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Digital Microfluidic Sample Preparation for Biological Mass Spectrometry

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A Thesis Submitted for the Degree of Doctor of Philosophy

School of Chemistry
College of Science and Engineering
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September 2010
Declaration

I declare that this thesis was composed by myself and that the work contained therein is my own, except where explicitly stated otherwise in the text.

(Adam A. Stokes)
I would like to thank the EPSRC/BBSRC Doctoral Training Centre in Cell and Proteomic Technologies, and the RC-UK Interdisciplinary Research Collaboration (IRColl) RASOR. Radical Solutions for Researching the Proteome for providing the training and funding for this work.

My sincere thanks go to my supervisors; Pat Langridge-Smith and Logan Mackay for their assistance and guidance, and their friendship, also to Anthony Walton, who introduced me to RASOR and the DTC.

I would also like to thank my colleagues both in the Scottish Instrumentation and Resource Centre for Advanced Mass Spectrometry (SIRCAMS) and the Scottish Microfabrication Centre (SMC). In particular I would like to thank Yifan Li for his assistance in manufacturing the silicon DMF devices, and to Jenna, Stefan, Daniel, Mo and Dave for making SIRCAMS a place I have enjoyed working in every day for the last three years.

Finally I would like to thank my wife, friends and family for their unfaltering support during my studies.
Abstract

The use of mass spectrometry in the biosciences has undergone huge growth in recent years due to sustained effort in the development of new ionisation techniques, more powerful mass analysers and better bioinformatic tools. These developments mean that it is now possible to introduce complex crude biological mixtures into a mass spectrometric platform and to obtain detailed information about the sample. The front-end sample handling techniques used for sample preparation have, for the most part, not changed despite the recent advances in hyphenation of liquid chromatography and mass spectrometry required to tackle the issue of increased sample complexity. In this thesis the possibility of using Digital Microfluidics (DMF) for front-end sample preparation prior to mass-spectrometric analysis of protein samples has been investigated. DMF is a micro-electromechanical system (MEMS) technology used for manipulation of sub-microlitre droplets. The movement of discrete droplets of liquid is exploited using the Coulombic forces arising due to free charge polarisation. Droplets can be split, joined, dispensed and moved over a sub-surface electrode array. In this thesis a range of DMF devices have been designed, manufactured and coupled with mass spectrometric platforms for protein analysis. A variety of techniques for mass spectrometry-based analysis of biological samples from the fluidic chips have been investigated. A robotic system has been developed to automate sample introduction, manipulation and removal. Finally the application of on-chip sample purification and enzymatic digestion have been demonstrated, providing proof of concept for digital microfluidic sample preparation in mass spectrometry-based proteomics.
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<tr>
<td>APCI</td>
<td>Atmospheric Pressure Chemical Ionisation</td>
</tr>
<tr>
<td>BCP</td>
<td>Bacterioferritin Comigratory Protein</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CID</td>
<td>Collisionaly Induced Dissociation</td>
</tr>
<tr>
<td>CMP</td>
<td>Chemical-Mechanical Planarisation</td>
</tr>
<tr>
<td>CRM</td>
<td>Charge Residue Model</td>
</tr>
<tr>
<td>DA</td>
<td>Bruker Daltonics DataAnalysis Software</td>
</tr>
<tr>
<td>DEP</td>
<td>Dielectrophoresis</td>
</tr>
<tr>
<td>DESI</td>
<td>Desorption Electrospray Ionisation</td>
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<tr>
<td>DMF</td>
<td>Digital Microfluidics</td>
</tr>
<tr>
<td>ECD</td>
<td>Electron Capture Dissociation</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray Ionisation</td>
</tr>
<tr>
<td>ETD</td>
<td>Electron Transfer Dissociation</td>
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<tr>
<td>EWOD</td>
<td>Electrowetting on Dielectric</td>
</tr>
<tr>
<td>FTICR</td>
<td>Fourier-Transform Ion Cyclotron Resonance</td>
</tr>
<tr>
<td>HCT</td>
<td>High Capacity Ion Trap</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>IEM</td>
<td>Ion Evaporation Model</td>
</tr>
<tr>
<td>IRMPD</td>
<td>Infrared Multiphoton Dissociation</td>
</tr>
<tr>
<td>LC</td>
<td>Liquid Chromatography</td>
</tr>
<tr>
<td>LC-MS</td>
<td>Liquid Chromatography - Mass Spectrometry</td>
</tr>
<tr>
<td>nLC</td>
<td>nano-Liquid Chromatography</td>
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<tr>
<td>MALDI</td>
<td>Matrix Assisted Laser Desorption Ionisation</td>
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<tr>
<td>MS</td>
<td>Mass Spectrometry or Mass Spectrometer</td>
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<tr>
<td>PAGE</td>
<td>Polyacrylamide Gel Electrophoresis</td>
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<td>PECVD</td>
<td>Plasma Enhanced Chemical Vapour Deposition</td>
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<td>PTM</td>
<td>Post Translational Modification</td>
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<td>PS-DVB</td>
<td>Polystyrene-divinylbenzene</td>
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<tr>
<td>TOF</td>
<td>Time of Flight</td>
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Chapter 1
Introduction, Overview and Aims

1.1 Introduction

In this thesis research work undertaken both in the Scottish Instrumentation and Resource Centre for Advanced Mass Spectrometry (SIRCAMS) and the Scottish Microelectronics Centre (SMC) is presented. This work reflects the interdisciplinary nature of the research, a combination of microelectronic engineering, mechanical engineering and biophysical chemistry. Funding for this research was provided via the EPSRC/BBSRC Doctoral Training Centre in Cell and Proteomic Technologies, and the RC-UK Interdisciplinary Research Collaboration (IRColl) RASOR, Radical Solutions for Researching the Proteome; a collaboration between the universities of Glasgow, Edinburgh, Dundee and Strathclyde. The motivation for this research work is detailed below, followed by an overview of the objectives of the project and a summary of the thesis content and layout.

1.2 Motivation

The underlying motivation for this work was the development of a new type of fluid handling technology to automate sample preparation prior to analysis using mass spectrometry\(^1\). The front-end sample handling technology that has been employed in biological mass spectrometry has not changed throughout the many recent advances in mass spectrometer design, sample purification and separation
protocols together with the associated growth of bioinformatic tools. The ability to miniaturise and automate sample processing of crude biological samples on a sub-microlitre scale will facilitate the development of multiplexed and parallel mass spectrometry based proteomic analysis. The parallel processing of multiple samples, and multiplexing of sample preparation steps, will lead to improvements in the number of samples that can be analysed in a given time. Advances in high-throughput techniques such as this are one of the driving forces in proteomics.

1.3 Project Objectives

At the outset of this research programme a list of objectives was drawn up to provide a reference for the ongoing development of each of the different aspects of the project. The overriding objective was to study whether it was possible to use DMF devices for sample preparation in biological mass spectrometry. The development was step-wise, starting with those objectives which appeared to be achievable in the near term and then moving towards more complex and powerful methods. Previous work by the group focussed on the development of single metal layer devices and these were characterised by manipulating droplets of water. Significant developments are presented in this thesis, including the manipulation of biological solutions, buffers and reagents. In addition a multi metal layer process has been developed and devices manufactured. The work that is presented on the integration of DMF and mass spectrometry builds on previous work by Wheeler et al.\textsuperscript{2-4} and the integration of DMF for protein analysis with high-field FTICR MS instrumentation is presented for the first time. The analysis of the time-to-failure of the DMF devices is presented and provides a framework for characterisation of the modes of failure of DMF devices. The final novel contribution is the development of screen-printed disposable DMF devices, which have been designed to be used once and discarded. The list below briefly summarises the main milestones that were achieved in this work.
1. The design and manufacture of silicon based DMF devices, both single and multi metal layer.
2. The design and manufacture of DMF devices using rapid-prototyping.
3. The development of DESI with the aim of direct mass spectrometric analysis from the surface of a DMF device.
4. The integration of DMF devices with mass spectrometric platforms by direct-analysis, DESI and MALDI.
5. The integration of DMF devices with hyphenated liquid chromatography mass spectrometry.
6. The development of on-chip enzymatic hydrolysis of proteins and analysis by LC-MS.
7. The development of on-chip purification using affinity binding of functionalised protein targets.
8. The development of proteomic methodologies using silicon devices.
9. The characterisation of DMF devices and studies of the underlying physics.
10. Experiments leading to the identification of future areas of work.

1.4 Summary of Thesis Content and Layout

This thesis has been written with the bulk of the experimental methods described at the end of the thesis. The thesis has been written in such a way as to provide a basic understanding of mass-spectrometry for engineers and, conversely, for chemists, a brief introduction to microfabrication and electrodynamics. A summary of the content of each chapter is given below:

- **Chapter 2 Introduction to Proteomics and Mass Spectrometry**
  A brief introduction to the concepts of proteomics and mass spectrometry, together with a literature review of proteomic methodologies and mass spectrometric technologies that are pertinent to this body of work.

- **Chapter 3 Theory of Digital Microfluidic Devices**
  A literature review
and introduction to the theory of DMF devices.

- **Chapter 4 Fabrication of Digital Microfluidic Devices** Results of the design, microfabrication and characterisation of a range of DMF devices. The control circuitry for driving the devices is also presented.

- **Chapter 5 Integration of Digital Microfluidic Sample Preparation and Mass Spectrometry** Motivation for using DMF devices for sample preparation in biological mass spectrometry, together with a literature review of previous work in this area. The results from the integration of a variety of analytical techniques that were investigated are presented.

- **Chapter 6 Summary and Conclusions** Summary and overview of the work, including conclusions and possible future developments.

- **Chapter 7 Methods and Materials** Microfabrication process flow, materials used, and an overview of the sample preparation and mass spectrometer methods employed.

- **Appendix & Electrodynamic Theory** Comprehensive summary of the electrodynamics underpinning not only the DMF, but also the interaction of charged particles and electromagnetic fields, as employed in mass spectrometers. Selected LabView programs are included and re-prints of published journal papers are provided.
Chapter 2

Introduction to Proteomics and Mass Spectrometry

2.1 Proteomics

The field of proteomics has rapidly developed following on from the work of the Human Genome Project and is widely regarded as the next chapter in biological discovery. Whilst the genetic blueprint that encodes protein sequences has been studied in detail, the dynamic cellular environment into which these proteins are introduced gives rise to a new level of complexity that is yet to be completely characterised. There are around 30,000 genes in the human genome giving rise to over a million unique proteins, once post translational modifications are taken into account. A major drawback in the analysis of these complex systems is the inability to amplify proteins in a similar way to the Polymerase Chain Reaction (PCR) used for DNA, and therefore highly specific and sensitive techniques are required. The word *proteome* was coined by Wilkins *et al.* in 1996 and reflects a shift in the understanding of the cellular environment. The move from functional genomics (an almost oxymoronic term) to the study of messenger-RNA and towards the complex environment of proteins gave rise to the term *proteomics*. Proteomics can be most optimistically thought of as the study of the total complement of proteins expressed in the cellular environment, and a complete characterisation of their spatial and temporal functions and interactions.
If one thinks of the cell as a ‘black-box’ one can use the engineering approach of perturbation to understand its internal structure and function. One could then derive transfer functions to relate inputs and outputs. It is possible to change the inputs by altering the external environment, by way of drugs or restriction of signalling molecules. The resulting effect on the cell and its outputs can be observed, and function deduced. In the era of genetic engineering it is even possible to change the machinery inside the ‘black-box’ and observe the effects thereof.

Mass spectrometry based proteomics uses a mass spectrometer as the detector for quantitative and qualitative analysis of the proteinious outputs of the cell. Metabolomics, phospho-proteomics, transcriptomics, epigenomics and a range of other so called ‘-omics’ studies look at a variety of the other outputs from the black-box, and employ appropriate separation and detection techniques to achieve their goals. In so far as proteomics is concerned, it remains a multifaceted, rapidly developing and open-ended endeavour.

2.2 Proteins and Protein Structure

Proteins are long single chain polymeric molecules composed of unit-building blocks called amino acids. The sequence of amino acids determines a proteins structure and function. The actual sequence is known as the primary structure, the folding of the amino acid chain in three-dimensional space determines the secondary and tertiary structure, and the combination of multiple proteins to form larger protein complexes is known as the quaternary structure. The twenty most common amino acid residues, their composition, mass and $pK$ values for the carboxylic acid and amino groups, as well as any side chains are listed in Table 2.1. This table has primarily been included for the engineer or non-specialist, who may have little or no knowledge of protein structure. Proteins are linear molecules and have two ends known as the C-terminus and the N-
terminus. Protein sequences start with the N-terminus and progress along listing each amino acid in turn until the C-terminus is reached. The C- and N-termini are so named due to the free −COOH or −NH₂ groups at the start and end of the chain respectively. Proteins range from ~30 to 4000 amino acids in length, the sequence (and the local environment) determines their structure, and the structure determines the function. The two main secondary structure building blocks are the alpha helix and the beta sheet. These structures are formed by specific hydrogen bond interactions of the amino acids, and are held in position by hydrogen bonding networks between carbonyl-oxygens and the N-H in the amide backbone. Tertiary structures are formed by interactions and associations of secondary structure elements. The structure and, therefore, the function of proteins is dynamic and changes both spatially and temporally. The complex environment of the cellular black-box is a challenging environment to probe.

2.3 Biological Mass Spectrometry

In proteomics, the tool of choice is the mass spectrometer. With careful experimental design this tool can yield qualitative and quantitative information about complex biological samples. Whilst proteomic analysis (the spatial and temporal characterisation of the full complement of proteins in a human cell) is not yet close to being realised, the techniques that facilitate mass spectrometric analysis of proteins are in a state of continual development, significant reviews in this field have been written by Aebersold and Goodlett, and Aebersold and Mann.

The invention and development of mass spectrometry began with J.J. Thomson in 1897, with his experiments studying cathode rays, ultimately leading to the discovery of the electron. His experiments involved studying the electrostatic deflection of electron beams in a cathode ray tube and inferring that this was due to the interaction of a negatively charged particle with the electrostatic field; an electron has a mass to charge ratio which is over a thousand times lower than a
Table 2.1: The twenty DNA encoded amino acids residues with their important properties.¹⁵

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Name and composition</th>
<th>Residue structure</th>
<th>Monoisotopic mass¹</th>
<th>Average mass¹</th>
<th>pK₁ α-COOH</th>
<th>pK₂ α-NH₂⁺</th>
<th>pKᵣ Side chain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala</td>
<td>Alanine C₃H₇NO</td>
<td></td>
<td>71.03711</td>
<td>71.07806</td>
<td>2.35</td>
<td>9.87</td>
<td></td>
</tr>
<tr>
<td>Arg</td>
<td>Arginine C₆H₁₂N₄O</td>
<td></td>
<td>156.10111</td>
<td>156.18608</td>
<td>1.82</td>
<td>8.99</td>
<td>12.48</td>
</tr>
<tr>
<td>Asn</td>
<td>Asparagine C₄H₈N₂O₂</td>
<td></td>
<td>114.04293</td>
<td>114.10288</td>
<td>2.14</td>
<td>8.72</td>
<td></td>
</tr>
<tr>
<td>Asp</td>
<td>Aspartic Acid C₅H₇NO₃</td>
<td></td>
<td>115.02609</td>
<td>115.08761</td>
<td>1.99</td>
<td>9.90</td>
<td>3.90</td>
</tr>
<tr>
<td>Cys</td>
<td>Cysteine C₅H₇NO₂S</td>
<td></td>
<td>103.00918</td>
<td>103.14414</td>
<td>1.92</td>
<td>10.70</td>
<td>8.37</td>
</tr>
<tr>
<td>Gln</td>
<td>Glutamine C₅H₈N₂O₂</td>
<td></td>
<td>128.05858</td>
<td>128.12950</td>
<td>2.17</td>
<td>9.13</td>
<td></td>
</tr>
<tr>
<td>Glu</td>
<td>Glutamic Acid C₅H₇NO₃</td>
<td></td>
<td>97.05276</td>
<td>97.11541</td>
<td>2.10</td>
<td>9.47</td>
<td>4.07</td>
</tr>
<tr>
<td>Gly</td>
<td>Glycine C₃H₇NO</td>
<td></td>
<td>57.02146</td>
<td>57.05144</td>
<td>2.35</td>
<td>9.78</td>
<td></td>
</tr>
<tr>
<td>His</td>
<td>Histidine C₆H₁₁NO₂</td>
<td></td>
<td>137.50891</td>
<td>137.13963</td>
<td>1.80</td>
<td>9.33</td>
<td>6.04</td>
</tr>
<tr>
<td>Ile</td>
<td>Isoleucine C₆H₁₁NO</td>
<td></td>
<td>113.08406</td>
<td>113.15791</td>
<td>2.32</td>
<td>9.76</td>
<td></td>
</tr>
<tr>
<td>Leu</td>
<td>Leucine C₆H₁₁NO</td>
<td></td>
<td>113.08406</td>
<td>113.1579</td>
<td>2.33</td>
<td>9.74</td>
<td></td>
</tr>
<tr>
<td>Lys</td>
<td>Lysine C₆H₁₂N₂O</td>
<td></td>
<td>128.09496</td>
<td>128.17260</td>
<td>2.16</td>
<td>9.06</td>
<td>10.54</td>
</tr>
<tr>
<td>Met</td>
<td>Methionine C₅H₉NO₂S</td>
<td></td>
<td>131.04048</td>
<td>131.19738</td>
<td>2.13</td>
<td>9.28</td>
<td></td>
</tr>
<tr>
<td>Phe</td>
<td>Phenylalanine C₆H₉NO</td>
<td></td>
<td>147.06841</td>
<td>147.17424</td>
<td>2.20</td>
<td>9.31</td>
<td></td>
</tr>
<tr>
<td>Pro</td>
<td>Proline C₅H₇NO</td>
<td></td>
<td>97.05276</td>
<td>97.11541</td>
<td>1.95</td>
<td>10.64</td>
<td></td>
</tr>
<tr>
<td>Ser</td>
<td>Serine C₅H₇NO₂</td>
<td></td>
<td>87.03203</td>
<td>87.07746</td>
<td>2.19</td>
<td>9.21</td>
<td></td>
</tr>
<tr>
<td>Thr</td>
<td>Threonine C₄H₇NO₂</td>
<td></td>
<td>101.04768</td>
<td>101.10408</td>
<td>2.09</td>
<td>9.10</td>
<td></td>
</tr>
<tr>
<td>Trp</td>
<td>Tryptophan C₁₁H₁₀N₂O</td>
<td></td>
<td>186.07931</td>
<td>186.21039</td>
<td>2.46</td>
<td>9.41</td>
<td></td>
</tr>
<tr>
<td>Tyr</td>
<td>Tyrosine C₅H₉NO₂</td>
<td></td>
<td>163.06333</td>
<td>163.17364</td>
<td>2.20</td>
<td>9.21</td>
<td>10.46</td>
</tr>
<tr>
<td>Val</td>
<td>Valine C₅H₇NO</td>
<td></td>
<td>99.06841</td>
<td>99.13129</td>
<td>2.29</td>
<td>9.74</td>
<td></td>
</tr>
</tbody>
</table>

¹Neutral mass
hydrogen ion ($H^+$). Continued work on the interaction of charged particles and electromagnetic fields by such significant scientists as Dempster\textsuperscript{18} and Aston\textsuperscript{19} along with work by Mattauch, Bainbridge and Neir led to the development of the modern mass spectrometer.

Figure 2.1 shows a block diagram of a typical mass spectrometer. Each block will be introduced in turn in the following sections. Each block has many variants although the overall sequence changes little from system to system. As a brief overview; the sample is prepared for analysis, this sample is then ionised and transferred into the gas-phase in the source region. The ionised sample is then filtered using the analyser before being identified in the detector. This information is then processed by a computer and a mass spectrum detailing the ionised gas-phase components of the sample can be obtained. The list of peaks in this mass spectrum can then be processed and, in the case of biomolecules, the list can be searched against a database for identification of the starting material.

Figure 2.1: Block diagram of a typical mass spectrometer showing the main source, analyser and detector blocks in a region of increasing vacuum. Also shown are the equally important sample preparation and computer analysis blocks.
Proteins are large macromolecules and the development of soft ionisation techniques for large molecules\textsuperscript{20} facilitated the development of \textit{biological mass spectrometry}. There are two predominant techniques for the soft-ionisation of proteins, namely: Matrix Assisted Laser Desorption Ionisation (MALDI)\textsuperscript{21} and Electrospray Ionisation (ESI)\textsuperscript{22}, though these are by no means the only two ionisation techniques for mass-spectrometry.

There are five basic types of mass analyser currently used in proteomics research. These are the ion-trap, time-of-flight (TOF), quadrupole, orbitrap and Fourier transform ion cyclotron (FT-MS) analysers. They are very different in design and performance, each with its own strength and weakness. These analysers can be stand alone or, in some cases, combined together in tandem to take advantage of the relative strengths of each. Factors such as mass-range and sensitivity led to trade-offs in the choice of detector.

\subsection{2.3.1 Source Ionisation Techniques for Biomolecules}

As previously mentioned the two predominant ionisation techniques in biological mass spectrometry are ESI and MALDI. The invention of these two "soft ionisation" techniques led to their inventors, John Fenn\textsuperscript{22} and Koichi Tanaka\textsuperscript{21} respectively, being awarded the 2002 Nobel Prize in Chemistry along with Kurt Wuthrich. A brief introduction to each technique is given below.

\subsubsection{2.3.1.1 Electrospray Ionisation (ESI)}

Figure 2.2 shows a schematic of a typical electrospray set-up. In this system the sample is kept in solution and is nebulised through a needle with an annular gas flow. A high potential is applied to the needle with reference to the MS-inlet. For positive mode ESI the sample is oxidised in the solution leading to predominantly positively charged species of the liquid phase analyte in the solution. Typical ESI liquid flow rates are between $\sim$3 and 1000 $\mu$l/min.

The mechanism of ESI (see Figure 2.3) can be broken down in to three main
stages. Initially, before any potential is applied, the only forces acting on the liquid-gas interface are the forces due to the liquid pressure and the surface tension. Upon the application of a potential an additional electrohydrodynamic force due to electrophoresis is added (see Section 3.2.3.1). Cations migrate towards the liquid-gas interface and anions are oxidised at the liquid-metal interface. The build up of charge at the liquid-gas interface causes a change in shape from a hemispherical cap to a Taylor cone. The accumulation of positive charges at the surface causes the tip of the cone to become unstable, and to form a jet which then forms parent and satellite droplets. Upon nebulisation of the solution, the droplets containing multiple charged ions begin to desolvate due to the nebulising gas and the solution phase ions enter the gas phase. The exact mechanism has been the subject of intense debate leading to two competing models for the transfer of ions from liquid to the gas phase, these models are the charged residue model and the ion evaporation model. The basic difference in the way these two models are used to explain the gas-phase ion-transfer is as follows; in CRM, the droplets split due to coulombic repulsion overcoming the liquid surface tension, whereas in IEM the ions are ejected directly from a droplet. There are arguments for each of these models though debate continues. Data collected in this thesis, detailing the interaction of liquid droplets and high electric fields, supports the CRM model as described in Section 4.3. Indeed there are many parallels which can be drawn between the mechanisms of ESI and DMF droplet
manipulation. Both involve the interaction of polar liquids and electric fields, and both are heavily dependent on the surface tension of the liquid. ESI is easily hyphenated with liquid chromatography for the MS analysis of complex samples, see Section 2.3.3. With the recent advances in nano-liquid chromatography and its inherent low flow rates, nanoelectrospray\textsuperscript{28} is nowadays commonly used to introduce the sample into a mass spectrometer. Nanoelectrospray differs from conventional ESI in the dimensions and volumes that are used. Typically the nanospray source consists of a pulled glass capillary, with an orifice size of 1-2 \( \mu \text{m} \) and typical flow rates of \( \sim 20 \text{ nl/min} \). A further advantage of nanospray is that no sheath gas is required.

Figure 2.3: Schematic of electrospray ionisation (ESI) process, the fission of droplets is a combination of the charge residue and ion evaporation models, ESI yields multiply charged protein ions.
2.3.1.2 Matrix Assisted Laser Desorption Ionisation

In Matrix Assisted Laser Desorption Ionisation (MALDI)\textsuperscript{21} the sample of interest is mixed with a matrix, and these are co-crystallised onto a target plate. A focused UV-laser (typically 337 nm) is made incident upon the sample. The energy is transferred which ionises the matrix resulting in charge transfer to the analyte molecule. The matrix facilitates ionisation whilst protecting the analyte from the laser. Figure 2.4 shows a schematic of a typical MALDI set-up. A high electric field (\(\sim 20 \text{ kV}\)) is applied between the target plate and the extraction grid. There is usually a delay between ionisation of the sample and acceleration by application of the potential to the extraction grid. This delayed extraction reduces the distribution of kinetic energies in the ion packet, which is of particular importance when using a time-of-flight mass analyser. MALDI is a very sensitive technique and typically it requires sample quantities of less than a picomole. In addition, once a sample has been crystallised into a matrix, it can be stored for a prolonged period of time and then re-analysed. This is a major advantage for samples which are expensive or in low-abundance.

2.3.1.3 Recent Developments in Ambient Ionisation Techniques

Desorption Electrospray Ionisation (DESI) is one of the most exciting developments in mass spectrometry in recent years. It allows the ionisation of solid phase samples deposited on almost any surface and at ambient temperature and pressure. DESI was invented in the laboratory of Professor Graham Cooks at Purdue University\textsuperscript{29}, with subsequent development and improvement by a number of groups around the world, including our own. Most groups have investigated the use of DESI for analysis of small inorganic molecules\textsuperscript{30}, and although some research has been conducted into analysis of biomolecules this has, for the most part, been confined to lipids and peptides\textsuperscript{31–33}.

The possibility of analysing biomolecules, ranging in molecular weight from hundreds to tens of thousands of Daltons and at biologically important tem-
Figure 2.4: Schematic of matrix assisted laser desorption ionisation (MALDI) process, typically the analyte ions are singly charged.
peratures and pressures, means that DESI is potentially a very important and powerful technique for analysing proteins. This could involve simple measurement of the molecular mass or, by using MSMS techniques, elucidation of the amino acid sequence and identification of sites of post translational modifications. Recent publications using ambient pressure ionisation techniques include, DART, ASAP, MALDESI, LAESI and IR-LADESI. Each of these techniques allows ionisation at ambient pressure with little or no sample preparation.

![Figure 2.5: Schematic of desorption electrospray ionisation (DESI) process, DESI involves a conventional ESI sprayer mounted at an angle to the sample surface and directed at the MS inlet](image)

Figure 2.5 is a photograph of the DESI source that was used in this work. It incorporates a conventional ESI sprayer and shows a typical experimental orientation of the critical geometric parameters. The basic operation of DESI is that an electrospray interface is inclined with respect to a sample surface, and highly charged droplets are incident upon the solid sample. These droplets ionise and pick up sample from the surface and then travel into the MS inlet. DESI analysis can be undertaken at atmospheric pressure in the ambient environment.
As in ESI, a potential of several kilovolts is applied between the spray tip and the MS inlet. The actual mechanism of sample ionisation and transport is not very well understood. Venter et al.\textsuperscript{42} studied the effect of nebulising gas pressure on droplet size and velocity using Phase Doppler Particle Analysis (PDPA). In addition they studied the effects of the applied voltage, gas flow rate and solvent flow rate. The results from their work suggested typical parameters of, 4-6 kV spray potential with a 2 l/min gas flow rate and a 2-4 µl/min solvent flow rate.

From an examination of the literature together with the experiments detailed in Section 5.2.1.2, the following list of critical parameters was identified. These parameters include, in no-particular order: $\alpha$ the incident angle, $\beta$ the collection angle, $d_1$ the tip-to-surface distance, $d_2$ the inlet-to-surface distance and, $d_3$ the inlet-to-tip distance. These are in addition to the normal ESI conditions. In Figure 2.6 a schematic for a typical DESI experiment is illustrated and the critical geometrical parameters are labelled.

DESI has been applied to the study of a number of different analytes, ranging from explosives\textsuperscript{30} to illegal drugs\textsuperscript{43–45}. In proteomic studies, however, one of the most significant advantages of DESI over other techniques is that there is no sample preparation required and analysis can be performed outwith a vacuum.
environment. Ambient and low sample-preparation techniques are beneficial in speeding up high-throughput analysis, this being a driving force in true proteomic studies.

2.3.2 Analyser and Detection Techniques

There are a huge variety of analysers and detectors that are used in proteomics based mass spectrometry. Further complexity arises with the addition of hybrid and hyphenated techniques to yield the benefits of multiple stages of separation and fragmentation. The reviews quoted earlier\textsuperscript{14,16} provide a good starting point for the non-specialist to obtain an overview of some of the possible permutations. In the following section a very brief introduction to the analysers that have been used in this of work is provided.

2.3.2.1 Quadrupoles

Wolfgang Paul’s group developed the quadrupole and the ion-trap techniques simultaneously\textsuperscript{46}, both techniques rely on the interaction of charged particles with a quadrupolar electric field. Ion beams entering the quadrupole experience a force determined by the DC and RF potentials applied to the four parallel rods (see Figure 2.7). The force imparted on the charged species can be used to eject ions selectively, allowing transmission of a defined range of m/z values. A linear quadrupole can, therefore, operate either as an ion guide or as a mass filter. Altering the electric field strength and frequency between the opposing hyperbolic rods has the effect of establishing a stability region at the centre of the device. Changing the electric field frequency or amplitude, in accordance with the Mattieu stability diagram allows selective ejection of ions from the device, and subsequent detection. Figure 2.7 shows an end-on view of a quadrupole. The applied potentials are $U + V\cos\omega t$ and $-(U + V\cos\omega t)$, where $U$ and $V$ are the magnitudes of the applied DC and AC potentials and $\omega = 2\pi f$, where $f$ is the RF frequency. Only ions with a particular m/z can travel down the axis of the device.
and enter either the detector or the next element of the mass spectrometer.

Figure 2.7: Schematic of quadrupole showing the applied DC and AC voltages, the resultant quadrupolar field and the direction of rotation of an ion traveling into the field.

