Mobility of Other Systems

6.2.1 Experimental

Melittin was obtained from Sigma Aldrich (Milwaukee, WI). All solvents were obtained from Fisher Scientific (Loughborough, UK).

The peptide was prepared at 40 μM in the following solvent conditions:

i. 100% H₂O
ii. 75:25 H₂O:MeOH
iii. 50:50 H₂O:MeOH
iv. 25:75 H₂O:MeOH
v. 100% MeOH
vi. 50:50 MeOH:10mM ammonium acetate pH 6.8

Each of these solutions was analysed on the MoQToF using ESI with a capillary voltage of 3.5 kV, a cone voltage of 60 V to 80 V and 35 V injection energy. Measurements were taken at 3.5 Torr He with a pusher period of 60 μs. Data were recorded at V_d = 60 V, 50 V, 40 V, 35 V, 30 V and 25 V.

6.2.2 Results

A typical mass spectrum obtained for melittin is shown in figure 6.7. The dominant charge state seen for all solvent conditions studied here is +4, +3 is seen under all conditions but +5 is only apparent at low methanol concentrations (0% and 25% MeOH). This suggests that in high aqueous conditions melittin has more exposed basic groups than it does in high organic conditions allowing higher charge states to be accessed under these solvent conditions. This finding is in good agreement with the CD data presented above.
6. Ion Mobility of Other Systems

The mass spectrum also shows the presence of the $Y_{13}$ fragment ion of melittin. This fragment is produced by nozzle skimmer dissociation. At high methanol concentration, the $+2$ fragment is the dominant species while the $+3$ ion is the dominant fragment species at high aqueous concentration. Once more, the data suggests the molecule is less structured in a high aqueous concentration solution than in a high organic concentration solution.

![ESI mass spectrum of melittin](image)

**Figure 6.7:** ESI mass spectrum of melittin.

Table 6.3 shows the collision cross sections measured for melittin under each solvent condition.
6. Ion Mobility of Other Systems

<table>
<thead>
<tr>
<th>Charge</th>
<th>0% MeOH</th>
<th>25% MeOH</th>
<th>50% MeOH</th>
<th>75% MeOH</th>
<th>100% MeOH</th>
<th>50% MeOH 50% AmAc</th>
</tr>
</thead>
<tbody>
<tr>
<td>+3</td>
<td>544 Å²</td>
<td>543 Å²</td>
<td>523 Å²</td>
<td>530 Å²</td>
<td>566 Å²</td>
<td>516 Å²</td>
</tr>
<tr>
<td>+4</td>
<td>584 Å²</td>
<td>576 Å²</td>
<td>572 Å²</td>
<td>561 Å²</td>
<td>587 Å²</td>
<td>578 Å²</td>
</tr>
<tr>
<td>+5</td>
<td>605 Å²</td>
<td>602 Å²</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Y₁₃ +2</td>
<td>340 Å²</td>
<td>339 Å²</td>
<td>328 Å²</td>
<td>332 Å²</td>
<td>350 Å²</td>
<td>332 Å²</td>
</tr>
<tr>
<td>Y₁₃ +3</td>
<td>359 Å²</td>
<td>351 Å²</td>
<td>-</td>
<td>337 Å²</td>
<td>356 Å²</td>
<td>362 Å²</td>
</tr>
</tbody>
</table>

Table 6.3: Collision cross sections of melittin from different solvent conditions.

6.2.3 Discussion

The trends in these data do not reveal a clear shift in cross section with solvent concentration that would indicate a large structural change caused by the transition from random coil to α-helix. Figure 6.8 shows the variation of the +3, +4 and Y₁₃ +2 cross sections with increasing methanol concentration.
Figure 6.8: Collision cross sections of melittin at varying methanol concentration.
It can be seen that there is a weak trend followed by each species where the cross section decreases slightly from 0% to 50% methanol before increasing to a maximum at 100% methanol. Interpretation of this trend is best related to molecular modelling structures obtained by the simulated annealing method outlined in section 4.1.4. Figure 6.9 shows the cross sections calculated for the crystal structure of melittin, a structure obtained...
from performing molecular dynamics on the crystal structure at 300K and two minimised simulated annealing structures of the +3 charge state – one compact structure and one extended structure. The simulated annealing structures were obtained by performing calculations in the solution phase using generalised born solvent (GB) to represent the presence of water. The figure also shows the experimental cross sections obtained for +3 melittin from 100% H₂O and 100% MeOH with error bars representing ±1-3%.