2.3.2.2 Ion Traps

The ion-trap is a folded version of a quadrupole. The theory behind the operation of this device is briefly introduced here though for the sake of clarity, though an excellent reference can be found in a paper by March et al. Figure 2.8 shows a basic schematic of a Paul ion trap, including the applied DC and RF potentials. Scanning through the DC potential \( U \) shifts the stability region of the Mathieu diagram, and has the effect of ejecting ions out of the trap and into the detector. In 1989 Wolfgang Paul and Hans Dehmelt were jointly awarded the Nobel prize in Physics for their work in the 1950s on the ion-trap technique. The first commercial ion trap mass spectrometer was manufactured by Finnigan in 1982 and since then over 10,000 ion trap mass spectrometers have been sold worldwide. A recent development in this field is the linear ion trap which allows
many more ions to be trapped in the larger volume as compared to the traditional 3D ion-traps. This analyser is a combination of a linear quadrupole together with trapping end plates, which allows ion packets to be trapped in the device.

Figure 2.8: Schematic of a Paul ion trap showing the applied DC and AC voltages ions in an unstable trajectory are ejected through the top of the device towards the detector.

2.3.2.3 Time of Flight

Time of Flight analysers operate by accelerating a temporally and spatially focused packet of ions to a specific kinetic energy, and then measuring the time required to fly a known distance. The technique was introduced by W. Stephens at the 1946 meeting of the American Physical Society. The fundamentals of the device can be easily explained in terms of simple physical concepts, namely force \((F = ma)\), kinetic energy \((E_k = 1/2mv^2)\) and speed \(v = \frac{\text{distance}}{\text{time}}\). The mass to charge ratio \((m/z)\) is determined by the charge \((e)\), the kinetic energy imparted from the source acceleration plates \((E_s)\), and the time \((t)\) taken to fly a known distance \((d)\), as shown in Equation 2.1.

\[
m/z = 2e(E_s)(\frac{t}{d})^2 \tag{2.1}
\]
TOF instruments typically employ a MALDI source as the two techniques are pulsed in nature and therefore complementary. Mass resolution in TOF analysers can be increased by the use of a reflectron (see Figure 2.9). This is a region in the flight tube which containing a stack of electrostatic lenses which act as an ion mirror. Ions enter the reflectron and, as the name suggests, they are retarded an then re-accelerated through nearly 180 degrees back towards a detector. Ions with a greater kinetic energy fly further into the reflectron, whilst those with lower energies penetrate only a short distance. This corrects to a large extent for any temporal inhomogeneity in the ion packets, thus increasing the resolving power of the instrument. TOF analysers are often combined in a linear fashion, with a collision cell between the two flight tubes. This is referred to as the TOF-TOF configuration. Again, the interested reader is directed to the previously cited review articles for an explanation of the TOF-TOF technique.

![Figure 2.9: Schematic of time of flight mass spectrometer with a reflectron to boost the resolving power](image)

Figure 2.9: Schematic of time of flight mass spectrometer with a reflectron to boost the resolving power
Fourier transform ion-cyclotron resonance (FT-ICR) mass spectrometry was devised in 1974 by Comisarow and Marshall\cite{49,50}. The technique works by exploiting the Lorentz force which acts perpendicularly to the motion of a charged particle. By holding charged particles in an ion trap, inside a high strength magnetic field, particles of a particular mass-to-charge ratio undergo cyclotron resonance at discrete frequencies. As the magnetic field strength is fixed by the design of the magnet, then an ion with mass $m$ (kg) with charge $q$ (C) travelling at a velocity $v$ (ms$^{-1}$), in a spatially uniform magnetic field $B$ (Tesla), will experience a force $F$ (N) with acceleration $[dv]/[dt]$ (ms$^{-2}$) given by Equation 2.2, known as the Lorentz force.

\[
F = m \frac{dv}{dt} = q v \times B \tag{2.2}
\]

The Lorentz force is a vector cross product of the magnetic field and the velocity, multiplied by the charge on the particle (the Lorentz force is discussed further in Appendix A). Equation 2.2 shows that there is a linear relationship between static field strength and the ion cyclotron frequency ($\omega_c$). Higher static field strength gives rise to higher cyclotron frequencies for ions of a particular mass-to-charge ratio. An ion travelling in a static magnetic field experiences a force vector dependant on its polarity. Figure 2.10 shows the direction in which the Lorentz acts on positively and negatively charged particles.

Figure 2.11 illustrates the required stages of an FT-ICR experiment. Once the analyte has been ionised it is accelerated along a flight tube and, optionally, through a series of mass filters. The ions enter the ICR cell and are accelerated around the cell in a circular fashion due to the combined cyclotron/magnetron motion. A frequency chirped radio frequency voltage is then applied to the excitation plates of the ICR cell. This RF voltage is quickly ramped from 100 kHz to 8 MHz. The application of this chirp pulse excites the ions out to a radius where the image current formed by the electrostatic repulsion of electrons in the
Figure 2.10: Cyclotron motion due to the Lorentz force acting on an ion in a homogeneous magnetic field

detection plates due to the passage of the ions can be measured. The transient ion image current is amplified by a very sensitive amplifier and then digitised. The Fourier transform part of the name of this technique refers to the fact that measurements are taken in the time domain. The fast Fourier transform (FFT) is a computationally efficient implementation. The FFT of the digitised transient yields the frequencies that were superposed to form the original ion current signal. These frequencies can then be related back to the corresponding m/z ratio of the trapped ion species via Equation 2.3.

\[ \frac{\omega_c}{2\pi} = \frac{1.535611 \times 10^7 B_o}{m/z} \]  

(2.3)

Increasing the static magnetic field strength \( B_o \) increases the cyclotron velocity and thus the difference between any two cyclotron velocities also increases. Higher magnet field strengths enable greater discrimination between similar species in the ICR cell and correspond to higher resolution mass spectra.

### 2.3.3 Liquid-Chromatography and Mass-Spectrometry (LC-MS)

The application of liquid chromatography for sample separation in mass spectrometric studies was first reported by Hunt et al. in 1992\textsuperscript{51} and reprinted as a
Figure 2.11: The operating principles of FT-ICR MS. Ions are trapped in the ICR cell and precess at their natural cyclotron frequency. Ions are then excited out to the detection radius and the transient ion current image is recorded. Finally a fast Fourier transform is taken and calibrated to yield a mass spectrum.
Pillars Article in Science in 2007\textsuperscript{52}. This technique is at the centre of mass spectrometry based proteomics\textsuperscript{53–55}. It allows the separation of multi-analyte samples by binding the analytes to a solid stationary phase. A liquid phase is then flowed through the column and the bound analytes elute from the column at a specific time point. Typically, a reverse-phase stationary medium is used, which allows the separation of analytes based on hydrophobic interactions. The mobile phase consists of a mixture of water and acetonitrile, the ratio of organic to aqueous solvent is increased in a linear fashion over time. The application of this gradient means that as the bound analyte experiences a change in the hydrophilicity of its local environment it will elute from the column at a specific time point, known as the retention time. Once the analyte elutes from the column the solution may be analysed by UV-vis spectroscopy or fed into an electrospray source where the analytes can be investigated by MS techniques. Recent advances in high performance liquid chromatography coupled with UV-vis and MS detection have established liquid chromatography as a highly reproducible and high resolution separation technique\textsuperscript{56}.

2.3.4 Fragmentation of Ions

Both Penning (ICR) and Paul traps allow ions to be held for extended periods of time. Therefore, a complex sample can be selectively filtered according to the mass to charge ratio of the constituent analytes and then held in the ion-trap. The trapped ions can be fragmented inside the instrument and the resultant fragments measured. This process is known as tandem mass spectrometry, or MSMS. Any given ion will have particular fragmentation characteristics, thus allowing structural information to be deduced and the analyte identified. In the ion trap it is possible to isolate and fragment many times, provided there is a sufficient ion population left at each stage, this is known as $MS^n$. There are a range of fragmentation techniques that have been developed for use in mass spectrometry. Two specific techniques Collisionally Induced Dissociation (CID) and Electron
Capture Dissociation (ECD) are described below. Other techniques that can be employed include, Infrared Multi-Photon Dissociation (IRMPD)\textsuperscript{57}, in which a laser excites precursor ions to higher vibrational stability levels; Electron Transfer Dissociation (ETD)\textsuperscript{58}; Electron Detachment Dissociation (EDD)\textsuperscript{59}; Sustained Off Resonance Irradiation CID (SORI CID)\textsuperscript{60} and Skimmer Induced Dissociation (SID)\textsuperscript{61}. These techniques are complementary and are often used in combination.

2.3.4.1 Collisionally Induced Dissociation (CID)

The most commonly used activation method in tandem mass spectrometry (MS) of peptides and proteins is energetic collisions with an inert gas. The overall process of collisional activation, followed by fragmentation of the ion, is referred to as collision induced dissociation (CID), or sometimes collision activated dissociation (CAD). The structural information that results from CID of a peptide or protein ion is highly dependent on the conditions used to effect dissociation\textsuperscript{62}. These include, for example, the relative energies of the ion and target gas, the composition of the target gas, the number of collisions that occur, and the physical construction of the collision cell and its location in the mass spectrometer. Figure 2.12 shows a polypeptide with the functional amino acid groups represented by residue labels $R_1 - R_5$. CID predominantly cleaves at the amide bonds producing b and y ions. CID can also result in extensive side-chain and unspecific cleavages, which can prove to be undesirable if side-chain information is of interest.

2.3.4.2 Electron Capture Dissociation (ECD)

Electron capture dissociation (ECD)\textsuperscript{63} is a new fragmentation technique commonly used in Fourier transform ion cyclotron resonance mass spectrometry and is complementary to more traditional tandem mass spectrometry techniques such as CID and IRMPD. Due to the shorter time scales involved in ECD as compared to the vibrational dissociation of CID and IRMPD, fragmentation is fast and specific, producing predominantly c and z ions. Disulfide bonds, normally
stable to vibrational excitation, are preferentially cleaved in ECD\textsuperscript{64}. Labile post-translational modifications and non-covalent bonds often remain intact even after backbone bond dissociation\textsuperscript{65}. ECD often provides more extensive sequence coverage in polypeptides than CID. The improvement in sequence coverage is due to the fact that electron capture events occur randomly along the peptide backbone whereas in CID the peptide backbone fragments at the weakest point. In biological mass spectrometry, the main application of ECD is in top-down analysis of proteins and poly-peptides, de-novo sequencing, disulfide bond analysis as well as the identification and characterisation of post-translational modifications.

2.3.4.3 Protein and Peptide Nomenclature

The process of performing repeated fragmentation on selected ions in a mass spectrometer is referred to as tandem mass spectrometry or MSMS\textsuperscript{66}. Figure 2.12 shows the nomenclature for fragments arising from different cleavages of the polypeptide backbone.

![Polypeptide chain showing fragmentation sites](image)

Figure 2.12: Polypeptide chain showing fragmentation sites, CID typically yields b & y fragments whereas ECD yields predominantly c & z fragments, a & x fragments are seen in high energy CID and ECD experiments.
2.3.5 Mass Spectrometric Proteomic Methodologies

There are three main types of mass spectrometric proteomic methodologies, each with relative advantages and disadvantages.

2.3.5.1 Bottom-Up

Bottom-up proteomics is a common method to identify proteins and to characterise their amino acid sequences and post-translational modifications. Traditionally, the complex mixture is first separated by size on a 1D gel, or by isoelectric point and then size using a 2D gel. After staining, selected protein spots are excised and digested, typically using trypsin. The digested proteins from the excised and digested gel-bands are then analysed by peptide mass fingerprinting, or by LC-MSMS approaches. In peptide mass fingerprinting the intact mass of a variety of tryptic-peptides from a sample is used to obtain a statistically based identification of the precursor protein ion from a database. In addition to LC-MSMS typical bottom-up approaches, shotgun proteomics is often employed. In shotgun proteomics, the crude mixture of proteins is proteolytically digested as a whole, prior to analysis by LC-MS this removes the need for separation on a 1 or 2D PAGE gel. In LC-MSMS a purified protein sample is digested using an enzyme and separated, typically using reversed phase chromatography. The resultant peptide fragments are then analysed by MS to generate a precursor mass, the most prominent peaks are then selected for tandem mass spectrometry. This is known as a data-dependent technique, and since each peptide has both a precursor mass and a series of fragment masses, this allows protein identification with much greater confidence.

2.3.5.2 Top-Down

Top-down proteomics is a method whereby the intact protein is ionised and introduced into the mass spectrometer. Fragmentation of the intact protein takes place in the gas phase using one of the fragmentation techniques mentioned previ-
ously, typically CID or ECD. Figure 2.13 is a schematic showing the methodologies for top-down protein mass spectrometry as compared to the bottom-up approach. The use of ECD has proved particularly powerful for sequencing proteins, whilst preserving post translational modifications.\textsuperscript{70–73}

Figure 2.13: Comparison of top-down and bottom up proteomic methodologies, adapted from wikicommons schematic by Magnus Palmblad

2.3.5.3 Middle Down

In middle-down proteomics, the protein is typically cleaved into three or four polypeptides in the solution phase by using enzymes which cleave after rare amino acids or by chemical cleavage agents. These fragments can then be sequentially isolated using a mass resolving quadrupole and before being fragmented and sequenced in a typical top-down type experiment.\textsuperscript{74} In common practice the top-down approach has an upper mass limit of approximately 60 kDa.\textsuperscript{75} With middle down techniques this range can be extended by cleaving a large protein into sequences of a manageable length for top-down type analysis.
2.3.6 Instrumentation Used in this Work

FT-ICR MS instruments with superconducting magnets at field strengths of 3, 9.4 and 12 Tesla were used in this work. These high-performance analytical instruments offer a variety of ionisation techniques (MALDI, ESI, APCI). In addition to the FT-ICR instruments, ion-trap and MALDI-TOF mass spectrometers were used. The instrumentation is introduced in detail in the following sections, photographs of the instruments are provided in Section D.

2.3.6.1 Bruker-Daltonics HCT Ultra Ion Trap and Dionex Ultimate 3000

The Bruker-Daltonics HCT ultra (see Appendix D.1) is a high capacity ion trap instrument, equipped with CID and Electron Transfer Dissociation (ETD) fragmentation techniques and \( MS^n \) capabilities. ETD is a similar technique to ECD but it uses an ionised fluoranthene molecule in place of an electron beam. The HCT ultra is interfaced for reversed phase nano-LC separations using a nano-ESI source for bottom-up experiments. Figure D.1 shows the HCT ion trap alongside the Ultimate 3000 (Dionex, UK), a HPLC system used primarily in this work for reversed phase chromatography prior to MS analysis. Figure 2.14 shows a schematic representation of the HCT ion trap. Ions are generated in the ESI source, where the heated counter flow drying gas aids in desolvation. The ions then move through the transfer hexapole optics and into the ion-trap where CID occurs on the introduction of an inert gas, typically nitrogen or helium. Scanning through the DC potential applied to the ring electrode selectively ejects ions out of the trap onto the detector, a conversion dynode followed by a channeltron.

2.3.6.2 Bruker-Daltonics 12T Apex-Qe FT-ICR MS

The Apex Qe FT-ICR mass spectrometer shown in Appendix D.2, is a Fourier transform ion cyclotron resonance instrument equipped with a 12 Tesla helium cooled super-conducting magnet. Figure 2.15 shows a schematic of the instru-
Figure 2.14: Schematic of Bruker-Daltonics HCT Ultra ion trap, analytes move from the atmospheric pressure interface through a series of skimmers and into the high vacuum region in the ion-trap.

2.3.6.3 Perseptive Biosystems Voyager DE STR MALDI TOF MS

The Perseptive Biosystems Voyager DE STR (see Appendix D.3) is a MALDI TOF mass spectrometer fitted with a reflectron. The internal configuration of
the instrument is very similar to that provided in Figure 2.9. The DE STR was typically used for peptide mass fingerprinting (PMF) experiments due to its high sensitivity and MALDI source.

2.4 Summary

A brief introduction to mass spectrometry based proteomics has been presented. Relevant mass spectrometry theory has been introduced along with a range of current proteomic strategies. Finally, the instrumentation used in this body of work has been briefly described.
Chapter 3

Theory of Digital Microfluidic Devices

3.1 Introduction

Microfluidics is the study and use of fluid behaviour on the microscale. Over the last twenty years a growing community of researchers have been investigating microscale fluid behaviour, and designing systems that exploit the advantageous effects of scaling. Microfluidic systems are part of the Micro-Electro-Mechanical-System (MEMS) research field, and this area is a rapidly expanding new area of engineering. Provided here is a brief introduction to scaling laws, conventional pressure driven microfluidics and those microfluidic systems which operate using the interaction of electric fields and fluids. Digital Microfluidics (DMF) is introduced and a variety of devices that have been designed and tested are presented. Work on characterising the devices is presented along with the control circuitry required to drive the devices.

3.2 Microfluidics

3.2.1 System Scaling

Scaling fluidic systems down to use nanolitre volumes of liquid means that different forces govern behaviour, compared to those at the macroscale. This can
Table 3.1: Scaling of different parameters on a unit volume of length \( l \)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Behaviour</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inertial forces</td>
<td>( l^2 )</td>
<td>Equation 3.1</td>
</tr>
<tr>
<td>Viscous forces</td>
<td>( l )</td>
<td>Equation 3.1</td>
</tr>
<tr>
<td>Surface tension</td>
<td>( l )</td>
<td>Equation 3.14</td>
</tr>
<tr>
<td>Contact angle</td>
<td>( l^0 )</td>
<td>Equation 3.14</td>
</tr>
<tr>
<td>Capillary rise height</td>
<td>( l^{-1} )</td>
<td>Equation 3.4</td>
</tr>
<tr>
<td>Reynolds number</td>
<td>( l^2 )</td>
<td>Equation 3.2</td>
</tr>
<tr>
<td>Diffusion time</td>
<td>( l^2 )</td>
<td>Equation 3.3</td>
</tr>
<tr>
<td>Young-Laplace pressure</td>
<td>( l^{-1} )</td>
<td>Equation 3.15</td>
</tr>
<tr>
<td>Electric field</td>
<td>( l^{-1} )</td>
<td>See Appendix</td>
</tr>
<tr>
<td>Gravity</td>
<td>( l^3 )</td>
<td></td>
</tr>
<tr>
<td>Pressure</td>
<td>( l^2 )</td>
<td></td>
</tr>
<tr>
<td>Area</td>
<td>( l^2 )</td>
<td></td>
</tr>
<tr>
<td>Volume</td>
<td>( l^3 )</td>
<td></td>
</tr>
</tbody>
</table>

lead to a variety of interesting and strange phenomena, such as laminar flow and capillary action. In his comprehensive textbook “Theoretical Microfluidics”\textsuperscript{76}, Henrik Bruus describes a scaling law as one which \textit{expresses the variation of physical quantities with the size (l) of the given system or object whilst keeping other quantities such as time, pressure, temperature, etc. constant}. Volume forces (gravity and inertia) act upon the bulk of a fluid flow or droplet, whilst surface forces (surface tension and viscosity) act on the interface. The effect of scaling on a variety of system parameters is summarised in Table 3.1. The characteristic lengths of the DMF devices that are presented in Chapter 4 are one to two orders of magnitude larger than in typical channel microfluidic devices and will therefore not engender such efficient mixing, control or throughput, though the potential for the downscaling of the system is clear.

### 3.2.1.1 Gravity and Surface Tension

Surface tension is the tendency exhibited by liquids to reduce the exposed surface to the smallest possible area. Molecules of water, for example, exert forces on neighbouring molecules. Dipole-dipole interactions (Van der Waals-Keesom,
Debye and London) act in the bulk fluid of a droplet, and over time these forces reach equilibrium. Molecules at the surface of a liquid experience unequal attractions and are orientated towards the centre of the liquid by the resultant net force. This makes the liquid surface act like a thin membrane. For a static system in fluid dynamics, the method of determining the effect of gravity on the fluid is to evaluate the dimensionless Bond number $B_0$, defined in Equation 3.1, where $g$ is the acceleration due to gravity, $\rho$ is the fluid density, $r$ is the characteristic radius and $\gamma$ is the surface tension.

$$B_0 = \sqrt{\frac{g\rho r^2}{\gamma}}$$

(3.1)

If the Bond number is less than one, then the dominant force is that due to surface tension and the system is operating in the microscale. As a result, the volume forces which are common to our everyday sense of physics become largely unimportant and the less intuitive surface forces come to dominate.

### 3.2.1.2 Laminar Flow

Interesting effects happen in the microscale region, including the phenomenon of laminar flow. The Reynolds number is a dimensionless number which describes the ratio of inertial forces to viscous forces in dynamic fluid systems. This is defined in Equation 3.2, where $\rho$ is the fluid density, $\mu$ is the dynamic viscosity, $l$ is the characteristic length and $u$ is the velocity.

$$Re = \frac{\rho ul}{\mu}$$

(3.2)

It has been determined from empirical studies, that if the Reynolds number is less than 2000, the system is typically operating in the laminar flow regime. Evaluation of the Reynolds number in a typical microfluidic system, consisting of water ($\rho = 998 \text{ kgm}^{-3}$ and $\mu = 1.002 \text{ Nsm}^{-2}$) flowing in a 1mm diameter channel at 0.2 $\text{ms}^{-1}$ results in a value of $Re = 19.92 \times 10^{-3}$. This system is clearly
operating in the laminar flow region. Kenis et al. make use of laminar flow in their 1999 Science paper\textsuperscript{77} detailing the microfabrication of silver wires in the centre of a channel using an electroless silver plating solution. The diagram in Figure 3.1 first appeared on the front cover of that issue of Science, and is a defining image of the peculiarities of laminar-flow on the microscale. The coloured fluids entering from the side channels do not mix immediately; instead they travel in a laminar flow regime with mixing between the different fluids limited by diffusion.

Figure 3.1: Peculiarities of laminar-flow in microfluidic devices\textsuperscript{77}. Mixing between the coloured fluids entering from the side channels does not occur immediately; they diffuse together over time. Reprinted with permission from AAAS.

3.2.1.3 Diffusion

Diffusion, in the context of microfluidics, is the movement of solute in a solvent moving from a high to a lower concentration until an equilibrium is established. The movement occurs due to Brownian motion and other thermally induced flows in the bulk of a liquid. The root mean square distance ($l_{rms}$) a molecule with a given diffusion constant ($D$) will move in a given time period ($t$), is defined by the Einstein relation for molecular diffusion in a solution, Equation 3.3.
Combining laminar flow and diffusion led to one of the first commercial microfluidic devices, the H-filter. This device, developed by Brody and Yager\textsuperscript{78,79}, is designed to allow the continuous extraction of molecular analytes from fluids containing relatively large particles. The filter output becomes preferentially concentrated with smaller molecules of a larger diffusion constant. Their suggested applications of this device include blood-plasma separation. Small particles like haemoglobin in blood take $\sim$300 hours to diffuse 1 cm, but only $\sim$1 second to diffuse 10 $\mu$m. Larger particles, such as red blood cells take $\sim$10 minutes to diffuse 10 $\mu$m. Haemoglobin will, therefore, readily diffuse across the flow stream to the filter output and is, therefore, separated from the red blood cells. This diffusion-based particle separation device will, however, only work on the microscale due to the laminar flow regime and diffusion lengths.

3.2.1.4 Capillary Effects

Capillary action is an interesting effect of the force due to surface tension dominating over gravity. The phenomenon is most often seen when a thin tube draws liquid upwards against the force of gravity. This action arises because the adhesive intermolecular forces between the liquid and the solid exceed the cohesive forces within the bulk of the liquid. In a biological context, plants use this effect to draw liquid up their stems against gravity. The height that a fluid can rise ($h$) within a thin tube is given by Equation 3.4

$$h = \frac{2\gamma \cos \theta}{\rho gr}$$

(3.4)

Where $\gamma$ is the surface tension, $\theta$ is the contact angle between the liquid and the solid (see Section 3.3.2.2), $\rho$ is the liquid density, $g$ is the acceleration due to gravity and $r$ is the radius of the tube. Due to the fact that the geometrical parameters can be easily measured, one of the most accurate experimental
methods for determining surface tension is to evaluate a rearranged version of
Equation 3.4. Nordstrom et al. used the principle of capillary action to provide
a passive capillary-pump in a cantilever biosensor application\textsuperscript{80}, thus removing
the need for bulky and expensive external pumps as is typical in pressure driven
microfluidic devices. More recently Martinez et al. have pioneered extremely low
cost capillary action driven microfluidic networks made from paper and tape\textsuperscript{81}.

### 3.2.2 Pressure Driven Flow

Fluid in a microchannel can be forced to flow by the introduction of a hydro-
static pressure. The Navier-Stokes equation describes the velocity vector \( \vec{v} \) of
an incompressible (Newtonian) fluid having a density \( \rho \) and viscosity \( \eta \) due to
a pressure \( p \). The full theoretical underpinnings of fluid-dynamics can be found
in the aforementioned textbook, Theoretical Microfluidics\textsuperscript{76}. As a brief overview
however, the Navier-Stokes equations can be derived from such fundamental phys-
ical concepts as the rates of change of, and conservation of, mass, momentum and
energy.

\[
\rho \left[ \delta_t \vec{v} + (\vec{v} \cdot \nabla) \vec{v} \right] = -\nabla p + \eta \nabla^2 \vec{v} + \rho \vec{g} + \rho_{el} \vec{E} \tag{3.5}
\]

Equation 3.5 has been derived in a similar way to the derivation of the Maxwell
Stress Tensor (as shown in Appendix A), using tensor notation and extensive use
of Gauss’ theorem. The last term in Equation 3.5 describes the force due to
an external electric field acting upon the free charge in the bulk of the fluid,
and can be ignored for purely pressure driven flow in the absence of an electric
field. Though this term will be of interest in the following sections so it has been
included for completeness. The Navier-Stokes equation is fundamental to fluid-
dynamics, and can be used to describe both turbulent and laminar flow. Indeed,
the Reynolds number (Equation 3.2) can be derived directly from Equation 3.5.
This is a non-linear differential equation and, therefore, difficult to solve in an
analytical fashion. However in particular cases it can be simplified and solved by
making assumptions about the boundary conditions. There are two main types of pressure induced flow, these are Poiseuille and Couette. Couette flow equations describe the motion of a fluid between two parallel plates driven by the relative movement of the plates, whereas Poiseuille flow equations describe the pressure induced steady-state fluid flow in very long rigid channels\textsuperscript{76}. The motion of fluids in familiar microfluidic closed-channel devices can be described in terms of Poiseuille flow equations. In basic form a high-performance liquid chromatography system, an instrument that was used in this thesis, consists (ignoring the packed bed in the analytical column) of a pump that develops a hydrostatic pressure ($\Delta p$), which forces liquid (of density $\rho$ and viscosity $\eta$) through a capillary of a small radius ($r$) and long length ($L$). The flowrate ($Q$) can be determined by evaluation of Equation \ref{eq:poiseuille}, which comes directly from the Navier-Stokes equation.

$$Q = \frac{\pi r^4}{8\eta L} \Delta p \tag{3.6}$$

Other microfluidic devices which depend directly on pressure driven, Poiseuille-type flow include, hydrodynamic flow focusing structures\textsuperscript{82} for cell sorting in flow cytometry applications\textsuperscript{83,84}, DNA isolation and PCR\textsuperscript{85} as well as phase-transfer magnetophoresis\textsuperscript{86}. Another way of developing hydrodynamic flow without pumps is by using centrifugation as utilised in Madou et al.’s lab-on-a-CD\textsuperscript{87}. Excellent reviews on continuous flow microfluidics and their utility in lab-on-a-chip applications can be found in articles by Auroux \textit{et al.}\textsuperscript{88}, Chen \textit{et al.}\textsuperscript{89}, Kohler \textit{et al.}\textsuperscript{90} and Edel and deMello\textsuperscript{91}.

\textbf{3.2.3 Electric Field Driven Microfluidics}

\textbf{3.2.3.1 Electrophoresis}

Electrophoresis is the simplest and most intuitive form of electric field driven microfluidics. As previously shown in Equation \ref{eq:electrophoresis}, an external electric field can induce a simple coulombic force on free charge carriers in a liquid. Coulomb’s law is discussed in the Appendix and is an axiomatic theorem of electrostatics. The
force vector $\vec{F}$ experienced by an ion with charge $q$, due to an electric field $\vec{E}$, is given by $\vec{F} = q \vec{E}$, and the velocity $\vec{v}$ with which the ion moves is dependant on its mobility $\mu$ in the relationship $\vec{v} = \mu \vec{E}$. This effect is most commonly seen in Polyacrylamide Gel Electrophoresis (PAGE), whereby charged proteins migrate though a dense gel due to an applied electric field. Large proteins have low mobility and therefore only migrate a short distance in a given time. Smaller proteins and peptides migrate much further through the gel and in this way a complex protein sample can be separated on the basis of size. This is the traditional method of separation in bottom-up proteomics. If the separation occurs in a capillary it is referred to as capillary electrophoresis (CE). In this technique, ions elute from a long capillary at a time point proportional to their mobility.

3.2.3.2 Dielectrophoresis

Dielectrophoresis is a phenomenon in which a force is generated when a dielectric body is introduced into an non-uniform electric field. The inhomogeneous energy variation across the body due to the non-uniform field strength will exert an imbalance of free charge in the body, and hence create a directional force vector. The technique was discovered and developed by Pohl in 1978. The force ($\vec{F}_{dep}$) that acts upon a particle of radius ($r$) and relative dielectric constant ($\epsilon_p$) suspended in a medium of dielectric constant ($\epsilon_m$) and in a non-uniform electric field ($\vec{E}$) can be calculated by analysis of the equivalent dipole moment method as developed by T.B. Jones at Rochester University. In this thesis the expression is presented from a derivation using the Maxwell Stress Tensor method by Wang et al. This approach has been used to be consistent with the derivation of the DMF equations provided in Appendix A. The basic methodology of this approach is to integrate the Maxwell stress tensor (Equation 3.7) over the surface of the particle.
The complex dielectric constants of the particle \((\epsilon_p^* = \epsilon_p - \frac{j\sigma_p}{\omega})\) and the medium \((\epsilon_m^* = \epsilon_m - \frac{j\sigma_p}{\omega})\) are related through the Clausius-Mossoti factor, Equation 3.8.

The complex dielectric constants have a real and imaginary part, the imaginary part is dependent on the conductivity \(\sigma\) of the medium/particle and on the frequency of the applied field, \(\omega = 2\pi f\).

\[
k(\omega) = \frac{\epsilon_p^* - \epsilon_m^*}{\epsilon_p^* + 2\epsilon_m^*}
\]

In a first order approximation of the DEP force for a linear and homogeneous medium and particle the DEP force \((\vec{F}_{\text{dep}})\) can be calculated as shown in Equation 3.9, as first derived by Sauer et al.\(^{99}\).

\[
\vec{F}_{\text{dep}} = \pi r^3 Re[\epsilon_p^*](k(\omega)(\vec{E} \cdot \vec{\nabla}) \vec{E}^* + k(\omega)(\vec{E}^* \cdot \vec{\nabla}) \vec{E})
\]

The polarity of the DEP force is determined by the real part of the Clausius-Mossoti factor. Positive DEP is where the particle moves in the direction of the strongest field, \(Re[k(\omega)] > 0\) and conversely negative DEP occurs when \(Re[k(\omega)] < 0\).

Significant contributions to the application of DEP in biotechnology has been made by Professor Ron Pethig\(^{100}\) who recently published a comprehensive review of dielectrophoresis in biomicrofluidic applications\(^{101}\). In addition Fan et al. have reported the integration of electrowetting and dielectrophoresis\(^{102,103}\).

### 3.2.3.3 Electrocapillary Action

Electrocapillary action is an extension of the previously discussed capillary flow. Experiments by Pellat over 100 years ago showed that an insulating dielectric liquid rises upwards between two planar parallel electrodes, against the force of gravity, when a potential is applied between them. Wang and Jones\(^{104}\) revisited
this phenomenon in 2004. They found that if the electrode spacing \((D)\) is small compared with the width and height, then the height the liquid rises \((h)\) is given by Equation 3.10.

\[
h = \frac{(\epsilon_r - 1)\epsilon_0 E^2}{2\rho g}
\]  \hspace{1cm} (3.10)

Where \(\rho\) is the liquid density, \(g\) is the acceleration due to gravity and \(E\) is the electric field strength, given by \(V/D\). Comparing this expression with Equation 3.4 shows that the normal capillary height of rise can be increased by the application of the electric field. Clearly addition of the electric field induces a new force acting upon the liquid-gas interface. This system was taken a step further by Gabriel Lippmann in his analysis of the capillary depression of mercury in contact with aqueous electrolyte solutions. Lee and Kim analysed this system in detail\(^{105}\) and described the movement in terms of a change in surface tension \((\gamma)\) due to an applied potential \((V)\). Lippmann’s equation can be written in this form, shown in Equation 3.11.

\[
\gamma_v = \gamma_0 - \frac{C}{2}(V - V_0)^2
\]  \hspace{1cm} (3.11)

Where \(C\) is the capacitance of the induced Electric Double Layer (EDL). The EDL is a spontaneously induced region of surface charge due to the interaction of the electrolyte and the surface. This charge induces an electric field which attracts ions of opposite charge, and repels ions of like charge. The EDL can be modelled as a capacitor with a positive charge on one side, a negative charge on the other, and an effective insulating region in the middle. This phenomenon was originally studied by Helmholtz in the 1850s. Later studies by Stern, Gouy and Chapman showed that a simple capacitor model was too simplistic, and they modified the model to include terms for adsorption and solvent-dipole interactions. The new model included two layers, the diffuse layer and the outer-Helmholtz-plane layer (also known as the Gouy-Chapman layer). The significance of the EDL model is
that it shows that no direct current path is required to affect the force acting on the liquid interface. This distinguishes electrocapillary action from electrophoresis and injection induced flows. This paved the way for the development of the microfluidic systems which form the main focus of this thesis, those in which the liquid is electrically insulated from the electrodes by a thin solid dielectric layer. The electrocapillary force has been used to actuate micro-pumps and other MEMS type devices, primarily by Kim et al.\textsuperscript{105,107,108}.

### 3.3 Digital Microfluidics (DMF)

Before discussing the theory concerning devices with an insulating dielectric layer, it is important to introduce the concept of digital microfluidics, as this represents a significant conceptual change in thinking, compared to that underpinning traditional microfluidic systems. Digital microfluidic systems do not contain any moving mechanical parts, such as pumps and valves, and are characterised by the manipulation of discrete droplets of liquid. Liquid moves through the device as individual droplets, rather than in a continuous flow, and on a planar surface rather than in closed microchannels. The first reported movement of discrete droplets of liquid, with a view to creating a microfluidic system, was reported by Whitesides and Chaudhury in 1992\textsuperscript{109}. Their device exploited differential surface free energy due to a gradient in the spatial concentration of a fluorochlorosilane hydrophobic layer. The resultant change in contact angle of a droplet of water (from 97 to 25 degrees) resulted in an imbalanced Young force (See 3.3.2.4), sufficient to propel the droplet up a 15 degree incline. Another technique in which droplets of liquid are manipulated on a surface is Surface Acoustic Wave (SAW) technology\textsuperscript{110}. In this technique acoustic energy is employed, travelling as a surface wave, and some of this energy is coupled into droplets sitting on the surface. This technique is not discussed further here, as it does not rely on the interaction of electric fields and fluids. There is, however, scope for integration
of this technology which is discussed in the future work section. A new fusion
technology\textsuperscript{111} of closed channel fluidics with digital microfluidics has the unique
benefits of both. The surface is reconfigurable and the flow can be driven by
electrowetting or hydrostatic pressure. This may prove to be an interesting fu-
ture direction of microfluidics. The digital microfluidic technology which has been
presented and developed in the course of the work described in this thesis is char-
acterised by the interaction of electric fields with discrete droplets of liquid on a
planar reaction surface, consisting of microelectrodes beneath a hydrophobic and
dielectric layer\textsuperscript{112}. This technology has been referred to by many names during
its development including; MIST (metal-insulator-solution-transport)\textsuperscript{113}, DMFS
digital microfluidic system)\textsuperscript{114}, EICE (electrowetting on insulator coated elec-
trodes)\textsuperscript{115}, EWOD (electrowetting on dielectric)\textsuperscript{116,117} and finally DMF (digital
microfluidics)\textsuperscript{3}. It is the view of the author that droplet microfluidics (DMF)
is the most appropriate term. The term is inclusive of the other technologies
without specifically describing the physical construction of a system (MIST) or
making assumptions about the mechanism of action (EWOD). One of the main
advantages of DMF systems compared to closed-channel microfluidics, is that the
system can be reconfigured by changing only the driving signals. This could be
reconfiguration for a new function to be performed on the array, local reconfigu-
ration to prevent unnecessary long-distance transport of fluids, or reconfiguration
to work around points that have become contaminated or have broken electrodes.
The characteristic lengths of the DMF devices that are presented in Chapter 4
are one to two orders of magnitude larger than in typical channel microfluidic
devices and will therefore not engender such efficient mixing, control or through-
put, though the potential for the downscaling of the system is clear. There are
four fundamental droplet operations which are required for a complete digital
microfluidic system as defined by Kim \textit{et al.}\textsuperscript{116} . These are:

1. Creating droplets from a reservoir
2. Splitting droplets into two
3. Merging droplets
4. Transporting droplets

The definition of this set of operations has led to publications in which a theoretical approach has been taken to determine the optimal routes for movement of droplets on an $M \times N$ array\textsuperscript{118,119}. In addition to purely theoretical studies, DMF devices are now starting to find a use in a multitude of applications\textsuperscript{2–4}.

### 3.3.1 Applications

Digital microfluidic devices have been used for everything from displays\textsuperscript{120} to drug-delivery devices\textsuperscript{121} and medical diagnostics\textsuperscript{122,123}. Though the effects of the interaction of electric fields and fluids have been known since the studies of Lippmann in the late 1800s, it is only with the recent advances in thin film materials and micro manufacturing techniques that the realisation of DMF technologies as useful devices has become a possibility. Given the massive recent development in mobile phone and smart device technologies it is not surprising that manufacturers are looking for ever cheaper and lower-power technologies to extend the battery-life of devices and reduce manufacturing costs. Kuipera et al.\textsuperscript{124} introduced the concept of a liquid lens for miniaturised camera focusing. The liquid lens consists of an optically transparent liquid droplet sandwiched between two insulated electrodes. Upon the application of a suitable potential, the droplet deforms thus changing the focal length, as shown in Figure 3.2. These devices have been manufactured in a stacked configuration, and demonstrated utility for focusing\textsuperscript{125} and zooming\textsuperscript{126,127} in optical systems.

DMF technology has also been investigated for its utility in video displays, with the first publications appearing in the 1980s\textsuperscript{128,129}. Liquavista is a spin-out company that was formed following research conducted by Phillips\textsuperscript{120}. An example of Liquavista’s display technology is shown in Figure 3.3. These high contrast displays can be manufactured to operate in reflective, emissive and transmissive formats. Each pixel in the display consists of an aqueous-oil cell.
Upon the application of a suitable electrostatic potential, the coloured oil is forced into a smaller area of the pixel and this changes the optical characteristics of the cell. The response time is of the order of ten-milliseconds, and is thus more than fast enough for full-colour video displays. This low-power technology has potential for use in mobile devices and e-paper applications.

Recent work by the SMC in Edinburgh showed that DMF technology can be used for true MEMS applications in the development of a pond-skater device\textsuperscript{130}. This device is small enough to interact with the surface tension of a beaker of liquid in a similar fashion to a water-boatman. The device is powered wirelessly and can propel itself around on the surface of a body of water by the displacement of gaseous bubbles beneath it. Figure 3.4 shows the device. This is a proof of concept device, but could see utility in microrobotics applications with development of the bubble manipulation technology\textsuperscript{131}.

Another important application of DMF technology is in Lab-on-a-Chip (LOAC) and biomicrofluidic technologies. It is the development of this type of technology for application in these areas that forms the basis of this thesis. Figure 3.5 shows
Figure 3.3: DMF techniques being used for low-power display technology\textsuperscript{120}. Reproduced with permission from source: Electrowetting White Paper, Liquavista 2009

Figure 3.4: Wireless pond skating device with integrated receiver circuitry floating on DI water\textsuperscript{130}
a device constructed by Barbulovic-Nad et al.\textsuperscript{4,132}. This device can perform all of the steps required in a complete mammalian (HeLa) cell culture on 120 µl droplets. Cells are made to grow on ‘adhesion pads’ which are positioned over DMF actuation electrodes, culture media and reagents can then be exchanged around the cells by DMF actuation and finally the cells can be removed from the ‘adhesion pad’ and transported to a new site for sub-culturing. Digital microfluidics lends itself to applications such as this; each droplet is in effect a complete microenvironment with static concentrations and volumes due to the effective membrane side-walls. Droplets can be dispensed from a reservoir, split so that each smaller droplet is identical in composition, combined for dilution steps, or transported for storage\textsuperscript{133} or assays. DMF based LOAC technologies include; PCR\textsuperscript{134}, a platform for miniaturising lab processes\textsuperscript{3,135}, cell-assays\textsuperscript{136}, DNA hybridisation and detection by surface plasmon resonance\textsuperscript{137} as well as sample preparation for proteomic analysis\textsuperscript{138}.

Figure 3.5: Complete mammalian cell culture on a chip driven by DMF actuation\textsuperscript{4,132}. Cells are made to grow on ‘adhesion pads’ which are positioned over DMF actuation electrodes, culture media and reagents can then be exchanged around the cells by DMF actuation and finally the cells can be removed from the ‘adhesion pad’ and transported to a new site for sub-culturing. The inset shows a droplet containing a sub-cultured population. Reproduced by permission of The Royal Society of Chemistry

\textsuperscript{47}
The advantages of DMF manipulation for LOAC applications are manifest and form the motivation for the development of this technology in our research group. In order to design these devices successfully, it is important to review the fundamental physics. This is the focus of the following section.

3.3.2 Digital Microfluidic Theory

3.3.2.1 Surface Tension of Droplets

Whilst surface tension has already been introduced and discussed, the situation for droplets of liquid with a relatively high surface tension is of fundamental importance to the operation of DMF devices, and it is, therefore, briefly revisited here. A water droplet in the absence of external forces will tend towards forming a sphere since, according to the second law of thermodynamics, matter naturally seeks to be in a low energy state. Bonding inside the droplet reduces the chemical energy and since a spherical shape provides the largest volume with the minimum surface area, it is energetically the most favourable geometric configuration.

The force due to surface tension can be written as \( F_{SL} = 2\pi r \gamma \) where \( F_{SL} \) is the force acting between the liquid and the solid substrate (N), \( 2\pi r \) is the circumference of the interface and \( \gamma \) is the surface tension (Jm\(^{-2}\)), that is the total force acting on the contact line. If we consider the force acting on the droplet due to gravity we obtain, \( F_g = \frac{4}{3} \pi r^3 \rho g \) where \( F_g \) is the weight (N), \( \frac{4}{3} \pi r^3 \) is the droplet volume (m\(^3\)), \( \rho \) is the liquid density (kgm\(^{-3}\)) and \( g \) is the acceleration due to gravity (ms\(^{-2}\)). The limit at which the force due to surface tension dominates over gravity can be calculated by equating these two expressions to give the characteristic length as shown in Equation 3.12.

\[
r = \sqrt[3]{\frac{3 \gamma}{2 \rho g}} \quad (3.12)
\]

This can be evaluated using standard values for water of \( F_{SL} = 0.0728 \text{ Jm}^{-2} \), \( \rho = 998 \text{ kgm}^{-3} \) and thus \( r = 3.3 \times 10^{-3} \text{ m} \), i.e. 3.3 millimetres. Digital Microfluidic systems having a droplet radius of less than 3.3 mm (for water) can, therefore,
be designed to exploit changes in surface tension forces since these dominate over gravity.

### 3.3.2.2 Young’s Equation

Digital Microfluidic systems involve the interaction of three phases of matter, as shown in Figure 3.6. The liquid (droplet), gas (air) and solid (hydrophobic substrate) interfacial tensions determine the shape of the sessile droplet due to gravity and when there is no applied electrical potential\textsuperscript{141}.

![Figure 3.6: Interfacial tensions acting on the triple contact line](image)

The line where these three forces meet is referred to as the triple contact line (TCL)\textsuperscript{142}. The three interfacial energies that act upon this line are defined as:

1. The liquid-gas interfacial tension $\gamma_{LG}$
2. The solid-liquid interfacial tension $\gamma_{SL}$
3. The solid-gas interfacial tension $\gamma_{SG}$

The angle between the liquid-gas interfacial tension and the substrate is known as the contact angle. This is specific for any given system of solid, liquid and gas. If one considers a small displacement of the TCL it is possible to calculate the change in free energy as a function of the change in distance. Using Gibbsian interfacial thermodynamics one obtains Equation 3.13.
\[
\delta F = \gamma_{SL} 2\pi r \delta x - \gamma_{SG} 2\pi r \delta x + \gamma_{LG} 2\pi r \delta x \cdot \cos \theta
\]  
(3.13)

Hydrostatic equilibrium is reached when the change in free energy with respect to area equals zero, i.e. \( \frac{\delta F}{\delta A} = 0 \). It follows that the cosine of the contact angle is given by Equation 3.14.

\[
\cos \theta(V) = \frac{\gamma_{SG} - \gamma_{SL}}{\gamma_{LG}}
\]  
(3.14)

Equation 3.14 is known as Young's Equation. It relates the contact angle to the interfacial tensions of the three phases. It defines a specific contact angle for any given system. Note that this is a hydrostatic equation and it does not consider any additional energies due to motion.

### 3.3.2.3 Young-Laplace Equation

The Young-Laplace equation (Equation 3.15) describes the hydrostatic pressure difference (\( \Delta p \)) across the air-liquid interface (\( p \)) of droplet of principle radii of curvature \( r_1 \) and \( r_2 \) and with surface tension \( \gamma \). This equation is of particular importance when considering the phenomenon of droplet splitting in DMF systems. Further information can be found in papers by Cho and Kim\textsuperscript{143,144} and also in a thesis by Herberth\textsuperscript{145}.

\[
\Delta p = \gamma \left( \frac{1}{r_1} + \frac{1}{r_2} \right)
\]  
(3.15)

### 3.3.2.4 Lippmann-Young Equation

Young’s original paper from 1875 was re-printed at the end of Mugele and Baret’s review in 2005\textsuperscript{146}. From this paper we can derive the Lippmann-Young equation, which describes the change in contact angle of the liquid interface as a function of applied voltage in an electrocapillary system.

This equation can be derived using an energy minimisation approach. Although an alternative derivation will be shown in a later section, the energy
minimisation method has been included here as it provides the most logical and intuitive derivation of the Lippmann-Young equation. Working from Equation 3.13 and making reference to Figure 3.7; if one include terms for the energy stored in the capacitor $W$ (see Equation A.22) and the work done by the voltage source $W_{VS}$ to build up the potential between the droplet and the electrode, it can be shown that:

$$\delta F = \gamma_{SL}2\pi r\delta x - \gamma_{SG}2\pi r\delta x + \gamma_{LG}2\pi r\delta x \cdot \cos \theta + \delta W + \delta W_{VS}$$ (3.16)

As with the derivation of the Young Equation (Equation 3.14), if one considers a displacement of the TCL leading to a change in area $\delta A = 2\pi r\delta x$ then the a change in energy in the capacitor given by the expression:

$$\frac{\delta W}{\delta A} = \frac{1}{2} \frac{\epsilon_r \epsilon_0}{d} V^2$$ (3.17)

The additional energy coming into the system must come from the voltage source. Hence:

$$\frac{\delta W_{VS}}{\delta A} = \frac{\epsilon_r \epsilon_0}{d} V^2$$ (3.18)

These two terms can be combined into a single term $\gamma_{EW}$ with units $Jm^{-2}$.

$$\gamma_{EW} = \frac{\delta W_{VS}}{\delta A} - \frac{\delta W}{\delta A} = -\frac{1}{2} \frac{\epsilon_r \epsilon_0}{d} V^2$$ (3.19)
Writing this in terms of interfacial tensions and taking the partial derivative with respect to area leads to the equation:

\[ \frac{\delta F}{\delta A} = \gamma_{SL} - \gamma_{SG} + \gamma_{LG}\cos\theta - \gamma_{EW} \]  

(3.20)

Setting \( \frac{\delta F}{\delta A} = 0 \) to minimise the equation, and rearranging for \( \cos\theta \) leads to an equation for the contact angle as a function of applied voltage:

\[ \cos\theta = \frac{\gamma_{SG} - \gamma_{SL} + \frac{\varepsilon_{r}\varepsilon_{0}}{2d}V^{2}}{\gamma_{LG}} \]  

(3.21)

Young’s Equation (Equation 3.14) can be used to describe the system under zero applied potential. With some substitution and rearrangement one can obtain the Lippmann Equation:

\[ \gamma_{SL}(V) = \gamma_{SL}(0) + \frac{1}{2} \frac{\varepsilon_{r}\varepsilon_{0}}{d}V^{2} \]  

(3.22)

Again with some substitution, rearrangement and equating with Young’s Equation we obtain the full Lippmann-Young Equation. This describes the change in contact angle as a function of the applied voltage. It is helpful to define a quantity \( \zeta \) included in Equation 3.23, as \( \zeta = \frac{\varepsilon_{r}\varepsilon_{0}V^{2}}{2\gamma_{LG}(V)} \) as defined by Mugele and Baret.\(^{146}\) This is the dimensionless electrowetting number \( \zeta \), a a measure of the ratio of the electrostatic energy to the surface tension.

\[ \cos\theta(V) = \cos\theta(0) + \frac{1}{2} \frac{1}{\gamma_{LG}(V)} \frac{\varepsilon_{r}\varepsilon_{0}}{d}V^{2} = \cos\theta(0) + \zeta \]  

(3.23)

This equation has been verified experimentally and the results obtained are in good agreement with those predicted theoretically, see Figure 4.20. There are a few subtleties and drawbacks such as contact angle saturation\(^{147}\) and hysteresis\(^{148}\), these phenomena will be assessed with respect to the actual system designed in this work, which is in Section 4.3. If one empirically derives the contact angle change required for actuation it is possible to calculate the required driving voltage using Equation 3.23. Traditionally it was thought the change in
contact angle that occurs due to an applied voltage was the driving force for the translocation of mass in the droplet. It is now believed that the change in contact angle is a consequence of charge crowding at the triple-interface line and is not in fact a requirement for droplet movement\textsuperscript{146}.

3.3.2.5 Contact Angle Saturation and Hysteresis

Contact angle saturation is a phenomenon whereby the actual change in contact angle as a function of applied voltage differs significantly from the theory. There are a few competing theories on the mechanisms behind the saturation of the contact angle namely, charge trapping\textsuperscript{149}, polarisation of the fluoropolymer\textsuperscript{150}, finite conductance of the liquid\textsuperscript{141} and charge crowding.

The charge trapping explanation proposed by Verhiegen \textit{et al.} is the most intuitive explanation. These authors hypothesise that some charge is trapped as an additional layer after injection into the dielectric. This acts as a shield and reduces the effective electric field across the device, therefore limiting the contact angle change. The same effect would manifest itself if the surface was becoming polarised as in Chudleigh’s electret model\textsuperscript{150}. Shapiro \textit{et al.} provide an interesting electronics model based on the finite conductivity of the liquid\textsuperscript{141}. They state that even de-ionised water has a small amount of conductance and that this is sufficient to explain contact angle saturation. The concept is that in the equivalent circuit model of the device the droplet is represented by a parallel resistor and capacitor. The resistor represents the conductance of the droplet. As the droplet wets out, then the effective length of the resistor increases, measured from the centre of the droplet to the TCL. As this distance increases, so does this effective resistance and, therefore, this limits the effective electric field that can be dropped across the droplet, resulting in the phenomenon of contact angle saturation. The charge crowding theory fits with data predicted by the model of Shapiro and builds on the ideas presented in the previous section on electrospray. The total amount of charge that can occupy any given liquid-volume close to the
TCL is fixed by the Raleigh Limit and the surface tension of the liquid. Charge crowding that occurs due to the induced free-charge polarisation leads to coulombic repulsion and ejection of satellite droplets from the interface line. Evidence supporting this last theory is presented in Section 4.3.

Interestingly, and of particular benefit for the work described in this thesis, one of the conclusions that can be drawn from the finite conductance model is that the contact saturation angle is reduced by charge carriers such as salt and proteins.

### 3.3.2.6 Electrodynamical Interpretation of DMF

Amongst others, Jones\(^97\) has been a proponent of the electrodynamic interpretation of EWOD and DMF systems. The rationale is that these systems are in fact low-frequency DEP systems and can be modelled as such. If this is in fact the case, then the equations describing DC-DMF, AC-DMF and DEP could all be derived from the same fundamental equations. This section briefly introduces just such a derivation using an electromechanical approach, starting with the Maxwell Stress Tensor, Equation 3.24.

One can determine the total force vector acting on the droplet surface by evaluating Equation 3.24 (a derivation of which is provided in Appendix A).

\[
\vec{F} = \oint_s \vec{T} \cdot d\hat{a} \tag{3.24}
\]

It has been shown\(^{146}\) that, if one considers a volume element \(dV\) at the liquid air interface of a perfectly conductive liquid droplet, the tangential component of the electric field at the surface vanishes, and that the normal component is related to the local surface charge density by Gauss’ law (A.43). Evaluation of the Maxwell Stress Tensor shows that the only non-vanishing contribution is a force per unit surface area \(dA\) directed outward along the outward surface normal \(\vec{n}\).

\[
\frac{\vec{F}}{dA} = P_{el} \vec{n} = \frac{\varepsilon_0}{2} E^2 \vec{n} = \frac{\rho_s}{2} \vec{E} \tag{3.25}
\]

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Equation 3.25 introduces the electrostatic pressure \( P_{el} = \frac{\varepsilon_0 E^2}{2} \) acting on the liquid surface. The term \( P_{el} \) is therefore a negative contribution to the total Young-Laplace pressure within the liquid. Working from Mugele’s 2005 paper\(^{146}\), one can determine how the local pressure change affects the contact angle. Far away from the contact line, the charge density at the solid-liquid interface is \( \rho_{sl} = \frac{\varepsilon_0 \varepsilon_r V}{d} \) and the liquid-vapour surface charge density goes to zero. As the three-phase contact line is approached both densities increase due to sharp edge effects. The force arising from the charges at the solid-liquid interface leads to a normal stress on the insulator surface, which is balanced by the elastic stress. The forces at the liquid-vapour interface, however, contain both a vertical and a horizontal component pulling on the liquid. Remembering the dimensionless electrowetting number; \( \zeta = \frac{\varepsilon_0 \varepsilon_r V^2}{2d^2 \gamma_{LG}(V)} \), the net horizontal force acting on the droplet can be evaluated as:

\[
F_x = \frac{1}{2} \frac{\varepsilon_0 \varepsilon_r V^2}{d} = \gamma_{lg} \zeta
\]

Equations 3.23 and 3.26 have been derived in two different but complementary ways. They are equivalent but for one fundamental difference. This is the fact that the energy minimisation method gives the contact angle change, the MST method defines the force acting on the droplet without invoking the requirement of a contact angle change. The shape independence therefore means that the change in contact angle should be regarded as an independent phenomenon and not one which is required for actuation of droplets in DMF devices\(^{151}\).

### 3.3.2.7 Relationship between DC-DMF, AC-DMF and DEP

Jones’s\(^{96,97,104,151}\) papers have similar derivations based on the MST method for DEP devices, and further work by other research groups\(^{152}\) are beginning to provide an integrated approach to the electrodynamics of the interaction of electric fields with liquids, and over the full range of frequencies from DC to RF. The fundamental interactions of charged particles and electric fields cannot change.
Therefore, all observed phenomena from Lippmann to Pethig must be facets of the same underlying physics. The problem of analysing these systems using a frequency sweep from DC to RF is a complex problem to solve, for a number of reasons. Firstly there exists a characteristic frequency \( \omega_c \), whereby liquids change from being mostly conductive to being mostly dielectric; \( \omega_c = \frac{\theta_1}{\epsilon_1 \epsilon_0} \), (see reference 146). This frequency marks the boundary, when \( \omega_c = 0 \) one has DC-DMF systems, far below \( \omega_c \) one has AC-DMF systems where the applied AC field can reasonably be modelled by the equivalent rms voltage as \( V_{rms} = \frac{V}{\sqrt{2}} \). Far above this value of \( \omega_c \) dielectric body forces dominate, and one is dealing with dielectrophoretic phenomena. The Maxwell stress tensor model incorporates all of these phenomena. In the general solution of the MST, \( \vec{f} = \frac{1}{2} \epsilon \vec{\nabla} \vec{E}^2 \), the frequency dependence is included in the \( \epsilon \) term in accordance with the Clausius-Mosotti factor, as the complex dielectric constant is frequency dependent. The electric field gradient and, therefore, x and y-axis device geometry is included in the \( \vec{\nabla} \) term, the z-axis device geometry and applied voltage is included in the \( \vec{E}^2 \) term, as \( E = \frac{V}{\sqrt{2}} \).

There is no single commercial software computer package available to solve the finite-element analysis of the time-dependant electric field gradients, computational fluid dynamics and equivalent circuit analysis simulations. Therefore design of the devices needs to be tackled with care, and with a complete overview of the implications of each variable on system performance.

### 3.4 Summary

The background theory of microfluidics and DMF has been introduced. The electrodynamic interpretation of DMF has been derived using consistent notation, and with reference to the fundamental electrodynamic theory. The development of this background theory enables physical devices to be designed and characterised as described in Chapter 4.
Chapter 4

Fabrication and Characterisation of Digital Microfluidic Devices

4.1 Design of Microfabrication Process and DMF Devices

The design and manufacturing process for digital microfluidics devices has led to some interesting technology, ranging from very sophisticated semiconductor type microfabrication to cheap, disposable devices\(^{153}\). A variety of devices have been designed and tested for their utility in digital microfluidic sample processing, each having their own relative benefits and drawbacks. The silicon based devices used in this work were manufactured in the Scottish Microelectronics Centre, using standard microfabrication techniques such as photolithography, plasma etching and spin coating. The background theory to these techniques is not introduced here in explicit detail, since the processes are commonplace and industry standard. The full microfabrication process flows are provided in the experimental section of this thesis. Rapid prototyping processes are described in Section 4.1.6.

4.1.1 Design Considerations for DMF Devices

The main design considerations and constraints for DMF devices can be deduced by a close examination of the fundamental governing equations of these systems. Consideration of the Lippmann-Young Equation (Equation 3.23) shows that for
the dielectric layer covering the metal electrode it should have a very high dielectric strength and (to facilitate lower driving voltages) be as thin as possible. The approach that has been taken in designing the devices follows from the approach of Moon et al.\textsuperscript{138}. Following from the experiments by Moon a contact angle change of 40 degrees has been used as a starting point for inclusion in initial design calculations using the Lippman-Young equation. The next stage was to work with the available manufacturing processes and material characteristics, such that a driving voltage could be calculated. These factors formed the basis of the initial device design, which was then iterated following device characterisation. The final devices described in the following sections owe much of their initial design characteristics to previous work in the group completed before this body of work was undertaken. Notable contributions to this preexisting work are from; Peng Li’s Masters Thesis, Design of backplane chip for active EWOD device\textsuperscript{154}, Keith Muir’s Masters Thesis\textsuperscript{155}, and Yifan Li’s PhD Thesis\textsuperscript{156}. Keith Muir’s thesis, in particular, focused on the study of the device geometry and the outcomes provided a starting design for the electrode geometry and spacing. The materials investigation by Yifan Li provided the starting point for the low-voltage devices resulting in a publication in Solid State Devices\textsuperscript{157}.

Two device configurations can be used with DMF devices, those with a top-coverplate (a closed configuration), as shown in Figure 4.3, and those without a coverplate (known as open or co-planar), as shown in Figure 4.7. The advantage of closed designs is that droplets can be split or dispensed and the droplet volume is at least an order of magnitude lower than in the equivalent open-device. However, access to the reagents on the device surface is restricted, for the initial studies presented here the co-planar geometry was used unless otherwise stated. Co-planar devices allow much easier access to the droplets on the chip-surface although the droplets cannot be split or dispensed in this configuration, in addition the evaporation of the droplet is more pronounced as compared to the closed configuration.
4.1.2 Silicon DMF Devices

The silicon based DMF devices that have been designed were based on industry standard CMOS processing techniques. There was no requirement to use silicon for the DMF devices as it merely acts as a substrate upon which the metal and dielectric layers are deposited and patterned. The substrate could easily be doped silicon coated with an oxide layer, silicon-over-insulator (SOI) wafers, or glass. 3-inch undoped (1,0,0) silicon wafers, with an insulating thermally grown oxide layer, have been used in this study as these are cheap, readily available and compatible with all of the processes required for device fabrication used in the SMC. Figure 4.1 shows the physical layout of the chip-design on a 3-inch wafer.