It is immediately apparent that the extended GB structure is not sampled by the experiment, the more compact GB structure, however, is in good agreement with the cross section obtained for 100% water. This structure represents the loss of almost all α-helix content of the peptide compared to the crystal structure. From the CD data we expect this to be the case. This suggests that for the low methanol content experiments, the solution phase conformation is preserved into the gas phase. This is perhaps unsurprising, as melittin is fairly unstructured in aqueous solution.

The larger cross section seen for 100% MeOH is more difficult to interpret in terms of the structures shown in figure 6.9. It is clearly more extended than the structure seen for 100% water but not as extended as the crystal structure. This suggests some sort of intermediate structure. Comparing the crystal structure to the compact GB structure we see that the α-helices have unravelled and the molecule has collapsed in on itself to create a more compact geometry. The structure seen from 100% methanol could therefore represent a state where some of the helical structure is lost on transferring the molecule to the gas phase, the unravelling of the helix then allows a more compact geometry to be adopted.

If this is the case then it should be expected that part of the molecule will be more extended than other parts. From previous CID work it is known that the +2 Y₁₃ fragment is a dissociation product of the +3 ion of the full peptide. The presence of the fragment ion therefore allows us to determine the degree of foldedness of the c-terminus of the peptide.

* H.V. Florance & P.E. Barran, unpublished data
Returning to table 6.3 we see for 100% H₂O the +2 Y₁₃ fragment has a cross section of 340 Å², 62% of the cross section of the +3 full length peptide. For 100% MeOH the +2 fragment has a cross section of 332 Å², 59% of the cross section of the +3 full peptide. For both it is clear that the n-terminus is more unfolded than the c-terminus. The cross sections obtained from each solvent are comparable (within 3%) and can be assumed to represent globally similar structures.

Examining the structure obtained for the compact GB calculation, there can be seen to be some retention of the helix in the n-terminus, it is lost entirely in the c-terminus. This agrees nicely with the data obtained for the Y₁₃ fragment.

It is proposed therefore that in 100% MeOH, melittin exists in a crystal-like alpha helical structure; on transfer to the gas phase, the peptide gains three charges – one on the n-terminus, two on the c-terminus. The presence of two charges on c-terminal half of the peptide cause it to lose its helicity, the n-terminal helix is at least partly maintained. To fully test this hypothesis, further molecular modelling must be performed.

Clearly for 100% MeOH, the solution structure is not transferred to the gas phase. To achieve this it may be necessary to use nano-electrospray ionisation. This technique allows a more gentle transfer from the solution to gas phase increasing the chances of efficiently sampling the solution structure, it may also allow access to the +2 charge state of melittin which may be more ‘native-like’.
6.3 Calmodulin

Calmodulin (CaM) is a calcium binding protein expressed in all eukaryotic cells. It is a 148 amino acid protein which has been shown to bind four calcium ions\(^ {17} \). CaM is involved in a huge number of processes within the body including cell growth, nitric oxide synthesis and immunosupression\(^ {18} \). Most of these processes involve the binding of CaM to one or more other proteins within the body, the binding is often calcium dependent e.g. calmodulin will bind to and activate the enzyme nitric oxide synthase only when calcium is present\(^ {19} \). The sequence is shown in figure 6.10 with the calcium binding regions highlighted in blue.

Apo CaM (i.e. calmodulin without calcium present) is comprised of two globular domains joined by a flexible linker; each domain consists of two helix-loop-helix metal-binding motifs known as EF hands\(^ {20} \). The binding of calcium to these sites induces a structural change in the molecule in which the anti-parallel helices in the domain linking region become parallel and form an \( \alpha \)-helical linker region\(^ {21} \). The NMR structures of the apo\(^ {20} \) and holo\(^ {21} \) (i.e. with four calcium ions) forms of CaM are shown in figure 6.11.
The large change in conformation from apo to holo should be easily observable by ion mobility; this section will focus on some preliminary work to probe this structural change.

### 6.3.1 Experimental

CaM was obtained from the research group of Dr. Simon Daff (Edinburgh, UK). It was desalted and purified on a PD1O column, lyophilised and brought up in 10mM ammonium acetate, pH 6.8 (Sigma Aldrich, Milwaukee WI) to a concentration of 150 μM.