![Image](image)

Figure 4.1: Left figure shows the physical layout of two silicon DMF devices on a three-inch wafer, the right figure shows the pad-ring numbering and the greyed box shows the location of the active electrode array.

The physical size of the devices and the pitch of the electrodes in the pad-ring was inherited from previous designs of device that had been manufactured in the group. These dimensions were re-used in order that the new-devices be compatible with pre-existing driving and testing hardware. It became apparent very early on in the design of these devices that there had been a lack of consistency in
device size in previous studies. To avoid alignment problems in the design of test hardware, a ‘Group-Standard’ document was prepared and deployed on the SMC tool-box (an internal manufacturing reference site). The standard device layout and dicing lanes are shown in Figure 4.1. The standard dimensions are: L1=50.26 mm, L2=25 mm, L3=21 mm, L4=2 mm, d1=1.6 mm, d2=0.55 mm, d3=0.55 mm, d4=0.54 mm. Allowance must be made for the width of the saw cut, therefore a 100 µm dicing lane is recommended. The overall chip dimensions are \((L2+d2+d1/2−0.05)\times(L1+2\times d3) = 26.3 \text{ mm} \times 51.36 \text{ mm}\). This standard was then used consistently in all silicon device designs. In addition to the physical device dimensions, the bond-pad layout was kept consistent, with 2000 µm by 2000 µm pads using a 2545 µm pitch.

The mask designs for the devices were drawn in the CAD package, Cadence Virtuoso Layout Manager. This is an industry standard design package in microelectronics, and allowed the resultant design files to be sent to commercial companies for fabrication of the photolithographic masks. The masks used in this work were constructed of chromium on quartz. These masks are made using an e-beam technique and are of high quality and resolution. There are two different types of mask design, known as light-field and dark-field. A light-field mask is one in which the majority of the mask is optically transparent, with the features defined by chromium covered areas. A dark-field mask is the exact opposite, with the majority of the mask being covered in chromium, and the features defined by the optically transparent, chromium free areas. The choice of mask depends on the photoresist being used. A positive resist is one in which exposing the resist to UV light weakens the bonding, and exposed areas can be removed using a chemical etchant. A negative resist would protect any non-exposed areas. The processing used in this work uses only positive photoresists. As a brief overview to photolithography, the main steps involved in patterning a deposited metal layer are:
Figure 4.2: The Group-Standard for silicon DMF device design, the standard dimensions are: L1=50.26 mm, L2=25 mm, L3=21 mm, L4=2 mm, d1=1.6 mm, d2=0.55 mm, d3=0.55 mm, d4=0.54 mm.
1. The photoresist is deposited by spin-coating onto the material that is to be patterned.
2. The mask is aligned over the device.
3. The device is exposed to a UV light source.
4. The optically exposed areas of weakened photoresist are removed.
5. The exposed underlying material is removed by chemical etching or plasma etching.
6. The photoresist is removed.

4.1.3 Single Metal Layer Devices

Figure 4.3 shows a schematic cross section of a typical single metal layer, DMF device, including the droplet and top-coverplate. Working from the bottom upwards, the cross sectional layers in the SML devices are described in Table 4.1. The layer thicknesses shown in the table are typical, although detailed process flows are provided in Section 7.1. The design of these devices requires only one mask layer, thus making them relatively cheap and quick to manufacture as compared to the more complicated devices presented later.

Figure 4.3: Schematic cross section of a single metal layer DMF device including the top-coverplate, the top-plate is typically glass coated with a thin conductive layer of ITO.

In Figure 4.4 the design for a ‘W-shaped’ device is shown. This device was designed as a first step towards the 8x5 array, it uses only a single metal layer and thus some of the electrodes from the full 8x5 array were removed to allow space for the interconnects, thus leaving a ‘W-shaped’ device. The full 8x5 array, discussed
Table 4.1: Composition of cross sectional layers in the SML devices

<table>
<thead>
<tr>
<th>Layer</th>
<th>Material</th>
<th>Typical Thickness</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate</td>
<td>Silicon</td>
<td></td>
</tr>
<tr>
<td>Bond pad and electrode metal</td>
<td>Aluminium or Tantalum</td>
<td>~300 nm</td>
</tr>
<tr>
<td>Optional Dielectric layer</td>
<td>SiO$_2$ or Ta$_2$O$_5$</td>
<td>~100 nm</td>
</tr>
<tr>
<td>Optional Dielectric layer</td>
<td>Parylene</td>
<td>~500 nm</td>
</tr>
<tr>
<td>Hydrophobic layer</td>
<td>Teflon or Cytop</td>
<td>~30 nm</td>
</tr>
</tbody>
</table>

in the following section, is a complex device that required many iterations in the development of the process design. In order to characterise the electrode geometries, and to investigate if it was indeed necessary to spend the development time on the full array, the design was flattened into a single layer and those electrodes which impeded the routing of interconnects were removed. The electrode size was roughly 1 mm by 1 mm, this size was chosen for two reasons. Firstly, droplets with a volume of 1 $\mu$l are roughly 1 mm in diameter. Secondly 1 $\mu$l droplets are approximately the smallest size that can be manually pipetted. The castellated edge design is derivative of a previous study$^{158}$, following work by previous group members$^{155}$ in which the geometry of the electrode edge was investigated for its role in droplet actuation. Interdigitated electrodes facilitate droplet actuation by providing an electric field gradient between electrodes. A droplet which is sitting mainly on one electrode but slightly overlapping with another is sitting in an area of electric field gradient and feels an electrohydrodynamic force. A photograph of two W-shaped arrays is shown in Figure 4.4.

4.1.4 Multi Metal Layer Devices

In Figure 4.5 a cross section for a typical multi-metal layer DMF device is shown, including the droplet and top coverplate. Working from the bottom upwards, the cross sectional layers in the MML devices are defined in Table 4.2. This is a significantly more complex design than the single metal layer devices, and required the use of three masks. The first mask defines the interconnect layer, the
second defines the via area for connection between the lowest and topmost metal layers. The third mask defines the electrode patterns. This device requires many more processing steps and the use of a chemical mechanical planarisation (CMP) machine. The CMP step removes any unwanted hillocks due to the underlying topology, and provides a flat surface onto which the dielectric and hydrophobic layers can be deposited.

Figure 4.5: Schematic cross section of a multi metal layer DMF device

Figure 4.6 shows the layout and pad-ring connections for each of the electrodes in the 8x5 multimetal layer array. Further information on the three masks is
Table 4.2: Composition of cross sectional layers in the MML devices

<table>
<thead>
<tr>
<th>Layer</th>
<th>Material</th>
<th>Thickness</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate</td>
<td>Silicon</td>
<td></td>
</tr>
<tr>
<td>Metal interconnect</td>
<td>Aluminium</td>
<td>∼300 nm</td>
</tr>
<tr>
<td>Insulating layer</td>
<td>$SiO_2$</td>
<td>∼1 µm</td>
</tr>
<tr>
<td>Bond pad &amp; electrode metal</td>
<td>Tantalum or Aluminium</td>
<td>∼300 nm</td>
</tr>
<tr>
<td>Optional dielectric layer</td>
<td>$SiO_2$ or $Ta_2O_5$</td>
<td>∼100 nm</td>
</tr>
<tr>
<td>Optional dielectric layer</td>
<td>Parylene</td>
<td>∼500 nm</td>
</tr>
<tr>
<td>Hydrophobic layer 2</td>
<td>Teflon or Cytop</td>
<td>∼30 nm</td>
</tr>
</tbody>
</table>

provided in Appendix 7.1. As with the single metal layer devices, two multi-metal layer devices were designed. These included devices with a ground line for co-planar operation as well as those without a ground-line for use in a top-plate configuration. Figure 4.7 shows a cross section of a typical co-planar multi metal layer DMF device.

Figure 4.6: Multi metal layer array showing the completed device and the pad-numbering referenced to the pad ring, the seemingly random numbering configuration is an artifact of the routing of the interconnect layer.
4.1.5 Tantalum Pentoxide

Anodic $Ta_2O_5$ is very well established in the electronics industry, being widely used as the dielectric in electrolytic capacitors. In addition it has been used for gate insulators in metal-oxide-semiconductor (MOS) devices, optical coatings, anti-reflection coatings and coatings for hot mirrors$^{159}$. It produces a dense, smooth, high relative permittivity and homogeneous oxide of well-defined and reproducible thickness at room temperature. When formed in an electrolyte solution or gel, this layer can be produced pin-hole free, making it very attractive for DMF applications where any porosity in the dielectric layers can cause electrolysis. This technology has been developed and implemented in both the single and multi-metal layer devices, where the top-metal layer is replaced by tantalum$^{157}$. Tantalum pentoxide is formed by anodisation, which is a partially sacrificial technique. The oxide forms on top of the tantalum whilst also eating into the surface slightly. Due to the anodisation process and the characteristics of tantalum pentoxide it acts as a good dielectric in one direction only, having a breakdown strength of $\sim$1 MV per centimetre$^{160}$. Anodic tantalum pentoxide has been well studied in the literature for use in capacitors$^{159-161}$. The dielectric constant varies with the thickness of the layer, ranging from $\epsilon_{Ta_2O_5} = 12$ at 38 nm to $\epsilon_{Ta_2O_5} = 26$ at 180 nm$^{160}$. The majority of the fluidic processing tasks that are presented in Chapter...
4 were performed on one of the single-metal layer W-shaped designs shown in Figure 4.4. Two devices, based on very similar geometries were designed. One incorporating an embedded ground plane for co-planar operation, and the other without a ground plane for cover-plate designs. The full mask designs are exactly the same as the previous single and multi-metal layer aluminium devices described previously, and are provided in Appendix 7.1.

4.1.6 Disposable Chips and Rapid Prototyping of DMF Devices

As can be seen from the preceding discussion of silicon based microfabrication, it is a highly specialised technique requiring millions of pounds worth of equipment and expertise. Wheeler\(^2\), from the University of Toronto, has pioneered a technique whereby the development and manufacture of digital microfluidic devices can be performed in any laboratory, quickly, cheaply and easily\(^1,162–164\). This technique allows both cheap manufacture and rapid prototyping. Devices can be drawn, printed, etched and coated in a matter of hours and requiring only common laboratory supplies. Whilst these devices cannot match the silicon microfabricated devices, in terms of repeatability or low-driving voltage, their low-cost, high-throughput manufacture and disposability means that they are of significant interest if DMF is to become a commercial and mainstream technology. Other groups have investigated rapid-prototyping using soft-lithography in the manufacture of digital microfluidic devices\(^{165}\). In addition to developing these copper-clad devices, a new technique for the manufacture of DMF devices has been developed by our research group. This technology is in its infancy but promises to deliver an even cheaper and higher throughput manufacture of devices. It is based on screen printing of conductive inks and is described at the end of this section. A clear advantage of this technique is that the devices can be used once and then disposed, this provides a fresh surface at the outset of each experiment thus mitigating problems due to biofouling and other modes of failure.
of the devices.

### 4.1.7 Copper Clad Polyimide Devices

The copper-clad polyimide devices are manufactured using sheets of a material called Pyralux®, manufactured by DuPont UK. The Pyralux sheets consist of a thin polyimide layer laminated between two sheets of 9 µm copper. This material is typically used for flexible circuit boards and in ribbon cables for LCD displays. Abdelgawad *et al.*¹ pioneered the use of Pyralux for use in DMF-devices as described in their 2009 paper titled ‘All-Terrain-Droplet-Actuation’. There are a variety of techniques that have been developed for the patterning and etching of these sheets. These are summarised in Table 4.3.

<table>
<thead>
<tr>
<th>Patterning Technique</th>
<th>Printer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toner transfer</td>
<td>Any laser printer</td>
</tr>
<tr>
<td>Laser printer direct printing</td>
<td>Brother HL-5270DN, 1200 dpi printer</td>
</tr>
<tr>
<td>Solid wax direct printing</td>
<td>Xerox Phaser 8560N, Solid ink printer</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Etching Solution</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferric-chloride</td>
<td>Cheap but messy and single shot</td>
</tr>
<tr>
<td>Cupric-chloride</td>
<td>Can be regenerated but slower etch times</td>
</tr>
</tbody>
</table>

#### 4.1.7.1 Patterning Techniques

There are relative advantages and disadvantages of each patterning or etching technique and each has been evaluated, albeit somewhat subjectively. The toner transfer technique is easy and allows piece of mind, since no copper sheeting has to pass through an expensive printer. However it is messy and provides low-resolution resists. This technique involves printing a design onto a glossy sheet of paper using a laser printer. The toner is then transferred by pressing and heating this sheet against the copper sheeting. Once cool the paper can be removed using water, leaving only the toner on the sheeting. The direct printing technique, by
comparison, requires that the copper sheeting goes directly through the printer. It provides high-resolution patterning, although the toner coverage can lead to pinholes in the devices after etching. Typically devices were designed using a vector graphics programme (Inkscape, open source) to provide high-quality images. The laser printer method proved difficult, as the image charge that is transferred to the sheet in the printer dissipated in the metal leading to poor quality image transfer. Figure 4.8 shows a photograph of a sheet of Pyralux that has been patterned by direct printing with toner in a laser printer. The solid wax direct printing technique provides a high quality resist with few or no pinholes, once the devices were printed the wax was melted briefly using a hot-plate to re-flow the mask over any potential holes or blemishes.

Figure 4.8: Pyralux sheets patterned with toner from a laser printer, one device has been cut out, etched and coated with pyralux as shown in Figure 4.9, this technique allows the co-manufacture of multiple devices on a single A4 sheet.

4.1.7.2 Etching Methods

The etching solutions employed were ferric chloride or cupric chloride. Whilst ferric chloride is cheap, it is a messy solution and requires careful disposal. The
cupric chloride can be regenerated by oxidation, and as such, never needs to be disposed of. However it does have slower etch times and requires the addition of 10M hydrochloric acid and 30% hydrogen peroxide, which are hazardous chemicals. The procedure employed was as follows. Ferric chloride crystals ($FeCl_3$, Maplin, UK) were dissolved in 250 ml warm tap water, which was then made up to one pint (468 ml). Pyralux sheeting masked with either toner or wax was immersed in this solution until all of the unmasked copper had reacted, Figure 4.9 shows a photograph of a device that has been masked using toner from direct printing, in a laser printer, followed by a ferric chloride etch. Once the etching is complete the toner can be removed by scrubbing the surface with acetone. Cupric chloride solution was made by adding one part hydrochloric acid (10 M) to two parts hydrogen peroxide (diluted to 3%), and etching copper sheeting in the resultant solution. The initial reaction; 

$$Cu + 2HCl + H_2O_2 \rightarrow CuCl_2 + 2H_2O$$

makes copper (II) chloride, and this should be a bright green colour. There is a secondary reaction; 

$$CuCl_2 + Cu \rightarrow 2CuCl$$

which produces a dark brown (copper(I)) precipitate, which eventually turns the whole solution a dark green colour and becomes unreactive. The major benefit of using $CuCl$ solution is that by bubbling oxygen through the solution, copper (I) can be oxidised back to copper (II) as shown in the reaction 

$$2CuCl + 2HCl + O \rightarrow 2CuCl_2 + H_2O.$$ 

The oxidation is complete when the solution returns to a bright green colour. This means that (with the periodic addition of more acid and peroxide) the solution can be re-used indefinitely, thereby removing the problems of disposal and cost associated with ferric chloride. The cupric chloride solution provides etch times that are approximately double those of the standard ferric chloride solution.

4.1.7.3 Cross Sectional Layout of Copper Devices

The majority of devices were manufactured using the solid wax printer and etched using ferric chloride. The composition of the cross sectional layers in the Pyralux devices, working from the bottom upwards, are given in Table 4.4.
Figure 4.9: Ferric chloride etched copper clad polyimide device, this device has been coated with parylene as a dielectric and hydrophobic layer.

Table 4.4: Composition of cross sectional layers in the Pyralux devices

<table>
<thead>
<tr>
<th>Layer</th>
<th>Material</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate</td>
<td>Polyimide</td>
</tr>
<tr>
<td>Metal interconnect and electrode metal</td>
<td>Copper</td>
</tr>
<tr>
<td>Insulating layer</td>
<td>n/a</td>
</tr>
<tr>
<td>Dielectric layer</td>
<td>Parylene</td>
</tr>
<tr>
<td>Hydrophobic layer</td>
<td>Teflon or Cytop</td>
</tr>
</tbody>
</table>
An interesting point to note is that the devices need to be polished before they will work reproducibly. The surface roughness affects the contact angle hysteresis. A variety of techniques were investigated for polishing the surface until it was as flat as possible. These included sandpaper, wet-and-dry paper, diamond lens polishing sheets and scrubbing with acetic acid. The best polish, however, was found to be a commercial metal polish (Brasso, Reckitt and Sons, UK). This can be thought of as a significantly cheaper analog of the CMP process used in microfabrication. The devices were polished for 3-4 minutes, until the copper was flat and reflective.

4.1.8 Screen Printed Devices

In more recent work, an even higher throughput and cheaper manufacturing process has been designed. This is based around screen-printing of conductive inks onto a flexible substrate. Two inks and two substrates have been investigated. The devices presented here are being developed in conjunction with an industrial partner (The Ants Nest), with a view to commercialisation. For this reason much of the physical data regarding the inks and varnishes is commercially sensitive and is not reproduced here. This technique is very much in its infancy but holds great promise for cost effective production of custom DMF devices. The speed and relatively low cost of this process, together with the ability to move rapidly from custom designs to functional devices promises significantly lower unit costs. This production process is presented here for completeness, although comprehensive device characterisation has yet to be fully assessed, this is due to the current availability of devices and the current state of the project. Future work will focus on a characterisation of the devices using the methodologies presented in this thesis as applied to the silicon devices. The screen printed device construction is similar to the previous silicon designs, but for the omission of a metal layer and the use of a conductive ink in its place. The composition of cross sectional layers in the screen printed devices, is given in Table 4.5 below.
Table 4.5: Composition of cross sectional layers in the screen printed devices

<table>
<thead>
<tr>
<th>Layer</th>
<th>Material</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate</td>
<td>Polycarbonate or fibreboard</td>
</tr>
<tr>
<td>Interconnect and electrodes</td>
<td>Carbon ink or silver epoxy</td>
</tr>
<tr>
<td>Insulating layer</td>
<td>n/a</td>
</tr>
<tr>
<td>Dielectric layer</td>
<td>Parylene</td>
</tr>
<tr>
<td>Hydrophobic layer</td>
<td>Teflon or Cytop</td>
</tr>
</tbody>
</table>

Shown in Figures 4.10 and 4.11 are photographs of the first batches of these devices. The two devices in Figure 4.10 only differ in that two different inks have been used, conductive carbon ink in black and conductive silver epoxy in white. The initial devices had only a screen printed ink layer, and the dielectric layers were then post-processed in the SMC. One major problem with these devices was that the ink formed tiny cracks, due to the flexible substrate, and this could lead to device failure. For this reason the substrate was changed to a much more rigid fibreboard, as shown in Figure 4.11. Current work is focusing on the feasibility of screen printing the dielectric layers using an epoxy and Teflon mixture. Although only at an early stage, this manufacturing process holds much promise for low-cost high-throughput DMF devices.

Figure 4.10: Prototype screen-printed polythene DMF devices
4.2 Implementation of Computer Control for DMF Devices

Control of the DMF devices was initially accomplished using a switch box to manually switch successive electrodes between low and high voltages. A 40 way switch box was designed and manufactured to allow control of the arrays, see Appendix D.4. Though this method was slow, and required a degree of dexterity by the operator it is none-the-less a very simple technique and was essential for debugging as well as providing a benchmark against which an automated control system could be compared. Automation of the DMF devices was essential to allow integration with the MS and LC-systems as well as allowing an objective analysis of system performance.

The switches utilised in the switchbox were single pole double throw (SPDT) to allow switching only between high and low voltages, see Figure 4.12. Previous designs had employed single pole triple throw switches, which allowed a ‘floating’ pole at the centre point of the switch. This design was rejected since it didn’t mimic the automated designs which would be used in later iterations, and could give rise to different system performance.

The system has now been fully automated using National Instruments’ LabView as the basis of the control system software. The following sections provide
details of the control circuitry which has been designed to allow control of low-voltage DC devices and high-voltage AC devices. The control system software-design is discussed in Section 5.2.2.

4.2.1 Control of Low-Voltage, DC Devices

Low voltage DC devices can be controlled directly from the U2653A 64-channel digital I/O box (Agilent, Santa Clara, USA). This device was chosen specifically because it can handle up to 35 volts per channel, meaning that no extra circuitry was required to drive the $Ta_2O_5$ devices. This device acts as a current sink, and an array of pull-down resistors is required to allow switching between high and low voltages.

The Agilent U2653A digital I/O box, shown in Appendix D.5, connects to a break-out screw-terminal board via a high-density 100-pin SCSI cable. The pull-down resistor array has a common positive voltage applied to one end and the other end of each resistor goes to a particular current-sink output. Figure 4.13 shows one element in the array. The control electrodes on the DMF arrays are connected via the clamps on the jig, then through an IDE ribbon cable and finally, are terminated individually in the screw-terminal break-out board.

The pull-down resistor has the effect of limiting the current to the electrodes, which may have been of significance in the operation of early DMF devices. This will be discussed further in Section 4.3. Figure 4.14 shows a number of consecutive
Figure 4.13: Circuit diagram of LabView controlled I/O box for DMF device control, this is one of forty repeated elements, the connection to the I/O box is made at the current sink.

still-frames from a video of a $\sim 500$ nl droplet moving from right to left and back again on a coplanar, tantalum pentoxide DMF array under complete computer control.

4.2.2 Control of High-Voltage, DC Devices

High voltage DC devices were controlled using a series of magnetically coupled reed relays (DIP05-1A75-11D, Meder) connected to the output of the Agilent digital IO box and to a high voltage supply. An initial bread-board system is shown in Figure 4.15. The relays separate the 5 volt control circuit from the high-voltage DC driving voltages.

Figure 4.16 shows the system diagram for one electrode in the HV-DC DMF control circuit, this simple design is repeated in the form of a device drive bus with up to forty elements.

4.2.3 Control of High-Voltage, AC Devices

The same system shown in Figure 4.15 was used to control the HV-AC devices, although for these devices a high voltage AC signal was also required. Initial
Figure 4.14: Frame by frame video of droplet movement, frames are separated by \(~750\) ms and the droplet is \(~500\) nl
Figure 4.15: System setup of HV-DC DMF device control. Shown, from left to right, the IO breakout board, the relay switching board and the chip connection block.

Figure 4.16: Circuit diagram of HVDC DMF device control, this is one of forty repeated elements, the connection to the I/O box is made at the current sink.
designs for the system involved use of an ENI 2100L power amplifier connected to a custom-designed, hand-wound transformer to act as a voltage-controlled voltage source and to amplify the output of a Gould DWG-7000 function generator. This system worked well, but due to the high-current in the primary winding, the transformer tended to generate excess thermal heating and the health and safety implications meant that it had to be abandoned. Instead, a current-limited high-voltage high-frequency amplifier with a safety switch cut-out was used. This device, a PZD700A amplifier, (Trek, New York, USA) is shown in Appendix D.6. This amplifier provides a voltage gain of 200, with a maximum output swing of plus or minus 700 V.

Figure 4.17 shows the system diagram for driving one electrode in the HV-AC DMF control circuit. This switching system is fundamentally the same as the HV-DC devices and, as such, moving between the two systems consists of merely switching the high-voltage DC supply for the function-generator and amplifier. Therefore, the control software and physical location of the relay circuitry can be fixed and consistent between the two driving regimes. Typically, AC devices were driven with an 18 kHz sinusoidal signal of amplitude $\sim 400-500$ volts peak to peak.

Figure 4.17: Circuit diagram of HV-AC DMF device control, this is one of forty repeated elements, the connection to the I/O box is made at the current sink.
4.3 Characterisation of Digital Microfluidic Devices

The fundamental relationship between contact angle and voltage has been experimentally investigated using a contact-angle measurement system. Whilst similar studies have been documented in the literature\textsuperscript{146}, there are few reports which have examined the reasons behind the failure of the devices over prolonged periods of time. In this work three types of failure have been observed; 1. Contact-angle saturation, where the change in contact angle deviates significantly from the theory. 2. Contact angle hysteresis, where the observed contact angle differs on the leading and receding edge of the droplet. 3. Biofouling, where proteins non-specifically adsorb to the surface and affect the hydrophobicity. The commonly observed mode of failure in each of these cases is the cessation of droplet movement after a period of time. There appears to be a significant dependence of the time to failure on the driving voltage and dielectric layer thicknesses. Several theories have been proposed in the literature in an attempt to explain this phenomenon, including; dielectric breakdown, charge trapping and polarisation\textsuperscript{147}.

In an attempt to define some metrics for the devices manufactured in this work a system was developed to provide an objective measure of performance.

4.3.1 Contact Angle Saturation Experiments

Working from Equation 3.23, and including the parameters from the devices that have been designed, one can plot the theoretical voltage-contact angle dependence curves according to Equation 4.1.

\[
\cos \theta(V) = \cos \theta(0) - \frac{1}{2 \cdot 0.072} (C_{\text{Tao}_5} + C_{\text{Teflon}}) V^2
\]  

(4.1)

The capacitance per unit area values in Equation 4.1 can be determined by evaluating \( C = \frac{\epsilon_0 \epsilon_r}{d} \). The relative permittivity for \( \text{Tao}_5 \) changes as a function of layer thickness\textsuperscript{166}. For the system that has been investigated here, the literature
values for the two layer thicknesses are $\epsilon_{Ta_2O_5} = 12$ at 38 nm and $\epsilon_{Ta_2O_5} = 26$ at 180 nm, for Teflon $\epsilon_{Teflon} = 1.9$ at 16 nm.

Figure 4.18: Contact angle measurement system

A simple contact angle measurement system was modified to include automated electrowetting and image acquisition as shown in Figure 4.18. Briefly, the system included a pulse train generator to trigger both the camera and a simple reed relay, which was used to deliver the voltage to the electrowetting system. Two images were captured per relay pulse event, and the contact angle was determined using the software package FTA (First Ten Angstroms, Virginia, USA). Figure 4.19 shows a typical output frame from the video that was acquired. This system was run for a variety of system parameters in order to fully characterise the performance of the tested devices.

A range of voltages were used to examine the dependance of voltage on the initial contact angle change. The results of these measurements are shown in Figure 4.20. The theoretical contact angle change calculated from Equation 4.1, is plotted along with the raw data. Data for two $Ta_2O_5$ layer thicknesses are shown. These results show that thicker dielectrics provide a larger range for the driving voltage. The deviation from the theoretical plot line is of interest. The modelling has been performed using the Lippmann-Young equation and using accurate values for the layer thicknesses and dielectric constants. Despite this, the results show a translational shift in the X-axis towards higher voltages. This
may indicate that the droplet is experiencing an additional voltage, due to polarisation or charging of the dielectric, this warrants further investigation. These results were collected by recording the contact angle change due to the applied voltage over one actuation. Looking at the response of the system over a series of actuations should give a clearer indication of the mechanism involved, and this has been performed as described in the following section.

4.3.2 Time to Failure Experiments

A well known phenomenon of DMF systems is their failure over time. In this work the captured video was processed using a custom piece of software, based on the OpenCV library. Essentially, any repeated frames are rejected and a reduced data set is created for analysis by the FTA contact angle software. The results from the contact angle determination are then further processed by calculating the left and right contact angles, these should be very similar and in the case where $\theta_l$ does not equal $\theta_r$ to within a defined tolerance the data point is ignored. The contact angle change between the off and on states is then calculated and an exponential function is fitted to the data. The exponential function used was $d_0 e^{\frac{\Delta \alpha}{\tau}} + d_\infty$, where $d_0$ represents the difference between the initial contact angle change and
Figure 4.20: Voltage vs contact angle results from CA measurement system, showing the comparison of the theoretical contact angle change with respect to voltage as predicted by the Lippman-Youn equation with the actual data collected. The equations use a $Ta_2O_5$ thickness of 38 or 180 nm, and a teflon thickness of 16 nm. Contact angle saturation is clearly shown where the experimental data deviates from the theory. This data is for the wafer batch numbers bos060811w09_38nm and bos060811w09_180nm
the saturated contact angle change, $d_\infty$. This exponential function was fitted to the data in order to try and identify the relationship between driving voltage and time to failure. The maximum rate of change was calculated by finding the gradient to a straight line tangential to the first non-zero data point.

![Figure 4.21: Typical data from the CA measurement system showing the exponential function that has been fitted to the data (black line) and the fitting parameters, $d_0$, $d_\infty$ and $\tau$. The green and red lines show the linear line of fit tangential to the first and second non-zero data points and result in the gradient factor $k$ (from $y=kx+c$), which gives the maximum rate of change of contact angle. This data is for the wafer batch number bos060811w09_38nm](image)

Figures 4.23 and 4.24 show the data for two devices under test, using the previously described procedure. The change in initial contact angle as a function of the number of actuations is plotted. Clearly the thicker $Ta_2O_5$ layer improved
Figure 4.22: Evidence for charging of the surface and the detrimental effect on data analysis. In region A the system behaves as expected. In regions B and C the droplet moves position thus changing the measured contact angle as the droplet is now sitting on a 'fresh' area. The initial contact angle is restored, this motion is observed to be periodic, indicating a charge threshold. This data is for the wafer batch number bos060811w11
system stability over the range of driving voltages which have been investigated. The data in Figure 4.23 shows that for a 38 nm dielectric driven at 21 V the time to failure is extremely rapid. The time dependant factor, $\tau$ may prove to be related to the ‘RC’ charging time-constant although significantly more data is required before this can be validated. In a standard RC circuit the time constant ($\tau = R \times C$) is the time taken for the capacitor to charge to $\frac{2}{3}$ of its final value. In this context, $\tau$ is the time taken for the device to reach $\frac{2}{3}$ of the saturated contact angle change, $d_\infty$. Due to the fact that the contact angle change is dependant on the capacitance, it is possible that these two time constants are related by a simple constant of proportionality. To validate this, the actual device capacitance would need to be measured accurately. This could be done by using the DMF device as a capacitor in an oscillator system, and taking accurate measurements of the oscillator frequency. The initial results shown here show a clear correlation between high electric field in the dielectric and the time to failure. The higher the field the lower the time to failure. The most obvious feature of the data from these test devices is that the stability of the device with a 180 nm dielectric is clearly evident, when compared to the 38 nm dielectric. This demonstrates that there must be a trade-off between keeping the dielectric thin, to lower the driving voltage, whilst having it sufficiently thick to allow an acceptable time-to-failure.

In Figure 4.25 the change in the initial contact angle change as a function of voltage is shown for both devices. This data shows the loss in contact angle change per actuation, clearly demonstrating that for the device with a 38 nm dielectric, driven at 21 V, nearly 2 degrees of contact angle is lost per actuation. If the required change in contact angle for droplet actuation is defined as $\sim 40$ degrees (as per Shapiro et al\textsuperscript{141}) then the time to failure is less than ten actuations. Figure 4.22 shows an interesting phenomenon which arises during the acquisition of these measurements. The droplet sometimes moves during the experiment possibly due to charging of the surface, the new location of the droplet means that it is now sitting on an uncharged region and thus the initial contact angle
Figure 4.23: Contact angle versus time results from CA measurement system for 38nm thick $Ta_2O_5$. The figure shows that the higher the actuation voltage the faster the rate of decay of the contact angle change, with the worst case at 21 V. This data is for the wafer batch number bos060811w09_38nm.
Figure 4.24: Contact angle versus time results from CA measurement system for 180nm thick $\text{T}_{2}\text{O}_5$. The figure shows that the 180 nm dielectric provides a more stable platform with more consistent contact angle change over time than the 38 nm dielectric in Figure 4.23. This data is for the wafer batch number bos060811w09_180nm.
is restored. This oscillation in contact angle as a function of droplet position lends some weight to the theory of charge injection into the dielectric layer, this is discussed in Section 4.4.2.

4.3.3 High Speed Video Analysis of the Ejection of Satellite Droplets

The ejection of satellite droplets in DMF systems is a well reported phenomenon\textsuperscript{146}. It is thought to occur because of the instability of the TCL under high electric field gradients. However, if the driving force behind droplet actuation is due to a surface tension induced contact angle change, as described by the Lippmann-Young equation, then there is no explanation for the ejection of satellite droplets. In contrast, if droplet actuation is due to the polarisation of free charge collecting at the interface, then there should exist a Raleigh limit as in electrospray. Charge densities exceeding this limit would lead to Coulombic repulsion and ejection of droplets. This phenomenon has been observed using a high-speed camera and some of the data obtained is included here for interest as it supports the electrodynamic interpretation of DMF.

4.4 Direction for Future Experimental Work

It is clear that the fundamental physics behind DMF still requires further investigation. Future work should focus on; 1. Extending the time to failure experiments to include dynamic analysis of droplet movement. 2. Investigation of the contact angle saturation models of polarisation and charge trapping. 3. Further optimisation of screen printed and rapid prototyping devices. The time-to-failure experiments that have been designed and implemented in the previous section show some interesting initial results and these should be extended to more dielectric types and thicknesses. A similar approach should also be taken to characterise the screen-printed devices. In addition to the change in contact angle experiments, the time-to-failure for real-world applications is the number of ac-
Figure 4.25: The change in initial contact angle change versus voltage is shown, the points represent the gradient factors $k$ for data collected in the experiment detailed in Figure 4.21. These experiments have been repeated for fifteen voltages and on two devices with different tantalum pentoxide thicknesses. The solid lines are a line of best fit and have been fitted to an exponential function to show the parallels between the behaviour of this system and RC-charging. The worst performance is for the 38 nm tantalum pentoxide at 21 V, almost 2 degrees is lost per actuation. This data is for the wafer batch numbers bos060811w09_38nm and bos060811w09_180nm.
Figure 4.26: Still images captured using a Photron SA-1 high speed camera showing the three phase contact line of a droplet of water on a silicon DMF device. The stills show ejection of satellite droplets and support the electrodynamic interpretation of DMF fluctuations possible until the droplet can no-longer be moved. Section 4.4.1 details the development of the integral part of this new experiment which is yet to be performed, a dynamic machine-vision monitoring system for droplet actuation studies.

4.4.1 Extension of Time to Failure Experiments

The output from a ‘MachineVision’ script written in LabView, used to process the video previously shown in Figure 4.14 is presented in Figure 4.27. This visual tracking of the droplet position provides a means to dynamically monitor the droplet position and size. This will allow full-closed-loop feedback on droplet movement. The droplet radius measurement can also be used to account for evaporation. This system will provide a means non only to study the contact angle of the device but also to investigate the actual time-to-failure for a patterned array of electrodes. This is ultimately what assessment of the reliability of the devices will require. The script that has been written processes the video on a frame by frame basis and performs a sequence of processing steps to extract the
current co-ordinates of the droplet. Firstly the image of the frame is opened and then the red plane from the RGB image is extracted. This greatly simplifies the data set and makes an effective greyscale image. A 'Sobel’ type edge detection algorithm is run followe by a Fast Fourier Transform (FFT) to identify the major components of the image. The image is then tidied up by removing objects below a threshold size, and any holes that are enclosed within a boundary are filled in. In the final step a circle detection algorithm is run and the script updates the co-ordinates of the circle centre and the radius. This script has been tested and is robust provided that the droplet is not at the edge of the field of view. This system is now ready to be integrated into a feedback loop.

4.4.2 Investigation of Charging and Polarisation Effects

The data presented so far in this section, clearly demonstrates that there is a phenomenon acting in the device which is linked to the field strength in the dielectric, which is a cumulative effect. This can only be due to polarisation of the dielectric or charge injection into the fluoropolymer layer. In reality it is probably a combination of the two effects. However it should be possible to distinguish between the two effects. Polarisation should be a reversible linear-effect, and one which acts on a relatively short time scale compared to charging. If the additional electric field is due to trapped charge, then the effect will be cumulative, but should have a Boltzmann-type decay distribution since the only route available to trapped charges to recombine is by tunnelling. It should be possible to measure this decay by using the capacitor as a timing element in an oscillator circuit and measuring the frequency accurately.

4.4.3 Developments in Screen Printing of DMF Devices

The screen printed devices are now entering their third iteration of development, with the most recent devices (as yet untested) incorporating a screen printed dielectric and hydrophobic layer. These devices have the potential to make DMF
Figure 4.27: Machine vision analysis of droplet movement, the dark segments show the extracted image place before the circle finding algorithm is run. The red circles show the identification of droplets and provide an output of their location and size.
cheap enough to commercialise, since cost appears to be the main barrier to the implementation of DMF devices in commercial microfluidic products. Further developments in screen printed devices will include reverse printing the layers onto a peelable backing sheet to provide a very flat surface which protects the device from contamination until it is ready to be used.

4.5 Summary

Silicon and rapid-prototyping devices have been successfully developed and the manufacturing processes described. The development of screen-printing technology for manufacture of these devices represents a novel contribution to the field. In addition the significant technical challenges associated with manufacturing a multi-metal layer silicon device have been overcome and a functional 8x5 DMF array using $Ta_2O_5$ has been developed. The control electronics have been presented, and the use of both DC and AC driving regimes has been described. The control electronics which have been designed provide a robust and computer controlled approach to driving the DMF devices and facilitate a new robust and repeatable experimental platform. The development of the computer controlled AC driving regime represents a change in the control of DMF devices as compared to previous work in the group and provides a means of investigating the effect of time-varying electric fields on the actuation of droplets. Experiments that have been designed for characterisation of the devices have been described and the initial design of future experiments, which should be extended to the screen-printed devices, have been suggested. It is clear from these initial results that there is a charging or polarisation phenomenon in the dielectric layer which is responsible for the failure of these devices, this is the first such analysis of these modes of failure.
Chapter 5
Integration of Digital Microfluidic Sample Preparation and Mass Spectrometry

5.1 Introduction and Motivation

The DMF devices that have been described previously have a variety of practical applications for microscale chemistry and sample handling. In this work these DMF devices have been deployed for biological sample handling together with sample preparation for subsequent mass spectrometric analysis using a variety of MS platforms.

This is not by any means the first application of microfluidics in combination with mass spectrometry\textsuperscript{167–171}, nor is it the first integration of DMF with MS, though several key advances have been in the work reported here which improve upon previously described techniques\textsuperscript{172,173}, see Section 5.1.1.

The obvious advantages of sample reduction and automation, using microfluidics for sample manipulation and sample preparation, mean that this coupling has been extensively reported in the literature\textsuperscript{174,175}. A comprehensive review of microfluidic systems and their use in proteomics has been provided by Lion \textit{et al.}\textsuperscript{167}. The first coupling of microfluidics and mass spectrometry was arguably the pulled glass-capillary nanospray emitter\textsuperscript{176}. This idea has been ex-
panded on and developed into a commercial product in the form of Advion Biosciences’ silicon based ESI-chip, which is found in their NanoMate product. Before founding Advion Biosciences, Henion’s group at Cornell developed capillary-electrophoresis CE-MS\textsuperscript{177,178} and applications of this area have been further developed by Sweedler’s group\textsuperscript{179}. Jacobson, from Indiana University, pioneered the integration of Capillary Zone Electrophoresis (CZE-MS\textsuperscript{180}), and Capillary Electrochromatography\textsuperscript{181} with mass spectrometry (CEC-MS\textsuperscript{182}), for multidimensional separation of peptide and protein mixtures in a capillary before analysis by nanospray mass spectrometry.

Previous work in this area by previous members of SIRCAMS and the EMSG has included the development of CEC ion trap TOF MS, as described by Simpson\textsuperscript{183} as well as the more recent work by Clarke \textit{et al.}\textsuperscript{184} on using pressure-driven microfluidic devices with mass-spectrometry coupling for investigation of enzyme kinetics using a quench-flow system incorporating micromixers. A reprint of this work is given in the Appendix. A interesting paper, also on the study of pre-steady state enzyme kinetics, but using DMF-MALDI coupling, was published in 2007 by Nichols \textit{et al.}\textsuperscript{185}.

5.1.1 Proteomic Analysis incorporating Digital Microfluidics

Work on the coupling of digital microfluidic devices with mass spectrometric analysers has been driven by the requirements for miniaturisation and automation that have come with the development of proteomics and protein mass spectrometry. The first reported coupling of DMF-MS (MALDI) coupling was by Wheeler \textit{et al.}\textsuperscript{172} in 2004. Wheeler’s initial work focussed on using DMF to prepare samples for MALDI on a conventional MALDI target plate. The work presented in this thesis takes this to the next logical step and provides analysis of samples directly from the DMF chip surface. Significant advances have been made by Wheeler whilst at the University of Toronto on DMF-MALDI for proteomic anal-
ysis\textsuperscript{173,186,187} and other key investigators such as Moon et al.\textsuperscript{138}. The work that is discussed in the following sections starts with MALDI analysis before moving on to new techniques of DMF-MS coupling which are as yet unreported in the literature, such as DESI-MS direct analysis and the use of a DMF chip as a functional component in combining nLC and mass spectrometry. The use of magnetic beads with DMF devices has been previously reported\textsuperscript{188,189}, although the work described here in Section 5.1.1.2 and 5.2.3.2 represents the first application of DMF for sample purification using magnetic beads and analysis by LC-MS.

Figure 5.1 shows one of our recent W-shaped design of DMF chip being employed in a lab-on-a-chip configuration (see Section 5.2.2.) The two fused silica capillaries that can be seen are used for sample introduction or removal, and are connected to a nano-LCMS system. There are a number of parameters which become important when using mass spectrometric detection. Care must be taken to balance the requirements to facilitate mass spectrometry, such as sample concentration, purity and solvent composition, with those for DMF device operation such as biofouling and droplet-size. Luk et al. have combated biofouling in their devices by the addition of polyethyleneglycol (PEG)\textsuperscript{190}. However, the addition of PEG to sample solutions using mass spectrometric detection leads to ion-suppression and poor ionisation of the analyte.

5.1.1.1 On-Chip Enzymatic Hydrolysis

Since its inception, tryptic hydrolysis of proteins has been the mainstay of mass spectrometry based proteomics\textsuperscript{16}. This can be a laborious procedure and one which has been automated by the use of fluidic systems and robotics. The use of DMF devices to deliver reagents and to perform digestion on-chip is an attractive prospect, since it reduces the volumes of analyte and reagents required, whilst enabling exploitation of some of the scaling laws that come with microfluidics. In addition, it provides the possibility of developing multiplexed and parallel systems.
Figure 5.1: Single metal layer W-shaped tantalum pentoxide device as used in an analytical application. The piezo dispenser is used for deposition of samples onto the surface. The middle silica capillary provides a method for coupling to a nano-LC column, and the silica capillary on the right is used to remove the sample and to provide an interface to a nLC-MS instrument.
5.1.1.2 On-Chip Functionalised Magnetic-Bead Purification

Instead of removing the droplet from the chip surface for purification by nano-LC, the use of functionalised magnetic beads was investigated to see if it was possible to purify a protein from a crude lysate on-chip. This idea has been investigated previously\textsuperscript{188,189} using micro-particles in water\textsuperscript{191}, although the studies by Cho \textit{et al}\textsuperscript{191} and Dorvee \textit{et al}\textsuperscript{192} were purely conceptual, and did not use actual biomolecules and functionalised beads. In the present work, clarified cell lysate was pipetted onto the chip and a drop containing functionalised beads was mixed into it. Any proteins with the requisite binding site collect on the beads. The beads can then be drawn to the bottom of the droplet, using an embedded electromagnet, and the supernatant removed by pipetting. The bound protein can then be eluted from the beads or digested directly. The results from such an experiment are presented later, in Section 5.2.3.2.

5.2 Results

5.2.1 Coupling Digital Microfluidics to Mass Spectrometry

The hybridisation of DMF and MS has been studied in a variety of ways. The first two approaches that were attempted involve direct analysis of the solid-analyte from the chip-surface, after the solvent has been allowed to evaporate. The next two experimental approaches involved the retention of the analyte in the liquid phase, before direct infusion by ESI-MS. Finally experiments are described which involve separation and analysis of the droplets by nano-LCMS. Pure protein standards were used for method evaluation and optimisation, and on-chip tryptic digests of these proteins have been carried out. The final experiment that is describes the use of on-chip magnetic bead purification of a crude cell lysate, tryptic digestion of the bound protein on-chip, and protein identification by nano-LCMS analysis. These experiments demonstrate the utility of DMF-MS coupling
as an important future tool for proteomic analysis\textsuperscript{193}.

\subsection{DMF with MALDI-TOF MS Analysis}

The technique of MALDI was introduced in Section 2.3.1. At first glance it is not an obvious choice for mass spectrometric coupling to microfluidic systems, due to the fact that this technique involves ionisation of solid-phase samples at relatively low pressure. A major advantage of MALDI, however, is that it is an extremely sensitive technique (sub-picomole sensitivity), and that each spot is discretely separated from other sample spots on the target surface. Wheeler \textit{et al.}\textsuperscript{172} reported the first use of DMF-MALDI MS for proteomic analysis in 2004, with subsequent reports by Wheeler \textit{et al.}\textsuperscript{173}, Moon \textit{et al.}\textsuperscript{138} and Lee \textit{et al.}\textsuperscript{194}. As a first step towards integration of the digital microfluidic devices described in the previous section, a Perceptive Biosystems Voyager DE STR MALDI-TOF mass spectrometer was used to analyse a specially modified MALDI target, see Figure 5.2.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{image}
\caption{Modification of a stainless steel MALDI plate to include recessed chip, the chip is help in position using a very thin layer of vacuum grease}
\end{figure}

The stainless steel target was milled out to a depth of 500 µm so that the
Silicon DMF device would sit flush with the surface. The chip was secured into the recess using a very thin layer of vacuum grease. Care must be taken to avoid any trapped air bubbles as when the plate is introduced to vacuum any outgassing can dislodge the chip. The first proof-of-concept experiment involved mixing both matrix and analyte on-chip in a ‘closed’ configuration, that is under a top-coverplate. The coverplate has the effect of limiting the evaporation of the solvent-matrix mixture, until the two droplets have mixed and the plate is removed. The method is described fully in Section 7.3.1. Figure 5.3 A, shows the resulting mass spectrum for a digest of BSA, mixed with matrix using DMF actuation on-chip. In this experiment the sample was allowed to crystallise prior to analysis using the DESI STR TOF MS. The resultant mass list was extracted and searched against a non-redundant database using MASCOT. Figure 5.3 B, shows the sequence coverage obtained from a search using the MASCOT database.

Control data for the same experiment conducted on the standard target has not been presented here, although the confidence-score of 53 is typical for a peptide mass fingerprinting experiment and corresponds well to that obtained by other groups using DMF-MALDI. In the sequence map, of 30% coverage, the matched fragments are shown in red. Also shown in the figure is an inset zoom of the most abundant peptide peak in the mass spectrum. The $^{13}C$ isotopes of this peptide are clearly fully resolved. Although useful, MALDI-TOF PMF does have drawbacks. Using MALDI, the most predominant peaks of the spectrum correspond to single positively charged species, restricting the use of fragmentation techniques such as ECD or ETD. Protein identification relies on matching the intact mass of poly-peptides. Two or more peptide identifications are required for confident protein assignment.

5.2.1.2 Direct Analysis by Desorption Electrospray Ionisation (DESI)

DESI is a relatively new technique. This technique enables direct analysis of samples from a surface but, in contrast to MALDI it doesn’t require a matrix.
Figure 5.3: A. MALDI-TOF MS of BSA standard mixed with matrix on DMF chip. B. MASCOT results; Sequence coverage = 30%, MASCOT score = 53
For protein analysis the charge state distributions obtained are similar to ESI. The fact that the proteins or peptides are multiply charged means that either top-down or bottom-up techniques, based on fragmentation and sequencing, can be used. This can provide an inherently higher confidence in the identification of proteins using bioinformatic tools. Commercial DESI sources were not available at the outset of the work described in this thesis. Consequently a versatile DESI source was designed, built and tested for the feasibility of performing top-down protein analysis using a 12 Tesla Apex Qe FT-ICR mass spectrometer (Bruker Daltonics, UK). The resulting work has been published\(^{196}\), and a reprint is given in Appendix E.2. The development of this DESI source has enabled direct analysis of intact proteins to be performed with minimal sample preparation. In a previous study, Takats \textit{et al.}\(^{195}\) reported top-down DESI FT-ICR MS analysis of a synthetic polypeptide containing 36 residues. The work on myoglobin presented here builds on the work by Basile \textit{et al.}\(^{197}\) and demonstrates that DESI can provide sufficient signal abundance for the ionised intact protein, and over a sufficient period of time (\(\sim 10\) minutes), that top-down sequencing by ECD FT-ICR MS can be performed on proteins with a molecular weight of at least \(12\) kDa. Single scan DESI FT-ICR mass spectra for both myoglobin and cytochrome-c which correspond to picomole sample consumption, were obtained. In addition, for cytochrome-c, top-down DESI FT-ICR mass spectra were recorded using both collision induced dissociation (CID) and electron capture dissociation (ECD).

5.2.1.3 Design of DESI Source

Inventor (Autodesk Inc, CA) was used to the DESI source. Modelling the source design permitted rapid iteration and fault finding. Figure 5.5 shows the final design fitted to the mass spectrometer. The stepper controlled XY stage was sourced from an Ultraflex MALDI TOF mass spectrometer (Bruker Daltonics), and implemented as a structural component of the DESI frame. Software control of the XY sample stage was achieved using Enhanced Machine Controller 2\(^ {198}\),
to generate step and direction inputs to the motor control driver boards. As previously mentioned, there are a number of critical geometrical parameters for DESI, which greatly influence the ionisation efficiency. In order to provide a high degree of reproducibility, the solid frame was used to hold both the ESI sprayer and sample stage. The ESI sprayer was mounted to an insulated XYZ micromanipulator, equipped with vernier scales for accurate measurement of the incident spray angle, tip-to-surface and tip-to-inlet distances. In order to allow the XY sample stage to raster below the mass spectrometer source inlet, a hybrid glass-brass capillary, with a stainless steel extension was used. This allowed the sample stage to move beneath the inlet. Without the extension only the first millimetre of sample in the Y-axis direction could be interrogated.

![Glass Transfer Capillary](image)

Brass End Cap

Stainless Steel MS Inlet Extension Capillary

10 mm

Figure 5.4: Capillary modification for DESI FT-ICR MS showing the brass end-cap and stainless tubing extension, this modification to the atmospheric pressure inlet of the FT-ICR mass spectrometer is required to allow the movement of the sample surface below the inlet and is required for MS-imaging

Figure 5.4 shows modifications made to a conventional glass-capillary. This involved fitting a solid brass end-cap and a 1/16 inch outer diameter 0.020 inch inner diameter stainless insert (Upchurch.) The stainless insert was recessed 3 mm into the glass, and extended 80 mm out through the brass end cap. The
critical geometrical DESI source parameters, as described by Cooks et al. were co-varied empirically in order to maximise ion-abundance. Nebulising gas flow rate, electrospray voltage and solvent flow rate were kept constant at 2 l/min, 3.5 kV and 3 µl/min respectively. The insert in Figure 5.5 shows a close up view of the DESI source. Correct adjustment of the critical geometrical parameters for the incident and collection angles, and distances was determined empirically. The experimentally adjustable variables in DESI are not independent. For example, a change in the inlet-to-tip distance, changes the electric field strength from the electrospray tip to the inlet, and this in turn affects the stability of the spray. Indeed, each of the geometric variables is intrinsically linked to the others. Accordingly, optimisation of the system can only be performed empirically, or by following an experimental design workflow. Table 5.1 shows typical values for the DESI parameters that were used in this work, although variation of up to 10% was sometimes required in fine-tuning the system. Interestingly, the ion-abundance was observed to increase when increasing the distance from the spray-tip to the mass spectrometer inlet. This may be due to the increase in time for desolvation of the nebulised droplets.

Table 5.1: Empirically determined critical geometric parameters and ESI conditions

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>α</td>
<td>Incident angle</td>
<td>40-80 degrees</td>
</tr>
<tr>
<td>β</td>
<td>Collection angle</td>
<td>2-10 degrees</td>
</tr>
<tr>
<td>$d_1$</td>
<td>Tip-to-surface distance</td>
<td>0.1-1 cm</td>
</tr>
<tr>
<td>$d_2$</td>
<td>Inlet-to-surface distance</td>
<td>0.1 cm</td>
</tr>
<tr>
<td>$d_3$</td>
<td>Inlet-to-tip distance</td>
<td>3-8 cm</td>
</tr>
<tr>
<td>Spray voltage</td>
<td>Inlet-to-tip voltage</td>
<td>3-5 kV</td>
</tr>
<tr>
<td>Gas flow rate</td>
<td>ESI gas flow rate</td>
<td>2 l/min</td>
</tr>
<tr>
<td>Solvent flow rate</td>
<td>-</td>
<td>3 µl/min</td>
</tr>
</tbody>
</table>
Figure 5.5: Bespoke CAD designed DESI source mounted on a Apex Qe FT-ICR mass spectrometer, the inset shows a typical configuration of the DESI geometric parameters.
5.2.1.4 Protein Analysis by DESI FT-ICR MS

Samples were prepared as described in Section 7.3.2. The DESI source and instrument parameters were set-up as described earlier. In Figure 5.6 are shown, the intact mass spectra obtained for myoglobin (16.9 kDa) and cytochrome-c (12.4 kDa) under mild denaturing conditions. These spectra correspond to a single scan and show the charge state distribution. These spectra demonstrate the intrinsically high resolving power of FT-ICR mass spectrometry and show that it is possible to generate sufficient ion-abundance using DESI for FT-ICR mass spectrometry. Knowing the concentration and volume of sample deposited on the DESI target plates, together with the area over which the sample wets (ca. 500 mm²) and the DESI spot size (ca. 1 mm²), it is possible to calculate that these single scan spectra correspond to approximately 5 pmole of protein sample consumed.

5.2.1.5 Top-down Protein Analysis by DESI FT-ICR MSMS

In Figures 5.7 and 5.8 the ECD and CID tandem FT-ICR mass spectra obtained for cytochrome-c by isolating the $[M + 15H]^{15+}$ charge state in the mass resolving quadrupole of the instrument are shown. Both the ECD and CID spectrum represent the accumulation of 200 spectral acquisitions. Peak assignments were made using Bruker-Daltonics’ SNAP 2.0 algorithm\(^\text{199}\). The fragmentation maps obtained by searching Prosight PTM-2.0\(^\text{200–203}\) are also shown. 43 (30%) of the 140 ECD peaks picked by the SNAP 2.0 algorithm were matched, with an expectation of $2.45 \times 10^{-8}$. The SNAP 2.0 algorithm returned 377 peaks from the CID spectrum of which 50 (13%) were matched to the theoretical fragmentation map in the database, yielding an expectation of $1.42 \times 10^{-5}$. Again it is possible to estimate the amount of sample consumed to record these FT-ICR tandem mass spectra. Assuming that during the time required for 200 scans, the DESI probe is rastered over a maximum sample area of 200 mm². This would correspond to approximately 10 nmole of sample consumed. As others have shown\(^\text{204}\), the
Figure 5.6: Single scan DESI FT-ICR mass spectra for (A) myoglobin and (B) cytochrome-c from an etched glass substrates
quality of mass spectra obtained using DESI is hugely dependent on the source parameters, which are shown in Table 5.1. In particular, the relative geometrical orientation and position of the sprayer, sample stage and MS-inlet are critical.

5.2.1.6 Proof of Concept for DESI FT-ICR MS Imaging

Imaging mass spectrometry is a relatively new field. It is primarily performed by MALDI on samples coated in a thin layer of matrix and under very low-pressure. DESI imaging, by contrast, can be performed without the addition of a matrix, and at ambient temperature and pressure. The DESI source shown in Figure 5.5 included a computer controlled stage with two degrees of freedom. This meant that, given the correct control signals, and when integrated with an appropriate mass spectrometric platform, this source could be used for DESI imaging mass spectrometry. In order to verify this, a proof of concept experiment was carried out whereby the XY stage was rastered from left to right, in the X-axis, underneath the DESI spray needle. At the end of each sweep two events occurred. Firstly, the stage was moved back to the home position in the X axis whilst being incremented one step unit in the Y-direction. The system would then send a TTL-type trigger signal to the mass spectrometer to trigger the next LC-MS run. Triggering the mass spectrometer in that fashion meant that it was possible to record a time-dependant chromatogram. Linking this to the traversing speed of the XY stage, it was possible to calibrate back to the distance travelled. In this way a spatial map of the distribution of ion-species on the surface could be built up. In Figure 5.9 a photograph of a glass microscope slide is shown, inserted into the DESI imaging frame. The number one has been drawn on the slide using red overhead projector (OHP) pen. The OHP pen contains predominantly rhodamine-b (a red dye) dissolved in a solvent. The quadrupole mass filter on the Apex Qe FT-ICR mass spectrometer was set to only allow charged species corresponding to rhodamine-b into the ICR cell. The resultant total ion chromatograms were then analysed by a custom written LabView program. This
Figure 5.7:  A. DESI ECD FT-ICR mass spectrum obtained for the isolated \([M + 15H]^{15+}\) charge state of cytochrome-c, assigned fragments are labelled. B. Prosight PTM-2.0 fragment map.
Figure 5.8:  
A. DESI CID FT-ICR mass spectrum obtained for the isolated $[M + 15H]^{15+}$ charge state of cytochrome-c, assigned fragments are labelled.  
B. Prosight PTM-2.0 fragment map.
assigned a false colour scale to the chromatograms, and enabled construction of a three dimensional image of the ion-species on the glass slide. Figure 5.10 shows the results from this proof of concept experiment. The image drawn on the slide in the false coloured reconstruction is clearly visible. The spatial resolution is limited by the incident spot size of the DESI spray.

![Figure 5.9: Photograph of the DESI sample stage containing a glass slide with an image of the number 1, produced using a red overhead projector pen](image)

5.2.1.7 Limitations of DESI and Future Use

In this work it has been shown that top-down sequencing by DESI ECD FT-ICR mass spectrometry of intact proteins, at least a large as 12 kDa, is feasible. In addition proof of concept for DESI imaging has been demonstrated. The addition of air amplifiers to improve the ion collection efficiency from the DESI plume may well extend the mass range and sensitivity of this technique further.
Figure 5.10: Screen shot of LabView VI algorithm used to process the MS-data of the extracted ion chromatogram for rhodamine, this shows proof of concept for DESI-imaging
 Whilst DESI is a potentially powerful technique, in the current system the total amount of protein required for top-down protein sequencing (10 n mole) limits the possibility of using DESI to directly analyse DMF devices. The difference in the maximum protein concentration and volume that can be manipulated by DMF (empirically determined to be $\sim 10$ mM in a $1 \mu l$ droplet) is approximately three orders of magnitude less than the limits of detection using DESI on the current FT-ICR MS platform.

### 5.2.2 Robotic Fluid Handling for a World-to-Chip Interface

Direct analysis from the chip surface of DMF devices using MALDI-MS and DESI-MS has already been described and the respective advantages and disadvantages discussed. Both of these techniques require the analyte to be in the solid phase. It would, however, be useful if the analyte could be kept in the liquid phase, since this would facilitate hybridisation of fraction collection by liquid chromatography and subsequently mass spectrometric analysis. In principle, capillary electrophoresis and LC-MSMS could be directly integrated by using the DMF device to decouple the time scales of these two techniques. The ability to purify the sample, using magnetic beads and to hybridise separation techniques, means that the DMF chips could be used for fraction-collection, sample processing or as active 96-well plates, in which each collected fraction could be further separated and processed. In order to move from macroscale reagent and analyte volumes to those that can be manipulated by DMF it is important to have a robust ‘world-to-chip’ interface. The initial experiment described here required many individual items of equipment, connected to a variety of computers for control, and each sub-system was isolated. In Figure 5.11 a representative photograph of this initial configuration of the experimental equipment is shown.