The resulting solution was incubated at room temperature overnight with 5 mM calcium acetate (Sigma Aldrich, Milwaukee, WI) to allow the CaM to become fully holo (i.e. bind four Ca$^{2+}$). This solution was then diluted down in 49:49:2 H$_2$O:MeOH:CH$_3$COOH to a final concentration of 40 μM.
6. Ion Mobility of Other Systems

The solution was analysed on the MoQToF with a capillary volatage of 3.5 kV and a cone voltage of 75 V. The cell was held a 3.5 Torr He, 305 K using an injection energy of 40 V. Measurements were taken at $V_d = 60$ V, 50 V, 40 V and 35 V. A pusher period of 98 μs was used.

6.3.2 Results

Figure 6.12 shows a typical mass spectrum obtained for the holo-CaM sample.

The addition of acid to the sample denatures the protein. The species observed in the mass spectrum are therefore of higher charge than might have been expected for a holo-CaM spectrum. Further to this, the most abundant ions present correspond to Apo CaM; for all species there is evidence of $\text{CaM} + 1\text{Ca}^{2+}$; for +7 and +8 there is evidence of $\text{CaM} + 2\text{Ca}^{2+}$; for +7 there is evidence of $\text{CaM} + 3\text{Ca}^{2+}$. Clearly the CaM is no longer holo but these data does give insight into the conformational change on adding/losing 1 to 3 $\text{Ca}^{2+}$ to CaM.
Table 6.4 shows the cross sections measured for all observed species

<table>
<thead>
<tr>
<th>Charge</th>
<th>CaM + 0Ca$^{2+}$</th>
<th>CaM + 1Ca$^{2+}$</th>
<th>CaM + 2Ca$^{2+}$</th>
<th>CaM + 3Ca$^{2+}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>+7</td>
<td>1526 Å$^2$</td>
<td>1575 Å$^2$</td>
<td>1390 Å$^2$</td>
<td>(1371 Å$^2$)</td>
</tr>
<tr>
<td></td>
<td>1750 Å$^2$</td>
<td>1805 Å$^2$</td>
<td>1786 Å$^2$</td>
<td></td>
</tr>
<tr>
<td>+8</td>
<td>1655 Å$^2$</td>
<td>1762 Å$^2$</td>
<td>(1882 Å$^2$)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2068 Å$^2$</td>
<td>2088 Å$^2$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+9</td>
<td>1660 Å$^2$</td>
<td>1851 Å$^2$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2022 Å$^2$</td>
<td>2030 Å$^2$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+10</td>
<td>1963 Å$^2$</td>
<td>1912 Å$^2$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>+11</td>
<td>2434 Å$^2$</td>
<td>2285 Å$^2$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>+12</td>
<td>2895 Å$^2$</td>
<td>2830 Å$^2$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>+13</td>
<td>2999 Å$^2$</td>
<td>2998 Å$^2$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>+14</td>
<td>3093 Å$^2$</td>
<td>3072 Å$^2$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>+15</td>
<td>3270 Å$^2$</td>
<td>3254 Å$^2$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>+16</td>
<td>3333 Å$^2$</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 6.4: Experimental collision cross sections for camodulin. Data in brackets were calculated using only two arrival times, all other data were calculated using four arrival times.

6.3.3 Discussion

Figure 6.13 is a plot of cross section against charge for all observed species.
Figure 6.13: Collision cross sections of CaM with 0 (open squares), 1 (open circles), 2 (open triangles) and 3 Ca$^{2+}$ (open star). The dashed line represents the calculated cross section for the crystal structure of apo CaM.

For the lower charge states there are two resolvable conformations, at higher charge there is only one resolvable conformer. Focussing first on the +7 to +9 charge state of the 0 Ca$^{2+}$ CaM we see that the two conformers are separated by ~13% at +7 increasing to ~20% for the +8 and +9 charge states. The +8 and +9 charge states give similar cross sections for both the compact and extended form (within ~2%) indicating similar conformations for each charge state. For the +7 ion the measured cross sections of both geometries is less than the calculated cross section for the crystal structure of apo-CaM.
(1870 Å²). For the +8 and +9 ions the crystal cross section lies between those measured for the two conformers.

For the +10 charge state the cross section measured lies between the two values measured for the +9 ion perhaps indicating that the measured value here is the average of two unresolvable conformers. At higher charge - +11 to +14 – where only a single conformer is present we see the expected increase in cross section with charge.

Turning now to the CaM + 1Ca²⁺ data it can be seen that the measured cross sections for most charge states are similar to those found for the 0 Ca²⁺ CaM with some exceptions: for +7 to +9 we see for the compact form, the +1Ca²⁺ CaM species presents a larger conformation than the 0Ca²⁺ form. This pattern is not repeated for the more extended conformer indicating the calcium has a greater influence on the conformer adopted by the more compact form. For the +11 ion we see the +1Ca²⁺ is ~6% smaller than the 0Ca²⁺; this difference comes in the middle of the largest increase in cross section with charge for the apo-CaM – 32% from +10 to +12 c.f. 6% from +12 to +14 – perhaps indicating that the presence of the calcium partially restrains the peptide.

For the +2Ca²⁺ data it can be seen that once more the addition of a second calcium has a different effect on the more compact conformer as compared to the extended conformer. For the more extended conformer of +7 CaM +2Ca²⁺ we see that once more the calcium bound form has a similar cross section to the calcium free form. The cross section measured for the more compact form of this ion , however, is ~9% smaller than that measured for the 0Ca²⁺ form. This indicates a large conformational change on binding/losing a second Ca²⁺. Only a single resolvable conformer was present for the +8 ion of CaM + 2Ca²⁺; the cross section measured form this lies between the two values measured for the Apo form indicating this is an average cross section of two species.

Interestingly, the cross section obtained for 7+ Cam +3Ca²⁺ is very close to that measured for the more compact +2Ca²⁺ conformer; this was the only observed conformer for this species.
As discussed above, CaM has four binding sites split evenly between two domains. This means that for CaM $+2Ca^{2+}$ there are two possibilities for the location of the calcium ions: (i) both ions on the same domain; (ii) one ion on each domain.

Previous work has shown that the binding in each domain of CaM is cooperative$^{22}$ i.e. if one site on a domain binds calcium then the second site on this domain will also then bind calcium. However, we see from this work and that of others that ESI produces CaM with odd numbers of calciums. If the cooperativity in solution holds (i.e. CaM in solution has either: 0 $Ca^{2+}$; 2 $Ca^{2+}$ on the same domain; or 4 $Ca^{2+}$) then the $+1Ca^{2+}$ and $+3Ca^{2+}$ species observed must be produced in the ionisation process. It is therefore possible that using ESI, one may sample both combinations (described above) of calcium binding for CaM $+2Ca$.

For CaM $+3Ca^{2+}$ one domain must have calcium in both binding sites therefore inducing the associated conformational change in one domain. As the cross section measured for this species is close to that of the more compact $+2Ca^{2+}$ ion then it is clear that this species can be assigned to an ion with both ions bound in the same domain. If we then assume that the more extended form of CaM seen for $+2Ca^{2+}$ corresponds to a form of CaM with the calcium bound in different domains then we have a plausible explanation for the two conformers seen at lower charge. If we hold this hypothesis to be true then the presence of two conformers for the $0Ca^{2+}$ and $+1Ca^{2+}$ forms of CaM at low charge suggests these ions are formed from the $+2Ca^{2+}$ species, presumably in the ionisation process or in the region between the source and the drift cell.

Ultimately it will be desirable to extend these experiments to include the fully holo form of CaM. This was not possible due to the source problems outlined in section 2.6.1.
6.4 Conclusions

In this chapter ion mobility studies of three different systems were presented.

Four different mutants of Trp-cage - a synthetic ‘mini-protein’ - each varying from the next by the number, and position of the basic residues. At room temperature these mutations were seen to have a very minor affect on the cross sections observed. Measurements of Trp-cage DM were also taken at higher temperatures revealing the molecule to be have a stable conformation up to 395 K, in good agreement with computational studies.

Melittin has previously been shown to adopt a helical structure in methanol and a random coil structure in aqueous solvent. In order to understand the influence of solvent on gas phase structure, ion mobility data was obtained using varying amounts of methanol. While there are clearly observable trend in the cross-sections observed for the various species they are weak ones.

The cross sections observed at low methanol content gave good agreement with the compact generalised born solvent (aqueous environment) structure generated by simulated annealing – indicating this structure maintained upon transfer to the gas phase. This structure, as expected, has no \(\alpha\)-helical content.