The centre-point of this system was the DESI frame, described in the previous section. This provided a 2-dimensional XY stage, as well as a stable mounting
Figure 5.11: Initial ‘chip-in-a-lab’ system configuration showing the multiple computers and disconnected sub systems required in a typical experiment
Figure 5.12: The DESI frame from Figure 5.5 was used for the initial world-to-chip interface of DMF and nLC-MS, this provided a 2-dimensional stage and a stable platform for integration of the required subsystems
point for clamps and cameras. A close-up view of this initial setup of the system
is show in Figure 5.12. Each sub-system operated in isolation, and therefore
synchronisation of droplet deposition, droplet movement, droplet removal, as well
as video camera operation was performed by the operator.

A list of each sub-system in this experimental setup is given below:

1. DMF control system
2. Piezo electric droplet delivery control system
3. Piezo electric droplet delivery camera
4. System camera
5. Droplet removal
6. Manual 6-port valve
7. XY stage control
8. HPLC system
9. Mass spectrometer

Integration of the sub-systems was clearly desirable in order to remove the
requirement for multitasking by the operator, and to allow the use of a single
computer for the whole system. Automation of required a rationalisation of each
sub-system into a new control system.

5.2.2.1 Robotics and Automation

A 2700 MALDI spotting robot (Waters, UK) was used as the base upon which
the rest of the sub-systems could be integrated. The MALDI spotting robot
had a number of beneficial aspects including, a 3-axis robotic arm, robotic sy-
ringe pumps as well as an enclosed environment to limit particulates and regulate
humidity.

Figure 5.13 shows the finalised system, after all of the sub-systems were built
into the robot. Two further robots were required. These were kindly donated by
the School of Chemistry, Open Access Laboratory, and the Sir Henry Welcome

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Functional Genetics Unit at the University of Glasgow. The final system includes two XYZ robotic arms, two robotic syringe pumps, an XYZ piezo microdroplet delivery system, integrated DMF control, space for three 96-well plates, a robotically controlled 6-port valve as well as an integrated camera for machine vision feedback of the droplet position.

The whole system is controlled by a rackmounted computer running a custom LabView program. The control unit, shown in Figure 5.14, consists of a function generator (Gould, DWG-700), a high-voltage high-frequency amplifier (Trek DWG700A), a DC power supply (Maplin, 35 V switch mode), a custom voltage switching device and an oscilloscope (LeCroy) for debugging.

The whole system is controlled using a custom LabView program, the architecture is based on the concept of state machines, or finite state transducers. There are two main types of state machine, the Moore Machine\(^2\) and the Mealy Machine\(^3\). The Mealy Machine consists of a finite set of states that generate an output based on both its current state, and the current input. The Moore Machine, by contrast, generates outputs based purely on its current state, and does not depend on the input. The state machine implemented here, in the robot control program, consists of both Mealy and Moore type. The computer science terminology for such a system is an Algorithmic State Machine (ASM). The ‘front panel’ of the robot control program is shown in Figure 5.15. Also shown in this figure is the state transition diagram of the ASM. The ‘back panel’ of the control program is given in Appendix C.2.

The inputs to the robot control program come from two sources. Firstly, there is the runlist, which is in the form of a text file. This is read in one line at a time on each completed cycle of the ASM. The second input to the ASM consists of feedback signals from the robot. Outputs from the ASM consist of control signals to the robot, control signals to the DMF control circuitry and internal signalling flags which update the runlist position and state diagram indicators. The robot is controlled via a serial connection, providing a series of ASCII commands following
Figure 5.13: Rationalised robotic system configuration as compared to Figure 5.11, showing the integration of all sub-systems into one platform, the 3-axis robotic arms provide macroscale fluid handling. The 2-dimensional stage and piezo dispenser provide a world-to-chip interface.
Figure 5.14: The sub-system computers have been integrated into one rack-mounted robotic control system showing the control computer, amplifier, oscilloscope and function generator in a 19-inch rackmount case with wheels, this is connected to the integrated robotic platform via an umbilical
Figure 5.15: Front panel of robot control software showing the algorithmic state machine and user interface for control of the system.
a protocol as defined by Tecan, the robot’s manufacturers. Table 5.2 provides an explanation of each bit in the command structure. The checksum is determined by taking the bit-wise exOR of each of the preceding bits to give a two character ASCII code which is used to validate data transmission. The back panel for the generation of the checksum can be found in Appendix C.5. The command start and end-of-line characters are non-printing in the ASCII representation, and the checksum has been omitted for clarity in the reproduction of the control code, which is shown in Appendix C.2.

Table 5.2: Robot control command structure

<table>
<thead>
<tr>
<th>Example Command</th>
<th>Description</th>
<th>Device &amp; Address Alternatives</th>
</tr>
</thead>
<tbody>
<tr>
<td>FF</td>
<td>Command Start Character</td>
<td></td>
</tr>
<tr>
<td>02</td>
<td>Space</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Physical Device 1 = Device 1, 2 = Device 2</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Address 8 = Arm, 1 = Syringe</td>
<td></td>
</tr>
<tr>
<td>PA</td>
<td>Command</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>Operand 1</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>Operand 2</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>Operand 3</td>
<td></td>
</tr>
<tr>
<td>CHKSUM</td>
<td>Checksum Character</td>
<td></td>
</tr>
<tr>
<td>OD</td>
<td>End of Line</td>
<td></td>
</tr>
</tbody>
</table>

The list of robot control commands is given in Table 5.3. Each command is read in sequentially from a text file and added to the queue, using the ‘Add States to Queue’ sub VI shown in Appendix C.3. At the beginning of each iteration of the robot control state machine, the queue is parsed using the sub VI shown in Appendix C.4. This determines the output from the state machine.

The robot command lists were compiled using another custom LabView program the ‘RunList Generator’. The front panel of this is shown in Figure 5.16. This program contains the ‘Checksum Generator’ (see Appendix C.5) and outputs text files which can then be read into the robot control program. An example can be found in Appendix C.2. The various panels of the RunList generator allow the user to input pre-defined macros, to write DMF control sequences, to click on a picture of the 96 well plate and instruct the arm to go to that position and, finally,
Table 5.3: List of robot control commands

<table>
<thead>
<tr>
<th>Command</th>
<th>Operands</th>
<th>Address</th>
<th>Device</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>PI</td>
<td>N/A</td>
<td>1 or 2</td>
<td>8</td>
<td>Initialise arm</td>
</tr>
<tr>
<td>PA[op1][op2][op3]</td>
<td>X,Y,Z</td>
<td>1 or 2</td>
<td>8</td>
<td>Move arm in x,y and z</td>
</tr>
<tr>
<td>XA[op1]</td>
<td>X</td>
<td>1 or 2</td>
<td>8</td>
<td>Move arm in x</td>
</tr>
<tr>
<td>YA[op1]</td>
<td>Y</td>
<td>1 or 2</td>
<td>8</td>
<td>Move arm in y</td>
</tr>
<tr>
<td>ZA[op1]</td>
<td>Z</td>
<td>1 or 2</td>
<td>8</td>
<td>Move arm in z</td>
</tr>
<tr>
<td>S[op1][op2][op3]</td>
<td>Speed,Volume,Valve Position</td>
<td>1 or 2</td>
<td>1</td>
<td>Move syringe &amp; valve</td>
</tr>
<tr>
<td>RX</td>
<td>N/A</td>
<td>1 or 2</td>
<td>8</td>
<td>Readback x position</td>
</tr>
<tr>
<td>RY</td>
<td>N/A</td>
<td>1 or 2</td>
<td>8</td>
<td>Readback y position</td>
</tr>
<tr>
<td>RZ</td>
<td>N/A</td>
<td>1 or 2</td>
<td>8</td>
<td>Readback z position</td>
</tr>
</tbody>
</table>

to input comments into the code. The software interface has been designed to be user friendly and intuitive.

Figure 5.16: Front panel of RunList generator software showing the multiple methods of generating code for robot control

The RunList Generator program architecture is also based on a state machine structure and the back panel of this is shown in Figure C.1. This programme structure allows easy debugging and modular development of the software.

Using the pipetting robot allows true integration of the microfluidic and macroflu-
idic domains. The platform can handle liquid volumes from hundreds of microlitres to sub-microlitre droplets. Using a combination of the syringe-pumps, robotic arms and DMF manipulation allows a world-to-chip interface, use of the 6-port valve or 96-well plate enables a simple chip-to-LC-MS coupling. This provides a powerful platform for proteomic analysis of complex and sub-microlitre biological samples.

5.2.3 DMF to 6-port Valve to LC-MS

Keeping the analyte in the liquid phase provides the advantage that it can be introduced into a liquid-chromatography system, separated by reverse-phase and analysed by any type of mass spectrometric platform. This has a number of advantages over direct-infusion, or MALDI-TOF PMF. LC-MSMS based proteomics provides much greater confidence in protein identification than peptide mass fingerprinting alone. To facilitate sample pickup by the LC system, the fused silica capillaries were coated with Parylene (see Section 7.1) in order to provide a hydrophobic coating. This stopped the droplets wetting onto the capillaries, and meant that the pickup-probe could be pushed into the droplet and moved out of it again without physically moving the droplet. The following experiments were all carried out using the Dionex Ultimate 3000 and HCT Ultra Ion Trap LC-MS system as described in Section 7.3.3.

5.2.3.1 On-Chip Tryptic Digests of Protein Standards

On chip digests of protein standards were performed to investigate if the DMF platform could be used to perform bottom-up MS experiments. Pure protein standards were purchased from Sigma-Aldrich. Care had to be taken when designing the protocol for on-chip digestion, as the requirement for denaturing the protein to facilitate good digestion needed to be balanced with the requirement not to denature the trypsin, thus rendering it inactive. In addition the lyophilised trypsin has to be rehydrated at pH 4.0 to avoid autodigestion. The pH needs to
be increased once the trypsin solution has been mixed with the denatured protein solution in order to activate the protease. The final protocol adopted employed three initial droplets;

1. 1ul of 1uM protein (0.1% formic acid)
2. 1ul of 0.8uM Trypsin in Trypsin buffer, pH 4.0
3. 1ul of Tris buffer, 50mM Tris, 1mM \(\text{CaCl}_2\), pH 7.0

These three droplets were mixed using DMF actuation and left to incubate at room temperature for one hour. Since the droplet evaporates it was periodically rehydrated using 1ul droplets of the Tris-\(\text{CaCl}_2\) buffer. After 1 hour the droplet was diluted using 10ul of buffer supplied by the robot and the resultant solution was aspirated and dispensed into a well on a 96-well plate. This 96 well plate was loaded into the Dionex Ultimate 3000 and analysed using the Bruker-Daltonics HCT Ultra IonTrap as per the methods provided in Section 7.3.3. Figure 5.17 shows the results of the first proof-of-concept experiment using horse-heart myoglobin (P68082) as the model protein. In Figure 5.17 A the base peak chromatogram is shown, signal intensity as a function of time. Figure 5.17 B is a mass spectrum from the tryptic peptide eluted from the column at a retention time of 20.5 mins. The spectra is annotated with the \(b\) and \(y\) ions that were identified, which correspond to the sequence underlined in the sequence coverage map shown in Figure 5.17 C. These results demonstrate that there is sufficient digestion of the sample, and that enough material can be picked-up, loaded onto the column, separated and analysed by MS to allow positive identification of the starting material.

Figure 5.18 shows the results from a second experiment using cytochrome-c as the model protein. The mass spectrum shown in Figure 5.18 B displays an uninterrupted series of \(y\)-ions from \(y(4)\) to \(y(10)\). This series allows confident assignment of the sequence. The addition of 0.1% formic acid to the protein droplet denatures the protein and results in more efficient CID. This, coupled
Figure 5.17: On-chip tryptic digest of horse heart myoglobin. A. Total ion chromatogram. B. Representative mass spectrum of highest scoring peak. C. MASCOT results; sequence coverage = 41%, MASCOT score = 87
with the fact that cytochrome-c is a smaller protein, gives rise to the increase in sequence coverage compared to the results for myoglobin.

These data show that on-chip digestion of pure protein standards is possible. Careful design of the experimental requirements for high-surface tension, protein denaturation and ion suppression have been balanced in order to provide a robust procedure for DMF-based bottom-up analysis.

5.2.3.2 HIS-Tagged Protein Purification Using Nickel Beads

The concept of functionalised magnetic bead purification was introduced in Section 5.1.1.2. Here a proof of concept experiment for on-chip purification, digestion and identification of a recombinant fusion-protein is described. In previous work from this laboratory (Clarke et al\textsuperscript{209}) Bacterioferritin Comigratory Protein (BCP) has been investigated. This protein was recombinantly expressed in \textit{E-coli} and included a His-Tag binding domain. This protein was chosen for evaluation of on-chip magnetic bead purification. The protocol for the preparation of a clarified lysate from these cells is given in Section 7.3.4.1. The full protocol for the BioClone BcMag His beads is provided in Section 7.3.4. Examination of this protocol shows clear motivation for miniaturisation and automation. The total amount of protein sample required for MS-analysis is approximately one tenth of the final purified volume of protein produced by following the protocol. This means that the volumes described in the published protocol can be divided by a factor of ten. In addition, there are multiple repeated steps which would benefit from automation due to improved reproducibility. The jig that had been designed to hold the DMF chips was modified to include an electromagnet, which was purchased from RS-components. The surface of the jig was milled flat to provide a flush surface. The previously described LabView program was used for control, and a series of lines of control code were generated to follow the magnetic bead protocol. The silicon DMF chips were used for droplet actuation and the robot was used to aspirate the supernatant. An example of the control code is provided
Figure 5.18: On-chip tryptic digest of cytochrome-c. A. Total ion chromatogram. B. Representative mass spectrum of highest scoring peak. C. MASCOT results; sequence coverage = 81%, MASCOT score = 368
in Appendix C.2.

Figure 5.19: Magnetic beads are pulled down in an on-chip enrichment using an embedded electromagnet

Figure 5.19 shows a video captured time-sequence of the magnetic beads being ‘pulled down’ using the embedded electromagnet. The frames are one second apart, and show how the electromagnet can be switched on under computer control, and provide a high degree of clarification of the supernatant. The magnetic beads were washed before being combined by DMF manipulation with the clarified cell lysate. The nickel coating on the BcMag beads acts as a coordination centre to which the His-Tag binding domain on the BCP binds. The electromagnet is then switched to pull-down the beads with protein bound to them. After multiple washing steps the beads are combined with a droplet containing porcine trypsin (pH 4.0) and another droplet containing a buffer solution to make the trypsin active (pH 7.0), see Figure 5.20.

On-bead enzymatic hydrolysis was allowed to proceed for an hour at room temperature, with periodic rehydration using 1 ul droplets of the Tris-CaCl$_2$ buffer. After the incubation period the droplet was diluted, using the robot to dispense a 10 ul droplet of Tris-CaCl buffer which was then loaded onto a 96-well plate. This 96 well plate was loaded into the Dionex Ultimate 3000 and analysed using the Bruker-Daltonics HCT Ultra ion trap using the method described in Section 7.3.4. Figure 5.21 shows the results from the LC-MS analysis. The
Figure 5.20: On-chip tryptic digest of His-Tag protein bound to magnetic beads
total ion-chromatogram is shown, along with the spectrum for the highest scoring tryptic peptide and a sequence coverage map. It is possible that the low-volumes used in this experiment provided very high local concentrations of Trypsin as the solvents evaporated and this may have had a beneficial effect on the kinetics of the enzymatic hydrolysis, though this needs to be investigated thoroughly.

Although previous studies by Shah et al. have reported the use of DMF devices for purification of cell products\textsuperscript{210,211}, their analytical methods were limited. The proof-of-concept experiment presented here shows that it is possible to use magnetic beads on-chip to purify tagged proteins from a crude lysate prior to analysis and identification by LC-MS.

### 5.3 Summary

The motivation for sample preparation by DMF has been outlined, and previously reported work describing the coupling of DMF-MS has been reviewed. Results from the direct analysis from the DMF chips using MALDI MS has been presented. A DESI source was designed, manufactured and characterised for its suitability for direct analysis of proteins from DMF chips. DMF to LC-MS coupling has been presented and an integrated, automated, robotic system designed and implemented as a world-to-chip interface. The robotic system was used for on-chip tryptic digests of protein standards with analysis by LC-MS. Finally a magnetic bead protocol was developed to selectively purify his-tag fusion proteins from a crude lysate on-chip, balancing the requirements for both the DMF actuation and MS detection. High quality mass spectra were obtained for BCP, with very good sequence coverage.
Figure 5.21: On-chip magnetic bead purification and tryptic digest of BCP. **A.** Total ion chromatogram. **B.** Representative mass spectrum of highest scoring peak. **C.** MASCOT results; sequence coverage = 56%, MASCOT score = 215
Chapter 6

Summary and Conclusions

6.1 Summary

This thesis has described the development of a range of digital microfluidic devices, and the results obtained show that they provide a potentially powerful fluid handling method for sample preparation in biological mass spectrometry. The relevant literature and background theory of mass spectrometry and proteomic methodologies has been introduced. A full description of all the instrumentation used in this work has been given.

An overview of recently published work on microfluidic devices has been provided and the advantageous effects of scaling laws have been discussed. The classical theory behind the Lippmann-Young equation has been presented. This theory has been extended and presented specifically for understanding the operation of DMF devices. Following recent developments in the literature\textsuperscript{97,151}, a derivation of the electrodynamic interpretation of DMF has been derived. This derivation starts from fundamental electrostatics and, using consistent notation, the Maxwell stress tensor is derived. The relevant theory has been used to design physical DMF devices.

The open (coplanar) and closed configurations of DMF devices have been described and the relevant design considerations discussed. A range of silicon based DMF devices have been designed, starting with single metal layer devices and
moving to more complex multi metal layer devices. These devices have been manufactured in the Scottish Microelectronics Centre, and the full process flows that were developed are provided. The silicon devices have an anodic tantalum pentoxide coating, which provides a thin, pin-hole free dielectric having a high relative permittivity. The concept of device fabrication using rapid prototyping has been introduced and devices based on copper clad polyimide sheets have been manufactured using a range of patterning and etching techniques. A new rapid prototyping technology, based on screen printing, has been investigated. Screen printing of DMF devices will significantly reduce the unit cost and could provide a route to commercialisation. The AC and DC driving regimes have been discussed, and the control circuitry for droplet actuation that was developed has been described.

These DMF devices have been used in a variety of ways to prepare samples for biological mass spectrometry. Integration of the DMF technology with MALDI TOF mass spectrometry has been demonstrated. The new and potentially powerful method of DESI has been investigated as a means of analysing samples directly from DMF devices. A DESI source was designed and employed in several FT-ICR mass spectrometric studies. Initial experiments involving DESI mass spectrometry imaging were also carried out. A robotic system was developed to facilitate a world-to-chip interface for coupling of DMF and LC-MS. Tryptic digests were performed on-chip with bottom-up analysis by LC-MSMS. The most complex experiment carried out so far involved automated on-chip purification of a His-tag fusion protein from a clarified cell lysate, using functionalised magnetic beads. The purified protein was enzymatically hydrolysed on-chip using trypsin and then analysed using LC-MSMS.
6.2 Conclusions

Work presented in this thesis clearly demonstrates the utility of DMF devices for fluid handling and sample preparation technology to facilitate biological mass spectrometry. Although these experiments employed a serial workflow, a significant advantage of DMF sample handling is the potential for parallel and multiplexed analysis of samples. The 8x5 DMF array that was designed and tested is a significant milestone in the development of large arrays, which could process many samples in parallel and under full computer control. These DMF devices were used to move droplets with a volume in the region of 500 nl to 1 µl. For the most part these droplets were manipulated on co-planar arrays since this facilitated straightforward integration with other fluidic systems. The use of closed DMF devices, utilising a coverplate may enable this sample volume to be reduced by at least one, or possibly two, orders of magnitude. The coverplate provides a number of advantages. The full range of dropwise operations can be performed. Droplet evaporation is mitigated by providing an environment with high local humidity. Finally, the fluoropolymer surface is protected from airborne particulates.

The development of screen printing techniques as described herein represents a significant advancement in the manufacture of DMF devices. The ability to quickly and cheaply design, manufacture and implement custom DMF designs based on a low cost, but reproducible, technique provides a clear commercial advantage over silicon microfabrication. Indeed, by integrating SAWN nebulisation\textsuperscript{212} with DMF sample handling and preparation, there is real potential to develop a novel mass spectrometric interface.

The DMF theory presented here represents the first coherent derivation of the Maxwell stress tensor, using consistent notation, and with specific reference to DMF device design. Examination of the implications of the frequency dependance for droplet actuation, along with the results of the time to failure experiments
(Section 4.3) provides a robust theoretical and physical basis for the development the next generation of DMF devices.

6.3 Future work

The most complex experiment carried out so far, described in Section 5.2.3.2, involved the use of functionalised magnetic beads and automated on-chip purification of a His-tag fusion protein for bottom-up nano-LC-MSMS. There are clearly a number of logical avenues for further experiments. An attempt was made to carry out on-chip transcription and translation of a His-tagged protein from a plasmid, using an in-vitro kit (Expressway cell-free expression system, Invitrogen, UK). Unfortunately, the kit supplied by the manufacturers was damaged in transit, preventing this experiment from being attempted. This experiment should be carried out, since it would represent the first application of cell-free expression of protein with subsequent purification using DMF using mass spectrometric analysis.

Other obvious experiments using mass spectrometric detection involve the development of integrated techniques for sample nebulisation and ionisation, directly from DMF devices, such as the recent work by Heron et al. utilising SAWN\textsuperscript{212} - surface acoustic wave nebulisation. The authors have shown that droplets of sample containing polypeptides can be nebulised from a planar surface and introduced into a mass spectrometer. One drawback with SAWN is that the lithium niobate wafers required for SAW activation are relatively expensive. Previously published work in the literature would seem to suggest that nebulisation and ionisation can be achieved using cheaper alternative activation devices, such as piezoelectric drivers\textsuperscript{213}. This possibility is under current investigation.

Clearly there are many other detection modalities that can be employed, other that mass spectrometry. Some of these, such as optical, electrochemical and electromechanical, readily lend themselves to integration with DMF devices, par-
particularly in array format. For example, recent work in the SMC/COSMIC has involved the use of Super Photo Avalanche Diodes (SPADS) for miniaturised, fluorescence lifetime imaging. Obviously silicon based DMF devices offer integration with considerably more sophisticated MEMS technology and provide a route to incorporating a CMOS active backplane\textsuperscript{156}.

The DMF devices described in this thesis, namely DC-driven silicon chips, as well as AC-driven copper and screen printed devices, need to be evaluated objectively. Each type of device has obvious relative advantages and disadvantages, such as unit cost, reliability, re-configurability and time-to-failure. These devices need to be evaluated, not only with respect to the influences of driving frequency in the AC domain, but also the effect of electric field strength, in the dielectric and fluoropolymer layers, on such practical aspects as device time-to-failure and user reliability.

Despite these provisos, it seems clear that there is increasing demand for more sophisticated sample preparation, sample purification and sample manipulation, in numerous fields of active research, such as the life sciences, medicine, food, environment and military/defence. DMF devices offer one of the most promising routes for the delivery of a generic technology platform that can address these pressing areas of miniaturised exploratory measurement science.
Chapter 7
Methods and Materials

7.1 Microfabrication Process

The microfabrication process flows for the single and multi metal layer silicon DMF devices are presented in Tables 7.1 and 7.2. The process target thicknesses are dependant on a variety of conditions in the instrumentation employed. The instrument specific conditions have been fully defined in the SMC, and for clarity the exact details have been omitted here, although the SMC toolbox (an internal, online resource) provides information on each of the instruments. The processing for the silicon devices begins with a blank silicon wafer upon which a thermal oxide is grown to electrically isolate the metal interconnects from each other. The oxide thickness was initially thought to be irrelevant, although there is some evidence that in AC-DMF it may have an effect due to parasitic capacitance. This will be discussed further in Section 6.3. The oxide is typically a wet-oxidised SiO$_2$ formed by the reaction of silicon with steam at typical temperatures of $1000\, ^\circ\text{C}$. The reaction that occurs is $Si + 2H_2O \rightarrow SiO_2 + 2H_2$. The metal layer is sputtered over the oxide and is typically aluminium or tantalum. The method of sputtering used is a process whereby a target metal is bombarded by a focused ion-beam. The high energy ions liberate metal from the target and it condenses onto the silicon wafers which are rotating in the vacuum chamber. The metallised wafers are coated with photoresist using the 3-inch SVG track system. Firstly, the wafer
is heated in a vacuum and hexamethyldisilane (HDMS) adhesion promoter gas is applied. The wafer is then transported along the track and SPR350 photoresist is spun onto the metal surface at $\sim 4000$ rpm. Finally the wafer is soft-baked at $90^\circ C$ for 60 s to vapourise the solvents in which the photoresist was dissolved. The next photolithographic step is exposure. The Karl-Suss contact photomask-aligner puts the quartz-chromium mask (see Appendix B) in direct contact with the wafer and UV light is used to irradiate the photoresist. The photomask aligner utilised a proprietary SUSS micro-tec diffraction reducing system, and allows resolution down to $0.5 \mu m$. Once the wafer has been exposed, it is put back into the SVG track system to develop the photoresist. Here the wafer is spincoated with 26A developer and hardbaked at $155^\circ C$ for 60 seconds to harden the unexposed photoresist. The metal is then etched in the STS reactive ion etching (RIE) etcher. Reactive ion etching gives a highly anisotropic etch, meaning that the metal is preferentially etched in one specific direction. The STS RIE etcher uses an electric field to dissociate chlorine compounds into a plasma. Typically, $SiCl_4$ is used to etch metals, $CF_4$, and $CHF_3$ are used to etch silicon dioxide and silicon nitride, respectively. The highly energetic monoatomic chlorine forms compounds with the exposed metal surface, which are purged from the system using the vacuum. The barrel asher oxygen plasma machine is used to remove the remaining photoresist after the RIE etch. The oxygen plasma consists of monoatomic oxygen radicals. The 3-inch wafer is then diced in accordance with the ‘Gold Standard’ dimensions (See Figure 4.2) using the Disco wafer saw. The chips are then ready to be anodised (see Section 7.1.1). After anodisation, the chips are coated with parylene-c in a conformal deposition process using the SCS parylene machine. The parylene dimer is heated to $600^\circ C$ where it decomposes to become a momomer. This gas is fed into an evacuated chamber where it polymerised onto any available surface. The thickness is determined by the amount of dimer that is loaded into the machine, and has been characterised as approximately $1.1 \mu m$ per gram. Finally, the teflon layer is spin-coated onto
the chips using a 1:100 teflon-fluorinert solution at speeds of approximately 600 rpm.

Table 7.1: Single metal layer $Ta_2O_5$ microfabrication process flow.

<table>
<thead>
<tr>
<th>Process</th>
<th>Mask</th>
<th>Equipment</th>
<th>Process Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thermal oxidation</td>
<td>-</td>
<td>Furnace</td>
<td>10,000 A</td>
</tr>
<tr>
<td>Tantalum sputtering</td>
<td>-</td>
<td>Balzers</td>
<td>3000 A</td>
</tr>
<tr>
<td>Photoresist coating</td>
<td>-</td>
<td>SVG</td>
<td>1,1,1</td>
</tr>
<tr>
<td>Lithography</td>
<td>Multilevel</td>
<td>Karl-Suss</td>
<td>5 Sec</td>
</tr>
<tr>
<td>Developing</td>
<td>-</td>
<td>SVG</td>
<td>9,1,1</td>
</tr>
<tr>
<td>Tantalum etching</td>
<td>-</td>
<td>STS-Ali etcher</td>
<td>-</td>
</tr>
<tr>
<td>Photoresist stripping</td>
<td>-</td>
<td>Barrel asher</td>
<td>-</td>
</tr>
<tr>
<td>Wafer dicing</td>
<td>-</td>
<td>Disco wafer saw</td>
<td>-</td>
</tr>
<tr>
<td>Tantalum anodisation</td>
<td>-</td>
<td>-</td>
<td>95 nm</td>
</tr>
<tr>
<td>CYTOP spin coating</td>
<td>-</td>
<td>Polo spinner</td>
<td>26 nm</td>
</tr>
<tr>
<td>CYTOP baking @ 180 deg</td>
<td>-</td>
<td>Tenny oven</td>
<td>10 min</td>
</tr>
</tbody>
</table>

The multi metal layer devices require a few more processing steps, although these are merely repeats of previous steps. The first interconnect metal layer is deposited onto the oxide and is electrically isolated from the second metal layer by a silicon nitride film, deposited by plasma enhanced chemical vapour deposition (PECVD). In this process one or more gaseous species react on a solid surface and one of the reaction products is a solid phase material. Plasma enhanced CVD uses an RF electric field to generate a glow discharge to transfer energy into the reactant gasses, allowing the deposition onto the substrate at significantly lower temperatures than in other CVD methods. This reaction occurs in a chamber filled with nitrogen at approximately 300 °C and 60-70 Pa. Silane is reacted with the nitrogen to form a silicon nitride film as in the reaction; $3SiH_4(g) + 2N_2(g) \rightarrow Si_3N_4 : H(s) + 6H_2(g)$. The $H(s)$ term means that some of the hydrogen radicals that are formed react with the nitride and stay in the film. Silicon nitride films often have hydrogen concentrations up to 40%. PECVD deposition is performed on the STS PECVD tool, which can deposit $SiO_2$, $SiN$ and $SiON$ films of up to 6 μm. These PECVD deposited films have different stress
characteristics dependant on the frequency of the plasma. Furthermore, they can crack when subjected to changes in temperature. To make the connection between the interconnect and top-metal layer a hole, known as a via, must be made in the nitride film. The VIA mask is a dark field mask and is processed using the same photolithographic steps as previously describes for patterning the metal. After patterning the insulating layer and removing the remaining photoresist the second metal layer is sputtered. This layer is planarised using a CMP process to remove the relief-patterned hillocks formed by the buried interconnect layer. This tool uses a combination of an abrasive pad and an abrasive/corrosive slurry to remove unwanted material from the surface. After planarisation the top metal layer is patterned using photolithography. The chips are then diced, anodised and coated in the same fashion as the single metal layer devices.