The cross-sections obtained from 100% methanol are more extended than this but more compact than the calculated cross section of the crystal structure (completely helical) which is thought to be representative of the methanol solution structure. This suggest a degree of collapse of this structure, probably a loss of some of the \(\alpha\)-helical structure. Ion mobility measurements of the \(Y_{13}\) fragment of melittin (the c-terminal half of the molecule) show it to account for more than half of the cross section of the full length peptide. It is therefore concluded that the n-terminal half of melittin retains some helical content on entering the gas phase whilst the c-terminal half largely loses its helical structure.
Ion mobility studies of calmodulin provided a wealth of structural information on species carrying 0 to 3 calcium ions and 7 to 16 positive charges. At lower charge states ($Z < 10$) the protein was seen to adopt more than one conformation: a compact conformation - thought to have at least one domain in a 'native' conformation; and an extended conformation - with both domains 'disordered'. At higher charge, a single conformation is observed.

References:

5. J. Neidigh, R. Fesinmeyer and N. Andersen, Biochemistry, 2001, 40(44), 13188-13200
7. Conclusions

In this work, the development of a new ion mobility mass spectrometer – The MoQToF – has been described. The MoQToF is a modified Micromass Q-ToF 1 with a linear mobility cell between the source hexapole and quadrupole mass analyser. The instrument has been shown to be a reliable IMMS instrument capable of accurately reproducing mobility measurements on a number of previously studied molecules (sections 2.5 and 4.1).

Some improvements to the apparatus’ software, electronics, ion optics and to the cell itself are still required in order for the instrument to work as expected (section 2.6) but initial results are promising.

Studies of β-defensins using IMMS and mass spectrometry have also been presented. FTICR-MS has been shown to be a reliable technique for determining the oxidation states of the cysteines in the peptides and hence determine the number of disulphide bonds in the molecule (section 3.1). Using MS based methods, the disulphide bonding topologies of two β-defensins – DEFB107 and Defb14 – have been determined (section 3.2).

In section 3.3 the interaction between DEFB107 and an artificial membrane has been shown to involve the N-terminal section of the peptide. In published β-defensin structures\(^1\), the N-terminal section of the peptide has been shown to be helical – an ideal structure for membrane interaction. We believe this to support the carpet model of antimicrobial activity\(^2\). Using this model it is proposed that the N-terminus binds to the membrane, anchoring the peptide to the cell surface; when this occurs at high enough concentration the cell membrane integrity is disrupted, causing lysis. It would be desirable to extend this work in the future to include studies of more peptides and crucially more complex artificial membranes containing a variety of lipid and carbohydrate components allowing a better approximation of bacterial membranes.
In section 3.4 a heparin-defensin binding assay is presented. Measured dissociation constants are seen to provide a qualitative scale of defensin activity in that more active peptides bind more strongly to heparin. It has also been shown that the presence of heparin can inhibit the activity of some of the peptides studied indicating a possible method of pathogen defence against anti-microbial peptides\(^3\).

In chapter 5 the interaction has been studied in more detail using molecular modelling and IMMS. The binding of heparin has been shown to stabilise the defensin molecules.

IMMS studies of a number of \(\beta\)-defensins and relate peptides have been presented in chapter 4. This work primarily concentrates on the differences between related peptides. It reveals the influence of disulphide bonding on molecular flexibility. Generally speaking, disulphide bonds restrain the peptides. This may have implications in terms of their purpose. It has been reported that the presence of disulphide bonds can lead to increased protection against proteolysis\(^4\). We see that peptides without disulphides are less conformationally restrained; these peptides in general are more likely to degrade when left at room temperature.

In section 4.1 an IMMS study of DEFB107 is presented\(^4\), this work reveals that the combination of molecular modelling and IMMS to be promising as a method to discover disulphide topologies by analysing the flexibility of the molecule. While this technique will never allow absolute confirmation of a connectivity, it may allow some topologies to be eliminated.

Chapter 6 presents some preliminary studies of three different systems: Trp-cage; melittin; and calmodulin. Much further work is required to fully understand these systems, however, initial results are promising.

References:

7. Conclusions

Appendix 1: MoQToF Drawings

Figure A1.1: Cell housing drawing 1.
Figure A1.2: Cell housing drawing 2.
Figure A1.3: Instrument layout 1.
Figure A1.4: Instrument layout 2.
Figure A1.5a: First half of cell and lens power supply assembly diagram.
Figure A1.5b: Second half of cell and lens power supply assembly diagram.
Figure A1.6a: First half of cell voltage power supplies circuit diagram. This circuit controls the voltages applied to the drift cell (C1 & C2).
Figure A1.6b: Second half of cell voltage power supplies circuit diagram. This circuit controls the voltages applied to the drift cell (C1 & C2).
applied to the lens elements.

Figure A17: Lens voltage power supplies circuit diagram. This circuit controls the voltages.
Figure A1 & L4 circuit diagram.
Figure A1.9: Power supply buffer circuit. This circuit is used to buffer the input voltage from the OTOP (i.e., the collision voltage).
Figure A1.12: Pulser modification circuit. The pulse from the pusher is fed into the clock (lpm_counter0) which is set to output a pulse after a set number of inputs (e.g. 200 pushes). The output pulse from the clock is then fed through a buffer to the pulse generator.
## Appendix 2: Amino Acid Structures and Masses

<table>
<thead>
<tr>
<th>Name</th>
<th>Abbreviation</th>
<th>Molecular Weight (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>Ala</td>
<td>89.0933</td>
</tr>
<tr>
<td>Asparagine</td>
<td>Asn</td>
<td>132.1182</td>
</tr>
<tr>
<td>Cysteine</td>
<td>Cys</td>
<td>121.1593</td>
</tr>
<tr>
<td>Glutamine</td>
<td>Gln</td>
<td>146.1450</td>
</tr>
<tr>
<td>Glycine</td>
<td>Gly</td>
<td>75.0665</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>Ile</td>
<td>131.1737</td>
</tr>
<tr>
<td>Leucine</td>
<td>Leu</td>
<td>131.1737</td>
</tr>
<tr>
<td>Methionine</td>
<td>Met</td>
<td>149.2129</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>Phe</td>
<td>165.1909</td>
</tr>
<tr>
<td>Proline</td>
<td>Pro</td>
<td>114.1232</td>
</tr>
<tr>
<td>Serine</td>
<td>Ser</td>
<td>105.0923</td>
</tr>
<tr>
<td>Threonine</td>
<td>Thr</td>
<td>119.1191</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>Trp</td>
<td>204.2278</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>Tyr</td>
<td>181.1899</td>
</tr>
<tr>
<td>Valine</td>
<td>Val</td>
<td>117.1469</td>
</tr>
<tr>
<td>Aspartic Acid</td>
<td>Asp</td>
<td>133.1023</td>
</tr>
<tr>
<td>Glutamic Acid</td>
<td>Glu</td>
<td>147.1291</td>
</tr>
<tr>
<td>Arginine</td>
<td>Arg</td>
<td>174.2026</td>
</tr>
<tr>
<td>Histidine</td>
<td>His</td>
<td>155.1561</td>
</tr>
<tr>
<td>Lysine</td>
<td>Lys</td>
<td>146.1886</td>
</tr>
</tbody>
</table>

Table A2.1: Table of amino acid codes and molecular weights.
# Appendix 2: Amino Acid Structures and Masses

<table>
<thead>
<tr>
<th>R-Groups</th>
<th>Amino Acid Back-Bone Structure</th>
<th>R-Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine (Gly, G)</td>
<td><img src="image" alt="Glycine Structure" /></td>
<td>Threonine (Thr, T)</td>
</tr>
<tr>
<td>Alanine (Ala, A)</td>
<td><img src="image" alt="Alanine Structure" /></td>
<td>Cysteine (Cys, C)</td>
</tr>
<tr>
<td>Valine (Val, V)</td>
<td><img src="image" alt="Valine Structure" /></td>
<td>Methionine (Met, M)</td>
</tr>
<tr>
<td>Leucine (Leu, L)</td>
<td><img src="image" alt="Leucine Structure" /></td>
<td>Asparagine (Asn, N)</td>
</tr>
<tr>
<td>Isoleucine (Ile, I)</td>
<td><img src="image" alt="Isoleucine Structure" /></td>
<td>Glutamine (Gln, Q)</td>
</tr>
<tr>
<td>Phenylalanine (Phe, F)</td>
<td><img src="image" alt="Phenylalanine Structure" /></td>
<td>Aspartic acid (Asp, D)</td>
</tr>
<tr>
<td>Tyrosine (Tyr, Y)</td>
<td><img src="image" alt="Tyrosine Structure" /></td>
<td>Glutamic Acid (Glu, E)</td>
</tr>
<tr>
<td>Tryptophan (Trp, W)</td>
<td><img src="image" alt="Tryptophan Structure" /></td>
<td>Lysine (Lys, K)</td>
</tr>
<tr>
<td>Serine (Ser, S)</td>
<td><img src="image" alt="Serine Structure" /></td>
<td>Arginine (Arg, R)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Histidine (His, H)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Proline (Pro, P)</td>
</tr>
</tbody>
</table>