7.1.1 $T_a_2O_5$ Anodisation Process

Anodisation has been previously introduced and described in Section 4.1.5. The process for anodisation of the tantalum surface is briefly introduced here. Further detail can be found in Yifan Li’s PhD Thesis\textsuperscript{156}. The gel used for anodisation is composed of 30 ml diethylene glycol, 5 g sodium carboxymethylcellulose, 200 ml deionised water and 0.6 g citric acid.

Figure 7.1 shows the correct positioning of the wire for anodisation. It should be positioned as vertical as possible to ensure that the electric field strength and therefore oxide thickness, is homogeneous. The equation governing anodic oxidisation of the tantalum is $J = D e^{\frac{B V}{h}}$, where $J$ is the current density, $V$ is the potential across the oxide layer of thickness $h$. $D$ and $B$ are constants\textsuperscript{156}. The current density to grow the $T_a_2O_5$ is normally fixed between 1 and 10 mA cm$^2$ which sets the growth rate of the oxide. For a given temperature the resulting film thickness is defined by the applied voltage, which for this work was in the range of 1.6 to 1.9 nm V$^{-1}$. For the two devices that were tested as described in Section 4.3 using layer thicknesses of 38 and 180 nm, the anodisation voltages
Table 7.2: Multi metal layer $SiO_2$ microfabrication process flow.

<table>
<thead>
<tr>
<th>Process</th>
<th>Mask</th>
<th>Equipment</th>
<th>Process Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thermal oxidation</td>
<td>-</td>
<td>Furnace</td>
<td>10,000 A</td>
</tr>
<tr>
<td>Aluminium sputtering</td>
<td>-</td>
<td>Balzers</td>
<td>7500 A</td>
</tr>
<tr>
<td>Photoresist coating</td>
<td>-</td>
<td>SVG</td>
<td>1,1,1</td>
</tr>
<tr>
<td>Lithography</td>
<td>METAL1</td>
<td>Karl-Suss</td>
<td>5 sec</td>
</tr>
<tr>
<td>Developing</td>
<td>-</td>
<td>SVG</td>
<td>9,1,1</td>
</tr>
<tr>
<td>Aluminium etching</td>
<td>-</td>
<td>STS-Ali etcher</td>
<td>-</td>
</tr>
<tr>
<td>Photoresist stripping</td>
<td>-</td>
<td>Barrel asher</td>
<td>-</td>
</tr>
<tr>
<td>PECVD oxide</td>
<td>-</td>
<td>STS-PECVD</td>
<td>20,000 A</td>
</tr>
<tr>
<td>Clean wafers</td>
<td>-</td>
<td>Wet deck</td>
<td>-</td>
</tr>
<tr>
<td>Photoresist coating</td>
<td>-</td>
<td>SVG</td>
<td>1,1,1</td>
</tr>
<tr>
<td>Lithography</td>
<td>VIA</td>
<td>Karl-Suss</td>
<td>5 sec</td>
</tr>
<tr>
<td>Developing</td>
<td>-</td>
<td>SVG</td>
<td>9,1,1</td>
</tr>
<tr>
<td>Oxide etching</td>
<td>-</td>
<td>Plasmatherm</td>
<td>-</td>
</tr>
<tr>
<td>Photoresist stripping</td>
<td>-</td>
<td>Barrel asher</td>
<td>-</td>
</tr>
<tr>
<td>Aluminium sputtering</td>
<td>-</td>
<td>Balzers</td>
<td>10,000 A</td>
</tr>
<tr>
<td>Aluminium CMP</td>
<td>-</td>
<td>Presi CMP</td>
<td>-</td>
</tr>
<tr>
<td>Clean wafers</td>
<td>-</td>
<td>Wet deck</td>
<td>-</td>
</tr>
<tr>
<td>Photoresist coating</td>
<td>-</td>
<td>SVG</td>
<td>1,1,1</td>
</tr>
<tr>
<td>Lithography</td>
<td>METAL2</td>
<td>Karl-Suss</td>
<td>5 sec</td>
</tr>
<tr>
<td>Developing</td>
<td>-</td>
<td>SVG</td>
<td>9,1,1</td>
</tr>
<tr>
<td>Aluminium etching</td>
<td>-</td>
<td>STS-Ali etcher</td>
<td>-</td>
</tr>
<tr>
<td>Photoresist stripping</td>
<td>-</td>
<td>Barrel asher</td>
<td>-</td>
</tr>
<tr>
<td>Wafer dicing</td>
<td>-</td>
<td>Disco wafer saw</td>
<td>-</td>
</tr>
<tr>
<td>Parylene deposition</td>
<td>-</td>
<td>-</td>
<td>5237 A</td>
</tr>
<tr>
<td>CYTOP spin coating</td>
<td>-</td>
<td>Polo spinner</td>
<td>260 A</td>
</tr>
<tr>
<td>CYTOP baking @ 180 deg</td>
<td>-</td>
<td>Tenny oven</td>
<td>10 min</td>
</tr>
</tbody>
</table>

were 20 V and 100 V, respectively.

The basic steps in this $Ta_2O_5$ anodisation process are as follows; The system is set up as in Figure 7.1, the gel is then dispensed onto the surface. The probe is inserted without touching the underlying surface material. The voltage supply is turned on and the stopwatch started. The current and voltage change is monitored until the values correspond to known values for a given dielectric thickness\textsuperscript{156}.  

\textsuperscript{156}
7.2 Materials and Reagents

The materials used during the course of this work are listed in Table 7.3. Included in this list are samples which were purchased for studies or calibration, as well as consumables required for sample preparation, such as solvents. The name of the compound, the quoted quality and supplier information is also provided if applicable.

Table 7.3: Materials used, together with supplier information.

<table>
<thead>
<tr>
<th>Name</th>
<th>Quality</th>
<th>Supplier</th>
<th>Catalogue No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apo-myoglobin</td>
<td>N/A</td>
<td>Sigma Aldrich</td>
<td>10K7026</td>
</tr>
<tr>
<td>Cytochrome-c</td>
<td>N/A</td>
<td>Sigma Aldrich</td>
<td>065K7001</td>
</tr>
<tr>
<td>Trypsin</td>
<td>N/A</td>
<td>Sigma Aldrich</td>
<td>T1143</td>
</tr>
<tr>
<td>BcMag®, His-Tag Magnetic Beads</td>
<td></td>
<td>Bioclon</td>
<td>n/a</td>
</tr>
<tr>
<td>BugBuster</td>
<td>N/A</td>
<td>Novagen</td>
<td>n/a</td>
</tr>
<tr>
<td>ESI tuning mix</td>
<td>N/A</td>
<td>Agilent technologies</td>
<td>G2431A</td>
</tr>
<tr>
<td>alpha-cyano-4-hydroxycinnamic acid</td>
<td>97%</td>
<td>Sigma Aldrich</td>
<td>14,550-5</td>
</tr>
<tr>
<td>Pepmap100 PS-DVB 100 µm monolithic column</td>
<td></td>
<td>Dionex LC Packings</td>
<td>44212</td>
</tr>
<tr>
<td>Formic acid</td>
<td>N/A</td>
<td>Fluka</td>
<td>94318</td>
</tr>
<tr>
<td>2-Propanol CHROMASOLV®</td>
<td>≥99.9%LC-MS</td>
<td>Riedel-de Haën</td>
<td>34965</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>LC-MS</td>
<td>Fisher Scientific</td>
<td>A/0638/17</td>
</tr>
<tr>
<td>Methanol</td>
<td>LC-MS</td>
<td>Fisher Scientific</td>
<td>M/4062/17</td>
</tr>
<tr>
<td>Water</td>
<td>LC-MS</td>
<td>Fisher Scientific</td>
<td>W/0112/15</td>
</tr>
</tbody>
</table>
The peptides and proteins used are listed in Table 7.4, together with the calculated neutral mass of the molecules, amino acid sequence, amino acid count and the elemental formulae used for isotopic modelling.

Any features which were important for the mass measurement are also indicated.

7.3 Methods for DMF Sample Preparation for Biological Mass Spectrometry

7.3.1 DMF based Peptide Mass Fingerprinting by MALDI-TOF MS

Further information is provided here for the experiments described in Section 5.2.1.1. The matrix used for all MALDI MS was alpha-cyano-4-hydroxycinnamic acid. This was prepared by mixing approximately 500 milligrams of anhydrous alpha-cyano-4-hydroxycinnamic acid crystals with LC-MS grade acetonitrile and mixing thoroughly by vortexing until the solvent was observed to be fully saturated. The suspension was then centrifuged for 5 minutes at 13,000 rpm to precipitate any solid particulate. The resultant super-saturated alpha-cyano-4-hydroxycinnamic acid supernatant was transferred into a fresh Eppendorf tube and used immediately. No solutions of alpha-cyano-4-hydroxycinnamic acid were stored, as it is known to degrade under exposure to light. MALDI digest samples were prepared by hydrating the lyophilised digest standards with LC-MS grade water and dividing the 0.1 pM/ul solution into 10 µl alliquots. These were then frozen until ready to be used. The digest standards were mixed with the matrix on-chip using DMF actuation. The cover plate was then removed and the mixed solution left to co-crystallise onto the chip surface at room temperature. The samples were then analysed by MALDI-TOF mass spectrometry.
<table>
<thead>
<tr>
<th>Name</th>
<th>Calculated average mass /Da</th>
<th>Sequence information</th>
<th>Total amino acids</th>
<th>Elemental formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heme (myoglobin)</td>
<td>616.49</td>
<td>-</td>
<td>-</td>
<td>C_{44}H_{82}O_{4}N_{4}Fe</td>
</tr>
<tr>
<td>Heme (cytochrome c)</td>
<td>684.65</td>
<td>CH_{3}CO-GDVEKGKKKFQVQKCACQCHTVEKGGKHKTPGLNLHGLFGKRTGQAPGFTYTDAKNNKGTWKEETLMYENLPKKPYIPGTKAHIKKGTKTEDLHAL YLFPDSIADVHLYSHKHPGDF-</td>
<td>-</td>
<td>C_{34}H_{30}O_{4}N_{2}S_{2}Fe</td>
</tr>
<tr>
<td>Cytochrome c</td>
<td>12364.98</td>
<td>H-GLSDGEWQVNLNVGWKEADAGHQCYEVLRIFTHPETLKFDKFKHLKTEAEMKASEDLKKGHTLVTLGGILKKGHEAEALKPLAQSHATSHEIKPIK</td>
<td>104</td>
<td>C_{560}H_{876}N_{148}O_{156}S_{4}Fe</td>
</tr>
<tr>
<td>apo-myoglobin</td>
<td>16951.30</td>
<td>GADAQGAMTKALEFRNDAIAKYKELGFPQQOH + heme</td>
<td>153</td>
<td>C_{769}H_{1212}N_{210}S_{21}O_{21}</td>
</tr>
<tr>
<td>holo-myoglobin</td>
<td>17559.72</td>
<td>H-GLSDGEWQVNLNVGWKEADAGHQCYEVLRIFTHPETLKFDKFKHLKTEAEMKASEDLKKGHTLVTLGGILKKGHEAEALKPLAQSHATSHEIKPIK</td>
<td>153</td>
<td>C_{603}H_{1244}Fe_{214}O_{222}S_{2}</td>
</tr>
<tr>
<td>BSA</td>
<td>69248.44</td>
<td>H-MKWVTFSLLLSSAYSQVRDFDTHKSEIAHREFKDLGEHEHFKGLVLIAFSQYLVQCPFDHEKVLNLETFKatekTCVADSHAGCEKSLHFTLFGDELCKVASC RETTYGDMADECEKQEPERNELCFLSHKDAESPDPDKLPDPNLTCDFKADEKK</td>
<td>606</td>
<td>C_{307}H_{1826}N_{818}O_{927}S_{4}</td>
</tr>
<tr>
<td>Trypsin</td>
<td>24393.81</td>
<td>H-FPTDDDDKIVGGYTCAANSIPYQVSLNSGSHCFGGSLNSQWVSAAYCYSRIQVRSHNHIDVLENEQFINAAKITHPNFNGNTLNDMLKLSAPPAT LNRSRATVSPLRSRCAAAAGETCILSWSGWNTKSAGSYYPLQLCKAPVLDSSC</td>
<td>231</td>
<td>C_{1060}H_{1662}N_{298}O_{338}S_{14 }</td>
</tr>
<tr>
<td>BCP</td>
<td>17622.70</td>
<td>H-MNPLKAGDIAPKFLDPQDGQVNLTDFOGQRIVYFYPKAMTPGCYCTQAGLRDNMDLLKAGDVGLISTDKPEKLSFRAEKENFNTLIDSEHQCQFGV WGEKSFMAKTYDGHRSLDADGKIEHVDFDKTSNHHDVNLWLE</td>
<td>156</td>
<td>C_{587}H_{1210}N_{208}O_{235}S_{7}</td>
</tr>
</tbody>
</table>

*aCalculated using elemental formula and Bruker Xmass software.

*bUniprotKB/Swiss-Prot entry P00004

cheme binding residues marked in red.

*dUniprotKB/Swiss-Prot entry P68082; residues 2 to 154
7.3.1.1 MALDI-TOF MS on the Voyager DE STR

The DMF chip coated with the crystallised MALDI matrix and analyte was located into the recessed MALDI target plate and held in place using a thin layer of vacuum grease. This modified target plate was then inserted into the Voyager DE STR TOF MS. Once the source had reached the correct pressure the target plate was moved using the joystick until the laser spot was incident on the analyte of interest. Typical settings for spectra acquired using the Perseptive BioSystems Voyager DE-STR are as follows: Reflectron and delayed extraction mode; delay time = 100 ns, Polarity = positive, accelerating voltage = 20,000 V, grid voltage = 72%, Mass range = 750 - 6000 Da, low-mass-cutoff = 700 Da, Number of laser shots = 200, laser power = 2000-3000, Matrix = alpha-cyano-4-hydroxycinnamic acid.

7.3.2 FT-ICR DESI-MS and MSMS

Further information is provided here for the experiments described in Section 5.2.1.2. In order to record DESI FT-ICR mass spectra of the intact proteins, myoglobin and cytochrome-c were dissolved at a concentration of 1 mg/ml in $H_2O$ and MeOH (1:1). A volume of 50 $\mu$l of these solutions were applied to the etched side of a glass microscope slide, which was used as the DESI target plate. The slide was then incubated at room temperature to evaporate the solvent. For the tandem mass spectra presented for cytochrome-c, a protein concentration of 2 mg/ml dissolved in $H_2O$ and MeOH (1:1) was required. 200 $\mu$l of this solution was applied to the target plate and the solvent allowed to evaporate. The etched glass microscope slides were mounted on the XY stage of the DESI source using a spring loaded clamp. Further details of the DESI source are given below. The critical geometrical DESI parameters, as defined by Takats et al. were determined empirically, each being co-varied until the maximum signal was obtained. A solution of methanol, water and formic acid (1:1:0.001) was used as
the spray solution, a potential of 3.5 kV was applied to the spray needle, and, nitrogen was used for the nebulising gas at a flow rate of approximately 2.5 l/min. The geometrical source parameters used to obtain tandem mass spectra were identical to those used to record spectra for the intact proteins. The mass resolving quadrupole was set to transmit only those ions that corresponded to the \([M+15H]^{15+}\) charge state of cytochrome-c (m/z 824), and the sample target plate was rastered beneath the DESI probe. In order to acquire the ECD spectrum, the electron source (Heatwave Technologies) was conditioned by applying 1.8 A overnight to the cathode filament. To record the spectrum the ECD lens was set to 20 V, and a pulse length of 6 ms and ECD bias of 1.2 V was employed. For the CID spectrum the collision voltage was set between 20 and 35 V. Fragmentation spectra were acquired at an FID size of 512 kword and were the sum of 200 acquisitions. Data analysis was performed using DataAnalysis 3.4 software (Bruker Daltonics). The SNAP 2.0 algorithm was used to generate fragment mass lists and the resultant top-down fragment mass lists were searched against Prosight PTM-2.0.

7.3.3 DMF based TrypticDigests and LC-MSMS analysis

Further information is provided here for the experiments described in Section 5.2.3.1. The three droplets (1ul of 1uM protein 0.1% Formic acid, 1ul of 0.8uM trypsin in trypsin buffer at pH 4.0 and 1ul of Tris buffer, 50mM Tris, 1mM CaCl\(_2\) at pH 7.0) were mixed using DMF actuation and left to incubate at room temperature for one hour. A co-planar W-shaped single metal layer DMF array was used for this work. Droplet evaporation was mitigated by periodically rehydrating using 1ul droplets of the Tris-CaCl\(_2\) buffer. These were dispensed by the robotic arm and syringe pump. The rehydrating buffer was stored in one well of a 96-well plate. After one hour the droplet containing the digested sample was removed from the surface by one of two methods, either collection by the robotic arm, drawn through a sample loop in a 6-port valve which was then switched.
to become in-line with the LC-MS system, or the sample was deposited into a 96-well plate which was then placed into the autosampler of the LC-MS system. Whilst the former approach was developed, problems with the 6-port valve arose which meant that the latter method was usually adopted. The LC-MS analysis methods are detailed below.

7.3.3.1 LC-MS Ion-Trap Methods

On-line liquid chromatography was performed using an Ultimate 3000 HPLC system (Dionex, Sunnyvale, CA) equipped with a monolithic PS-DVB (100um by 15mm) reverse-phase analytical column (Dionex, Sunnyvale, CA) coupled to a nanoESI source on a Bruker Daltonics HCT Ultra ion trap mass spectrometer. Protein (5 pmoles) was loaded onto the column (maintained at 60°C) and eluted using a 42 minute linear gradient from 0 - 50 % acetonitrile. After 42 mins the mobile phase was increased to 100% acetonitrile to remove any tightly bound analyte from the column and to prevent the carry-over of sample to the next run. Typically, the efficiency of the system was checked prior to analysis of a DMF prepared sample by loading 5 pmole of a protein digest standard. The exact composition of the aqueous and organic buffers was as follows. Aqueous buffer: 98.2% LC-MS grade water, 1.8% formic acid. Organic buffer: 80% LCMS grade acetonitrile, 19.3% LCMS grade water, 0.7% formic acid. Typically, a trap column was used to protect the analytical column from large particulates.

7.3.4 DMF based Magnetic Bead Purification and LC-MSMS analysis

Further information is provided here for the experiments described in Section 7.3.4. The clarified cell lysate was prepared as described in Section 7.3.4.1 and pipetted into one well of a 96-well plate. The unwashed magnetic beads, washing and elution buffers were pipetted into the next three wells. The protocol described in Section 7.3.4.2 was encoded into machine readable code using the RunListGen-
erator tool. The first few lines of this code are reproduced in Appendix C.2. The robot then aspirated a volume of approximately 1 µl of the clarified lysate onto a W-shaped coplanar single metal layer DMF array. The unwashed beads were transferred by the robot onto another position on the array and the magnetic bead purification protocol started. This included synchronisation of the embedded electromagnet, DMF actuation of the droplets and washing of the fluidic tip of the robot between steps. Following the purification protocol, an on-chip on-bead digestion of the sample was performed by mixing the beads (plus bound protein) to a droplet of trypsin on the chip using DMF actuation. This solution was left to incubate at room temperature for 1 hour before being transferred to the 96 well plate using the robot. This 96 well plate was then analysed using the same LC-MS methods as described in Section 7.3.3.

7.3.4.1 Preparation of Clarified Cell Lysate

*E*. coli cells were thawed on ice and 100 mg wet mass was mixed with a commercial lysis detergent, BugBuster (Novagen) following the manufacturers instructions. The resultant mixture was centrifuged to remove cell debris. The resultant supernatant was removed and stored at −20°C until required.

7.3.4.2 Magnetic Bead Purification Protocol

The magnetic bead purification protocol, based on information from BioClone Inc, was performed as follows: The cells were lysed using BugBuster and centrifuged to yield clarified cell lysate. The stock solution of magnetic beads was prepared by dilution in the binding/washing buffer. The beads were washed by repeated magnetic pull-down using a strong electromagnet, aspiration of the supernatant and re-dilution in binding/washing buffer. The washed beads were combined with the clarified lysate on-chip by DMF actuation allowing approximately 100 µl of beads per 1 mg of expressed protein. The beads were allowed to mix with the supernatant for 5 mins with mixing enhanced by DMF actuation. The beads
were then pulled down using the electromagnet and the supernatant discarded. The beads were resuspended using 8 volumes of binding/washing buffer, pulled down and the supernatant discarded, this was repeated twice. The bound protein was eluted from the beads by a similar method of pull-down and removal of the supernatant, though with resuspension in 500 nl of elution buffer, the mixture was mixed using DMF actuation at room temperature for 5 minutes and then the beads were pulled down using the electromagnet. The 20 µl of the supernatant was pipetted off for analysis by SDS-PAGE and the remaining droplet was introduced to a 96-well plate and analysed by nLC-MS.

7.4 Analysis of Mass Spectrometry Data

All FT-ICR mass spectra were externally calibrated using ES tuning mix (Agilent Technologies, Santa Clara, CA), and analysed using DataAnalysis software (Bruker Daltonics). The SNAP 2.0 algorithm was used for automated peak picking of top-down data and the resulting mass lists were searched against the relevant primary sequences using ProsightPTM. Error tolerances were set to 10 ppm. The HCT ion-trap spectra were analysed using DataAnalysis software and the resulting mass lists were searched against a the MASCOT database. MALDI spectra were analysed using DataExplorer and the resulting mass lists were searched against the MASCOT database.
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Appendix A

Electrodynamic Theory

In this appendix the fundamentals and background theory of electrodynamics are presented, as it is relevant for the experimental work described in this thesis and, to provide a theoretical basis upon which the DC-DMF, AC-DMF and DEP theory is built.

The material presented in this appendix relies heavily on three textbooks, Electromagnetics with applications by Kraus & Fleisch\cite{Kraus}, Introduction to electro-dynamics by David J. Griffiths\cite{Griffiths} and finally Electromagnetic Theory by Julius Adams Stratton\cite{Stratton}.

A.1 Fundamental Electrostatics

A.1.1 Coulomb

Coulomb’s Law (Equation A.1) is the starting point for electrostatic analysis. This is an equation derived from empirical observations and describes the force acting on a test particle $Q$ due to a single point charge $q$, which is at rest a distance $\kappa$ away.

\[
\vec{F} = \frac{1}{4\pi\epsilon_0} \frac{qQ}{\kappa^2} \hat{\kappa} \tag{A.1}
\]

The constant $\epsilon_0$ is the permittivity of free space. Coulomb’s Law shows that the force is proportional to product of the charges and inversely proportional to
the square of the separation distance vector $\mathbf{\kappa}$.

The **principle of superposition** states that the interaction between any two particles is completely unaffected by the presence of others. Therefore if there are multiple charges, their force vectors can be added together $\mathbf{F}_t = \mathbf{F}_1 + \mathbf{F}_2 + \mathbf{F}_3 ...$ and the resultant force on the test charge $Q$ can then be determined.

Coulomb's Law and the principle of superposition represent the fundamentals of electrostatics. All that follows is a mathematical elaboration of these two axioms.

**A.1.2 Gauss**

Gauss's Law states that the flux (that is the "number of field lines") passing though a closed surface is proportional to the amount of charge enclosed within that surface. If one considers a point charge $q$ at the origin, the flux of $\mathbf{E}$ through a sphere of radius $r$ is:

$$\oint \mathbf{E} \cdot d\mathbf{a} = \int \frac{1}{4\pi\epsilon_0} \left( \frac{q}{r^2} \hat{r} \right) \cdot \left( r^2 \sin \theta d\theta d\phi \hat{r} \right) = \frac{1}{\epsilon_0} q$$

(A.2)

Thus, the radius of the enclosing sphere (or any other "Gaussian" surface) cancels out and so the product is constant. This can be extended to give a useful form of Gauss’s Law, Equation A.3

$$\int_v \nabla \cdot \mathbf{E} d\tau = \oint_s \mathbf{E} \cdot d\mathbf{a} = \frac{1}{\epsilon_0} \int_v \rho d\tau = \frac{1}{\epsilon_0} Q_{enc}$$

(A.3)

Therefore, charge enclosed in a volume can be described in terms of the enclosed charge acting on the surface. This can be extended to any closed surface, rewritten in terms of the charge density and simplified using the divergence theorem to give Gauss's law in a neater, differential form, Equation A.4.

$$\nabla \cdot \mathbf{E} = \frac{1}{\epsilon_0} \rho$$

(A.4)
In a similar way to the derivation of Equation A.2, where the divergence of the electric field due to a charged particle situated at the origin was used, we can also calculate the curl of the electric field.

\[ \int_{a}^{b} \mathbf{E} \cdot d\mathbf{I} = \frac{1}{4\pi\varepsilon_0} \int_{a}^{b} \left( \frac{q}{r^2} \right) \cdot dr = \frac{1}{4\pi\varepsilon_0} \left( \frac{q}{r_a} - \frac{q}{r_b} \right) = 0 \quad (A.5) \]

Where \( r_a \) is the distance from the origin to the point \( a \) and \( r_b \) is the distance to \( b \). The integral around this closed path is zero, as \( r_a \) then equals \( r_b \). Using the Kelvin-Stokes’ theorem one can write Equation A.5 as,

\[ \nabla \times \mathbf{E} = 0 \quad (A.6) \]

Equation A.6 is also known as the curl law.

### A.1.3 Electric Potential

The electric field \( \mathbf{E} \) is a special vector function which always has a curl of zero. An interesting point to note, but not one which will be proven or derived is that the gradient of the electric field at any point yields the electrical potential, see Equation A.7

\[ \mathbf{E} = -\nabla V \quad (A.7) \]

The electric field and the potential obey the superposition principle. Dividing \( \mathbf{F}_i \) by \( Q \) yields \( \mathbf{E}_i = \mathbf{E}_1 + \mathbf{E}_2 + \mathbf{E}_3 \ldots \) and integrating from a defined reference point to a defined point from the source charge one obtains \( V_i = V_1 + V_2 + V_3 \), due to the fact that \( V \) is a scalar quantity. It is merely an ordinary sum and not a vector sum.

### A.1.4 Laplace

If one rewrites Equations A.4 and A.6 in terms of the electric potential \( V \) then Gauss’s law becomes Poisson’s equation,
\[ \nabla^2 V = -\frac{\rho}{\epsilon_0} \quad (A.8) \]

In regions of zero charge, Poisson’s equation reduces to the Laplace equation,

\[ \nabla^2 V = 0 \quad (A.9) \]

When the curl law is rewritten in terms of \( V \) it becomes,

\[ \nabla \times \vec{E} = \nabla \times \left( -\nabla V \right) = 0 \quad (A.10) \]

This is in fact a mathematical identity and shows that the curl of gradient equals zero.

### A.1.5 Polarisation and Dielectrics

In order to consider the system shown in Figure 4.3 one needs to examine the way that electric fields interact with matter. Atoms and molecules are neutrally charged overall, although they do have an induced dipole moment. The induced dipole \( \vec{p} \) means that the atom or molecule has a distribution of charge, either due to the arrangement of its orbitals or because of the distribution of polarisable atoms along a molecule. Due to the externally applied electric field \( \vec{E} \), the atoms or molecules will become aligned with the direction of the field lines.

\[ \vec{p} = \alpha_{ij} \vec{E} \quad (A.11) \]

In Equation A.11, \( \alpha \) is a set of nine constants called the polarisability tensor. This is an experimentally derived quantity for an atom or molecule.

Polar molecules have a high dipole moment due to their structure and charge distribution. A molecule which can be described as a perfect dipole and one which is free to rotate will align with the electric field. The torque exerted about the centre of the dipole can be derived as,
\[
\overrightarrow{N} = \overrightarrow{p} \times \overrightarrow{E}
\]  \hspace{1cm} (A.12)

If the applied field is nonuniform, either by accident or design, such that the forces acting on either end of the dipole do not exactly cancel then there will be a net applied force. The net-force can be derived as,

\[
\overrightarrow{F} = \overrightarrow{F}_+ + \overrightarrow{F}_- = q \left( \overrightarrow{E}_+ + \overrightarrow{E}_- \right) = q \left( \Delta \overrightarrow{E} \right)
\]  \hspace{1cm} (A.13)

Where \( \Delta \overrightarrow{E} \) represents the difference between the field at the plus end and the field at the minus end. Assuming the dipole is very short one can write,

\[
\Delta \overrightarrow{E} = \left( \overrightarrow{d} \cdot \nabla \right) \overrightarrow{E}
\]  \hspace{1cm} (A.14)

Clearly the electric field has to be changing very abruptly for this to have a significant effect in the space occupied by one molecule. Indeed it is the force due to an abrupt change in applied non-uniform electric field that will be explored later on as a mechanism for moving droplets of polar liquids.

### A.1.6 Electric Displacement

The effect of polarisation is to produce accumulations of bound charge (dipoles with a fixed charge at either end). The bound charge can be shown to be equal to \( \rho_b = -\nabla \cdot \overrightarrow{P} \) within the dielectric, and \( \sigma_b = \overrightarrow{P} \cdot \hat{n} \) on the surface. Where \( \hat{n} \) is the unit vector normal to the surface and \( \overrightarrow{P} \) is the dipole moment per unit volume. If the charge density due to any free-charge in the material is defined as \( \rho_f \), then the overall charge density can be written as \( \rho = \rho_b + \rho_f \). If applied to Gauss’s law, and considering the total field due to polarisation and free-charge then it follows that:

\[
\epsilon_0 \nabla \cdot \overrightarrow{E} = \rho = \rho_b + \rho_f = -\nabla \cdot \overrightarrow{P} + \rho_f
\]  \hspace{1cm} (A.15)

Combining the two divergence terms yields the expression:
\[ \nabla \cdot \left( \varepsilon_0 \vec{E} + \vec{P} \right) = \rho_f \] (A.16)

The electric displacement is defined by the letter \( \vec{D} \), and is equal to the term within the brackets in Equation A.16. When Gauss’s law is expressed in terms of \( \vec{D} \) it reads,

\[ \nabla \cdot \vec{D} = \rho_f \] (A.17)

In integral form this gives,

\[ \oint \vec{D} \cdot d\vec{a} = Q_{enc} \] (A.18)

This is a particularly useful way to view Gauss’s law as one can control the free charge in the system shown in Figure 4.3 by a combination of chemistry and electronics. In addition this expression shows why a reductionist view of the system is unreliable, the curl of \( \vec{D} \) is not always zero, nor is that of \( \vec{P} \).