Table A2.2: Amino acid side chains.
Appendix 3: β-Defensin Sequences and Activities

Defr1: DPVTY IRNGG ICQYR CIGLR HKIGT CGSPF KCCK
Defr1 Y5C: DPVT C IRNGG ICQYR CIGLR HKIGT CGSPF KCCK
Defr1 1Cys: DPVT A IRNGG IAQYR AIGLR HKIGT AGSPF KCAK

DEFB107: RALIS KRMEG HCEAE CLTFE VKIGG CRAEL AFCCK NR

Defb14: FLPKT LRKFF CRIRG GRCAV LNCLG KEEQI GRCSN SGRKC CRKKK
Defb14 1Cys: FLPKT LRKFF ARIRG GRAAV LNALG KEEQI GRASN SGRKC ARKKK
Defb14 Ala: FLPKT LRKFF ARIRG GRAAV LNALG KEEQI GRASN SGRKA ARKKK
Defb14 DIP1: FLPKT LRKFF ARIRG GRAAV LNA
Defb14 DIP2: AcNH-LNALG KEEQI GRASN SGRKA ARKKK
Defb14 DIP3: AcNH-LNALG KEEQI GRASN SGRKC ARKKK

HBD-2: VFGGI GDPVT CLKSG AICHP VFCPR RYKQI GTCGL PGTKC CKKP

HBD-3: GIINT LQKYY CRVRG GRCAV LSCLP KEEQI GKCST RGRKC CRRKK

Figure A3.1: Sequences of β-defensins studied.
<table>
<thead>
<tr>
<th>Peptide</th>
<th>Anti-microbial Activity (µg/ml)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>P. aeruginosa</td>
<td>B. cepacia</td>
<td>S. aureus</td>
</tr>
<tr>
<td>Defr1</td>
<td>6</td>
<td>&gt;100</td>
<td>12.5</td>
<td></td>
</tr>
<tr>
<td>Defr1 Reduced</td>
<td>50</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td></td>
</tr>
<tr>
<td>Defl Y5C</td>
<td>50</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td></td>
</tr>
<tr>
<td>Defr1 ICys</td>
<td>6</td>
<td>n/a</td>
<td>n/a</td>
<td></td>
</tr>
<tr>
<td>Defb14</td>
<td>1.5</td>
<td>&gt;100</td>
<td>3.13</td>
<td></td>
</tr>
<tr>
<td>Defb14 Reduced</td>
<td>1.5</td>
<td>n/a</td>
<td>n/a</td>
<td></td>
</tr>
<tr>
<td>Defb14 ICys</td>
<td>1.5</td>
<td>&gt;100</td>
<td>3.13</td>
<td></td>
</tr>
<tr>
<td>Defb14 Ala</td>
<td>1.5</td>
<td>n/a</td>
<td>3.13</td>
<td></td>
</tr>
<tr>
<td>Defb14 DIP1</td>
<td>1.5</td>
<td>n/a</td>
<td>3.13</td>
<td></td>
</tr>
<tr>
<td>Defb14 DIP2</td>
<td>&gt;50</td>
<td>n/a</td>
<td>&gt;50</td>
<td></td>
</tr>
<tr>
<td>Defb14 DIP3</td>
<td>&gt;50</td>
<td>n/a</td>
<td>&gt;50</td>
<td></td>
</tr>
<tr>
<td>HBD-3</td>
<td>1.5</td>
<td>&gt;100</td>
<td>3.13</td>
<td></td>
</tr>
</tbody>
</table>

**Table A3.1:** Anti-microbial activities of β-defensins against three selected pathogens: *P. aeruginosa* (gram negative); *B. cepacia* (gram negative, highly defensin resistant); *S. aureus* (gram positive).