A.1.7 Capacitance

The system shown in Figure 4.3 can now be analysed as though it were a parallel plate capacitor. Working from Coulomb’s Law (Equation A.1) one can derive an equation that describes the capacitance of the system. Due to the fact that the electric field \( \vec{E} \) is proportional to the charge \( Q \), then so is the electric potential \( V \), the constant of proportionality between \( Q \) and \( V \) is defined as the capacitance \( C \), this relationship is formalised in Equation A.19.

\[ C \equiv \frac{Q}{V} \] (A.19)

In the device under investigation there is more than one dielectric layer. By applying Coulomb’s law and the principle of superposition it can be shown that the total combined capacitance in the system \( C_T \) can be calculated as,
\[
\frac{1}{C_T} = \frac{1}{C_1} + \frac{1}{C_2} + \frac{1}{C_3} = \frac{\varepsilon_0 \varepsilon_{r_1} A}{t_1} + \frac{\varepsilon_0 \varepsilon_{r_2} A}{t_2} + \frac{\varepsilon_0 \varepsilon_{r_3} A}{t_3} \quad (A.20)
\]

Where \( \varepsilon_{r_{1,3}} \) and \( t_{1,3} \) are the relative permittivities and thicknesses of the dielectric layers respectively. A discussion of the implications of this equation will follow, but for now it is sufficient to note that it is desirable to have thin layers of material with a high relative permittivity, since this leads to high capacitance. A capacitor stores an incremental energy given by the product of the voltage applied and the incremental charge. Thus;

\[
dW = Vdq = \frac{1}{C}qdq \quad (A.21)
\]

The total energy is;

\[
W = \frac{1}{C} \int_0^Q qdq = \frac{1}{2} \frac{Q^2}{C} = \frac{1}{2} CV^2 = \frac{1}{2} QV = \frac{1}{2} \varepsilon EAEh = \frac{1}{2} \varepsilon E^2 Ah \quad (A.22)
\]

Where \( Ah \) = capacitor volume \( m^3 \). Dividing by this volume, one obtains the energy density (assumed uniform) in the capacitor as;

\[
w = \frac{1}{2} \varepsilon E^2 \quad (A.23)
\]

**A.2 Fundamental Magnetostatics**

**A.2.1 Biot-Savart**

\[
\vec{B}(r) = \frac{\mu_0}{4\pi} \int \frac{\vec{T} \times \hat{z}}{r^2} \delta l' \quad (A.24)
\]

The Biot-Savart Law defines the magnetic field of a steady line current and is the starting point for magnetostatics. It plays an analogous role to Coulomb’s law in electrostatics, the \( \frac{1}{r^2} \) term, and the principle of superposition are common to both. The constant \( \mu_0 \) is the magnetostatic analogue of \( \varepsilon_0 \). It is known as the permeability of free space. These constants are linked, and defined by Equation A.25.
\[ \epsilon_0 = \frac{1}{\mu_0 c^2} = 8.854187817 \ldots 10^{-12} \text{F/m} \quad (A.25) \]

The choice of units for these constants gives \( \vec{B} \) units of Newtons per Ampere-Metre, or Tesla’s; providing consistency with the Lorentz equation.

### A.2.2 Lorentz

The Lorentz equation describes the force vector acting on a charged particle due to an applied electromagnetic field. Equation A.26 shows that the force acting on a volume with charge density \( \rho \) has a force acting on it due to the external electric field \( \vec{E} \) and the external magnetic field \( \vec{B} \).

\[
\vec{F} = \int_v \left( \overrightarrow{E} + \overrightarrow{v} \times \overrightarrow{B} \right) \rho \cdot \delta \tau = \int_v \left( \rho \overrightarrow{E} + \overrightarrow{J} \times \overrightarrow{B} \right) \delta \tau \quad (A.26)
\]

Integrating to give the force per unit volume gives Equation A.27, where \( \overrightarrow{J} \) is the volume current density.

\[
\overrightarrow{J} = \rho \overrightarrow{E} + \overrightarrow{J} \times \overrightarrow{B} \quad (A.27)
\]

The Lorentz equation is axiomatic to electrodynamic theory, thus there is no derivation from first principles. It was first developed to describe the magnetic repulsion or attraction of two wires carrying currents in either the opposite or the same direction respectively. It describes the circular magnetic field that arises around a current carrying conductor. The fact that a moving charge carrier produces a magnetic field led to the development of many of the most well known discoveries and theories in science, Maxwell’s equations, special relativity and radio communication, to name a few.

### A.2.3 Ampere

Amperes law, Equation A.28 describes the curl of the \( \vec{B} \) field.

\[
\nabla \times \overrightarrow{B} = \mu_0 \overrightarrow{J} \quad (A.28)
\]
Again, representing this in a more useful integral form one obtains Equation A.29.

\[
\int \left( \nabla \times \vec{B} \right) \cdot d\vec{a} = \oint \vec{B} \cdot d\vec{l} = \mu_0 \oint \vec{J} \cdot d\vec{a} = \mu_0 I_{enc} \tag{A.29}
\]

Ampere’s law is the magnetostatic equivalent of Gauss’s law in electrostatics and describes the total amount of current flowing through a closed surface.

### A.2.4 Magnetic Vector Potential

If, again, one draws parallels between electrostatics and magnetostatics, there should be an analogue of the electric potential. Indeed there is; the magnetic vector potential, though it is a vector not a scalar field,

Amperes law, Equation A.28 describes the curl of the \( \vec{B} \) field.

\[
\vec{B} = \nabla \times \vec{A} \tag{A.30}
\]

This brief introduction to the magnetic vector potential has been included for completeness, although it is not required in the derivations that follow.

### A.2.5 Magnetic Fields in Matter

In addition to the electric dipole moment, as described in Equation A.11, there exists a magnetic dipole moment, known as the magnetisation, \( \vec{M} \). This is the density of single dipoles \( \vec{m} \) per unit volume. The effect of magnetisation is to establish a magnetic field due to these bound currents. These loops of bound current give rise to a magnetic field inside the medium, called the \( \vec{H} \)-field. Written in terms of \( \vec{H} \), Ampere’s law becomes,

\[
\nabla \times \left( \frac{1}{\mu_0} \vec{B} - \vec{M} \right) = \nabla \times \vec{H} = \vec{J}_f + \frac{\delta \vec{B}}{\delta t} \tag{A.31}
\]

The \( \frac{\delta \vec{B}}{\delta t} \) term in Equation A.31 represents Maxwell’s correction to Ampere’s law for a magnetic field in a linear material. In a more convenient integral form this becomes,
The quantity $I_{enc}$ is the total free current passing through the 'Amperian loop'. The Amperian loop is a way of visualising the effective electric current loop around the edge of the material produced by the alignment of the magnetic dipoles. The $\vec{H}$-field can, therefore, be thought of as the total magnetic field produced by a combination of the field due to the free current and the field due to the magnetisation.

### A.3 Electrodynamics

#### A.3.1 Ohm

Using the equations derived in the previous sections one can now explore two fundamental laws of electrodynamics, Ohm’s law and Faraday’s law. If an electromagnetic force is employed to push charges around a circuit then, for the vast majority of materials, the current density $\vec{J}$ is proportional to the force per unit charge $\vec{f}$, and linked by the proportionality factor $\sigma$, known as the conductivity. The reciprocal of the conductivity is $\rho$, the resistivity; this is the more common unit. Provided that the charges are moving relatively slowly, Ohms law can be defined as in Equation A.33.

$$\oint \vec{J} \cdot d\vec{I} = \sigma \vec{E} \quad \text{(A.33)}$$

Consider a resistor of cross sectional area $A$, length $l$ and having a conductivity of $\sigma$. If an electric potential is established between the two ends, then the electric field is uniform within the wire. Equation A.33 tells us that the current density is also constant and therefore,

$$I = JA = \sigma EA = \frac{\sigma A}{L}V \quad \text{(A.34)}$$
Where $\frac{L}{\sigma A}$ is known as the resistance $R$. This leads to the ultimately more familiar version of Ohm’s law; $V = IR$. In contrast with the previous laws, Ohm’s law is only applicable under certain conditions and is therefore strictly not a fundamental law.

Due to the resistance, a certain amount of work has to be done to move charge from one end of the wire to the other. There are an associated number of collision events that occur as a charge carrier flows down the wire and this energy loss manifests itself as a dissipated power, known as Joule heating. This can be described as in Equation A.35,

$$P = VI = I^2R$$

With reference to the digital microfluidic system shown in Figure 4.3, this turns out to be of major practical significance. The resistance of the wires leading to the embedded electrode can lead to significant Joule heating, resulting, in the worst case scenario, in the device catching fire.

### A.3.2 Faraday

The system shown in Figure 4.3 is not normally subject to any applied external magnetic field, so, consideration of Faraday’s induction law seems to be unnecessary. It is, however, required for the derivation of the Maxwell Stress Tensor and as such it is very briefly introduced.

$$\nabla \times \mathbf{E} = -\frac{\delta \mathbf{B}}{\delta t}$$

Faraday recognised that a changing magnetic field induces an electric field. Therefore a conductor in the presence of a changing magnetic field will experience an electromotive-force acting on the free-charge within it. No further discussion of electromagnetic induction is required here, except to note the correction Maxwell made to Ampere’s law to describe how a changing electric field induces a magnetic
field. Maxwell introduced the concept of the displacement current, \( \vec{J}_d = \varepsilon_0 \frac{\delta \vec{E}}{\delta t} \) into Ampere’s law, although this will not be discussed here.

### A.4 Maxwell’s Equations

#### A.4.1 Electrostatic Equations

Maxwell’s equations as described below in Equations A.37, A.40, A.39 and A.41 together with the Lorentz Force, Equation A.27, summarise the entire theoretical content of classical electrodynamics. (Note that these equations must be accompanied by suitable boundary conditions, e.g. \( \vec{B} \) and \( \vec{E} \) go to zero at sufficiently large distances from a localised charge distribution).

\[
\nabla \cdot \vec{E} = \frac{1}{\varepsilon_0} \rho \quad (A.37)
\]

Equation A.37 is Gauss’s law. As before, it states that the divergence of the electrostatic field \( \nabla \cdot \vec{E} \) is equal to the reciprocal of the relative permittivity multiplied by the charge density. (One should note that the complex component of the relative permittivity is frequency dependant and this has implications for the operation of the device.) Gauss’s law allows one to calculate electric fields due to Coulumbic charge interactions. Written in terms of the electric displacement, assuming a linear medium Gauss’s law becomes,

\[
\nabla \cdot \vec{D} = \rho_f \quad (A.38)
\]

As previously stated Faraday’s Law describes how a changing magnetic field induces an electric field.

\[
\nabla \times \vec{E} = -\frac{\delta \vec{B}}{\delta t} \quad (A.39)
\]

#### A.4.2 Magnetostatic Equations

\[
\nabla \cdot \vec{B} = 0 \quad (A.40)
\]

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Equation A.40 is Maxwell’s second equation, and describes the divergence of a magnetostatic field $\nabla \times \vec{B}$ due to a steady current. As far as it is known there is no such thing as a point magnetic charge particle, so this equation applies to steady magnetic fields generated by a steady current. Analysis of the Biot-Savart Law, Equation A.24 reveals the divergence of the magnetic field to be zero. Ampere’s law with Maxwell’s correction for a *linear medium* is shown in Equation A.41.

$$\nabla \times \vec{H} = \vec{J}_f + \frac{\delta \vec{D}}{\delta t} \tag{A.41}$$

### A.5 Derivation of Maxwell’s Stress Tensor

Starting with the Lorentz equation, the force per unit volume is;

$$\vec{f} = \rho \vec{E} + \vec{J} \times \vec{B} \tag{A.42}$$

Gauss’s Law can be rearranged for $\rho$, giving,

$$\rho = \epsilon_0 \left( \nabla \cdot \vec{E} \right) \tag{A.43}$$

The Maxwell corrected Ampere’s Law can be rewritten in terms of $\vec{J}$ to give,

$$\vec{J} = \frac{1}{\mu_0} \left( \nabla \times \vec{B} \right) - \epsilon_0 \frac{\delta \vec{E}}{\delta t} \tag{A.44}$$

Substituting these into Equation A.42 gives,

$$\vec{f} = \epsilon_0 \left( \nabla \cdot \vec{E} \right) \vec{E} + \frac{1}{\mu_0} \left( \nabla \times \vec{B} \right) - \epsilon_0 \frac{\delta \vec{E}}{\delta t} \times \vec{B} \tag{A.45}$$

Using the fact that,

$$\frac{\delta}{\delta t} \left( \vec{E} \times \vec{B} \right) = \left( \frac{\delta \vec{E}}{\delta t} \times \vec{B} \right) + \left( \vec{E} \times \frac{\delta \vec{B}}{\delta t} \right) \tag{A.46}$$

and using Faraday’s Law (Equation A.39, we can write;
\[
\frac{\delta \vec{E}}{\delta t} \times \vec{B} = \frac{\delta}{\delta t} (\vec{E} \times \vec{B}) + \vec{E} \times (\vec{\nabla} \times \vec{E})
\]  
(A.47)

and thus the force per unit volume can be written as,

\[
\vec{f} = \epsilon_0 \left[ (\vec{\nabla} \cdot \vec{E}) \vec{E} - \vec{E} \times (\vec{\nabla} \times \vec{E}) \right] - \frac{1}{\mu_0} \left[ \vec{B} \times (\vec{\nabla} \times \vec{B}) \right] - \epsilon_0 \frac{\delta}{\delta t} (\vec{E} \times \vec{B})
\]  
(A.48)

Maxwell’s second equation states that,

\[
\vec{\nabla} \cdot \vec{B} = 0
\]  
(A.49)

so one can write,

\[
(\vec{\nabla} \cdot \vec{B}) \vec{B} = 0
\]  
(A.50)

This can then be expanded and simplified using a mathematical identity for \(\vec{\nabla} (X^2)\),

\[
\vec{\nabla} (B^2) = 2 (\vec{B} \cdot \vec{\nabla}) \vec{B} + 2 \vec{B} \times (\vec{\nabla} \times \vec{B})
\]  
(A.51)

Applying the product rule in Equation A.51 for \(\vec{B}\) yields an expression for the total electric and magnetic force per unit volume acting on the droplet.

\[
\vec{f} = \epsilon_0 \left[ (\vec{\nabla} \cdot \vec{E}) \vec{E} + (\vec{E} \cdot \vec{\nabla}) \vec{E} \right] - \frac{1}{\mu_0} \left[ (\vec{\nabla} \cdot \vec{B}) \vec{B} + (\vec{B} \cdot \vec{\nabla}) \vec{B} \right] - \frac{1}{2} \vec{\nabla} \left( \epsilon_0 E^2 + \frac{1}{\mu_0} B^2 \right) - \epsilon_0 \frac{\delta}{\delta t} (\vec{E} \times \vec{B})
\]  
(A.52)

The fourth term is known as the light radiation pressure, and contains the Poynting Vector where \(\vec{S} = \frac{1}{\mu_0 (E \times B)}\). The Poynting Vector describes the energy
per unit time, per unit area as transported by the electric and magnetic fields. It can be thought of as the energy flux density.

A.5.1 Integration and Simplification by Tensor Notation

Equation A.53 defines the force per unit volume, in order to simplify the mathematics we can introduce Tensor notation. If one defines the Maxwell Stress Tensor as,

\[ \vec{T} = \vec{T}_e + \vec{T}_m = \begin{bmatrix} T_{xx} & T_{xy} & T_{xz} \\ T_{yx} & T_{yy} & T_{yz} \\ T_{zx} & T_{zy} & T_{zz} \end{bmatrix} \]  \hspace{1cm} (A.53)

Where, if, the Maxwell Electric Stress Tensor is defined as,

\[ \vec{T}_e = \vec{E} \otimes \vec{B} - \frac{1}{2} (\vec{E} \cdot \vec{B}) \delta_{ij} \]  \hspace{1cm} (A.54)

The Maxwell Magnetic Stress Tensor is defined as,

\[ \vec{T}_m = \vec{H} \otimes \vec{B} - \frac{1}{2} (\vec{H} \cdot \vec{B}) \delta_{ij} \]  \hspace{1cm} (A.55)

where the Kroneker delta function is,

\[ \delta_{ij} = \begin{cases} T_{xx} = T_{yy} = T_{zz} = 1 \\ T_{yx} = T_{zx} = T_{xy} = T_{zy} = T_{xz} = T_{yz} = 0 \end{cases} \]  \hspace{1cm} (A.56)

One can now substitute for \( \vec{T}_e \) and \( \vec{T}_m \), collect terms, tidy up and re-express the equation in terms of the divergence of the two stress terms to give the combined electromagnetic force acting on a volume \( v \),

\[ \vec{f} = \nabla \cdot \left( \vec{T}_e + \vec{T}_m \right) = \rho \vec{E} + \vec{J} \times \vec{B} - \frac{1}{2} E^2 \nabla \epsilon - \frac{1}{2} H^2 \nabla \mu + \mu \epsilon \frac{d}{dt} \vec{S} \]  \hspace{1cm} (A.57)
Analysis of this equation shows that the first term is equal to the Lorentz force. The second and third terms in this equation describe the homogeneity of the material, and the fourth term is the light radiation pressure, which includes the Poynting Vector, $\vec{S}$. Replacing $\vec{T}_e + \vec{T}_m$ with $\vec{T}$ we can define the force per unit volume in tensor notation as,

$$\vec{f} = \nabla \cdot \vec{T} - \varepsilon_0 \mu_0 \frac{\delta \vec{S}}{\delta t}$$  \hspace{1cm} (A.58)

Thus the total combined force acting on the droplet shown in Figure 4.3, due to electromagnetic forces is the volume integral of the force per unit volume. This can be transferred to a surface integral by the proper application of Gauss’s Law, thus,

$$\vec{F} = \oint_s \vec{T} \cdot d\hat{a} - \varepsilon_0 \mu_0 \frac{\delta}{\delta t} \int_v \vec{S} \, d\tau$$  \hspace{1cm} (A.59)
Appendix B

Layout of Silicon DMF Arrays

Figures B.1 and B.2 show the physical layout of the brightfield mask that was used to pattern 3-inch silicon wafers. There is a slight difference between the design on the left and that on the right. Chip 1 has a continuous ground line array between each individual electrode, whereas chip 2 does not. This allowed evaluation of the efficiency of an embedded ground plane versus alternate driving strategies.

The pad-ring pinout of the coplanar W-shaped array (Figure B.1) is given in Table B.1. The devices labelled ‘fish’ are connected to a herring-bone test structure that was under evaluation, but never implemented in this body of work. Table B.2 shows the pad-ring connections corresponding to the electrodes shown in Figure 4.6, this table simply shows that each electrode in the pad-ring is directly connected to a corresponding electrode in the array. The top electrode is separately connected to ground.
Figure B.1: Layout of two single metal layer W-shaped arrays
Figure B.2: Layout of a multi metal layer open-DMF array
Table B.1: Pinout of W-Shaped array, G = ground, f=fishbone structure

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<thead>
<tr>
<th>Pad-Ring</th>
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Table B.2: Pinout of 8 by 5 array, Top electrode = ground.

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Appendix C

Selected Programs and LabView VI’s

C.1  Robot Run-List Generator and Control VI’s

C.2  Automated Magnetic Bead Purification Machine Code

This is a short extract of the machine code generated by the RunList Generator VI, which was used to run the StateMachine VI for part 3 of the magnetic bead purification protocol.

- //His Tag Purification
- 18 PA 0 0 0  //Arm 1 to Home Position
- 18 PA 248 66 1185  //Arm 1 to 96 Well Plate position A1
- 11 S11 A250 O R  //Syringe 1 aspirate 1ul of beads
- 18 PA 419 968 805  //Arm 1 to DMF Chip
- 11 S11 A0 O R  //Syringe 1 dispense 1ul of beads
- 18 PA 0 0 0  //Arm 1 to Home Position
- Magnet On  //Turn on Electromagnet
- 18 PA 48 445 850  //Arm 1 to Wash Position
- 11 S5 A2000 I R  //Syringe 1 Wash Routine
- 11 S5 A2000 O R  //Syringe 1 Wash Routine
Figure C.3: Add states to queue, inspired by JKI Statemachine (www.jkisoft.com)

Figure C.4: Parse state queue, inspired by JKI Statemachine (www.jkisoft.com)

Figure C.5: Checksum generation in LabView
• 11 S5 A0 O R //Syringe 1 Wash Routine
• 11 S5 A0 I R //Syringe 1 Wash Routine
• 11 S5 A2000 I R //Syringe 1 Wash Routine
• 11 S5 A2000 O R //Syringe 1 Wash Routine
• 11 S5 A0 O R //Syringe 1 Wash Routine
• 11 S5 A0 I R //Syringe 1 Wash Routine
• 11 S5 A2000 I R //Syringe 1 Wash Routine
• 11 S5 A2000 O R //Syringe 1 Wash Routine
• 11 S5 A0 O R //Syringe 1 Wash Routine
• 11 S5 A0 I R //Syringe 1 Wash Routine
• 18 PA 419 968 805 //Arm 1 to DMF Chip
• 11 S11 A250 O R //Syringe 1 remove supernatant
• 18 PA 48 445 850 //Arm 1 to Wash Position
• #Repeat wash cycle, removed in thesis for brevity
• 18 PA 328 66 1185 //Arm 1 to 96 Well Plate position A2
• 11 S11 A250 O R //Syringe 1 aspirate 1ul of 1x Binding/Washing Buffer
• 18 PA 419 968 805 //Arm 1 to DMF Chip
• 11 S11 A0 O R //Syringe 1 dispense 1ul of 1x Binding/Washing Buffer
• 18 PA 48 445 850 //Arm 1 to Wash Position
• #Repeat wash cycle, removed in thesis for brevity
• //End of MagBead Purification Protocol Part 3, Beads Dispensed and Washed.
Appendix D

Supplementary Photographs

This section provides photographs of instrumentation used in this work and are provided for identification and comparison with the schematics provided in Chapter 2 and 4.

Figure D.1: Bruker-Daltonics HCT Ultra ion trap
Figure D.2: Bruker-Daltonics 12T Apex Qe FT-ICR mass spectrometer

Figure D.3: Perseptive Biosystems Voyager DE STR

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Figure D.4: Manually operated switchbox for DMF device control

Figure D.5: LabView controlled I/O box for DMF device control
Figure D.6: Trek PZD700A high voltage amplifier; maximum voltage gain of 200 and output swing of plus or minus 700 V
Appendix E

Published Papers

E.1 Conference Papers and Presentations


3rd Annual RASOR Conference, Drymen; 24th - 25th November 2008. Poster: Development of Automated Protein Identification by nLC EWOD DESI FT-ICR ECD MS/MS


RASOR Lab-on-a-Chip Symposium, 5th March 2010: Delegate


5th EMSG Symposium, Ardgour 3-7th November 2008. Poster: Development of
Automated Protein Identification by nLC EWOD DESI FT-ICR ECD MS/MS.
Talk: Development and Integration of Digital Microfluidics with Mass Spectrometry.


56th ASMS Conference on Mass Spectrometry, Denver, CO, USA; 1st - 5th June 2008. Poster: Development of Automated Protein Identification by nLC EWOD DESI FT-ICR ECD MS/MS.


Edinburgh Chemical Biology PhD Meeting, Firbush; 13th - 15th April 2009. Poster: Development of Automated Protein Identification by nLC EWOD DESI FT-ICR ECD MS/MS.

Imperial College London Business School, Dragon’s Den, 16th March 2010: Presentation: Droplet Microfluidic Innovations.

CBC & RASOR Technology Showcase, Imperial College London, 29th June - 1st July 2010: Delegate

E.2 Journal Papers


Appendix F

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Online Quench-Flow Electrospray Ionization Fourier Transform Ion Cyclotron Resonance Mass Spectrometry for Elucidating Kinetic and Chemical Enzymatic Reaction Mechanisms

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SIRCAMS, School of Chemistry, University of Edinburgh, West Mains Road, Edinburgh, EH9 3JJ, U.K.

We have developed an automated quench-flow microreactor which interfaces directly to an electrospray ionization (ESI) mass spectrometer. We have used this device in conjunction with ESI Fourier transform ion cyclotron resonance mass spectrometry (FTICR MS) to demonstrate the potential of this approach for studying the mechanistic details of enzyme reactions. For the model system chosen to test this device, namely, the pre-steady-state hydrolysis of p-nitrophenyl acetate by the enzyme chymotrypsin, the kinetic parameters obtained are in good agreement with those in the literature. To our knowledge, this is the first reported use of online quench-flow coupled with FTICR MS. Furthermore, we have exploited the power of FTICR MS to interrogate the quenched covalently bound enzyme intermediate using top-down fragmentation. The accurate mass capabilities of FTICR MS permitted the nature of the intermediate to be assigned with high confidence.

Electron capture dissociation (ECD) fragmentation allowed us to locate the intermediate to a five amino acid section of the protein—which includes the known catalytic residue, Ser195. This experimental approach, which uniquely can provide both kinetic and chemical details of enzyme mechanisms, is a potentially powerful tool for studies of enzyme catalysis.

The introduction of electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) in the mid-1980s has made mass spectrometry (MS) an essential tool in many branches of protein chemistry. As a result, the use of MS as a detection technique in enzyme kinetic studies has become more widespread over the past few years. This technique was first employed in 1989 by Lee et al., who performed online continuous flow experiments using a triple-quadrupole mass spectrometer to directly monitor the activity of both lactase and α-chymotrypsin. Their experiments allowed the determination of Michaelis–Menten kinetic parameters (K_M and V_max) which were consistent with results obtained using conventional steady-state kinetic analyses. The use of MS over traditional spectroscopic detection techniques has several potential advantages. First, because virtually all enzymatic conversions (with the exception of epimerases and racemases) result in a change in mass, natural substrates can be used in monitoring enzyme-catalyzed reactions. This obviates the requirement for chromophoric or radiolabeled substrates. Bothner et al. have highlighted this advantage by analyzing several enzyme-catalyzed hydrolysis reactions, demonstrating significant differences in the kinetics between natural and chromophore-labeled substrates. Second, this experimental approach takes advantage of the high sensitivity and selectivity inherent in modern mass spectrometers, when compared to spectrophotometric detectors. Additionally, MS detection affords the user the ability to observe multiple species simultaneously. These advantages allow the kinetic analysis of enzymes which would be impossible to study using spectrophotometric detectors. For example, Leary and co-workers have successfully used MS to characterize the kinetics of enzymatic sulfate and phosphate group transfer.

The interfacing of a rapid mixing system, such as a stopped-or quench-flow apparatus, with a mass spectrometer was first proposed in 1997. Theoretically, if mixing systems operate at sufficiently high temporal resolution (typically milliseconds for enzyme reactions), this approach allows the direct detection of the relative concentrations of not only reactants and products but also transient intermediates. Therefore, this combination promises the exciting possibilities of determining both kinetic mechanisms (and the accompanying kinetic parameters) as well as chemical reaction pathways.

To date time-resolved mass spectroscopy has been achieved using a variety of instrumentation, including electron impact...
MS$^{12,13}$ and MALDI-MS.$^{14}$ However, rapid mixing systems are best suited in combination with ESI-MS, an approach which allows a direct “online” link between the solution phase reaction mixture and the ESI-MS inlet. This soft-ionization technique has been widely used to monitor analytes ranging from small molecules to peptides and large proteins, and it has also been successfully utilized to monitor noncovalent enzyme–substrate interactions.$^{15,16}$ Two early reports using ESI quench-flow MS detail the observation of transient intermediates bound to the iron binding glycopeptide bleomycin.$^{16,17}$ These species displayed half-lives on the order of several seconds. More recently, the temporal resolution of rapid mixing MS has been improved to some tens of milliseconds through the use of miniaturized mixers, capillaries, and ESI sources.$^{15,18–20}$

The first study in which time-resolved ESI-MS was used to determine the pre-steady-state kinetic parameters of an enzymatic reaction was published in 1998.$^{21}$ The authors monitored the appearance of a transient covalent enzyme intermediate within the active site of a mutant xylanase enzyme from *Bacillus circulans.* Data was collected using a basic mixing system attached directly to an ESI source, and the resulting kinetic parameters were in excellent agreement with those obtained using stopped-flow UV–vis spectroscopy. More recently, this research group has developed an elegant custom-built capillary mixer attachment which featured an adjustable reaction chamber volume.$^{22}$ This device allowed measurements with low-millisecond resolution and has successfully been used in conjunction with a triple-quadrupole MS to study the steady-state and pre-steady-state kinetics of chymotrypsin.$^{23}$

An alternative approach using an “off-line” rapid quench step is generally more time-consuming than “online” systems. However, it is technically simpler and allows greater flexibility in the workup of each quenched reaction. For example, high-performance liquid chromatography (HPLC) separation, desalting, or chemical digest can be employed prior to MS analysis. Furthermore, an “off-line” workflow does not limit the choice of MS instrumentation to ESI, and it has been effectively used in conjunction with both MALDI and desorption/ionization on silicon (DIOS) desorption/ionization instruments for analysis of pre-steady-state enzyme kinetics.$^{14,24–29}$ Kelleher and co-workers have successfully used “off-line” rapid-quench technology in conjunction with high-resolution Fourier transform MS (FTMS) in order to characterize the mechanism of a nonribosomal peptide synthetase (NRPS).$^{27,28}$ Several transient enzyme-bound intermediates in the enzymatic production of yersiniabactin were kinetically resolved “off-line” using a commercial rapid-quench instrument. These samples were then processed by chemical cleavage and HPLC before analysis on an ESI-Q-FTMS instrument. The percentage occupancies of the multiple active sites were calculated at various time points, allowing the authors to deduce the rate of accumulation of specific intermediates and propose a general kinetic model for NRPS systems.

The high resolving power of FTMS is ideally suited to the detection and interrogation of covalently bound enzyme intermediates. Mass resolution of 100 000–500 000 is achievable using these instruments, allowing the detection of a 1 Da mass shift ($\Delta m$) at ~30 kDa with high confidence. Moreover, the inherent high sensitivity permits detection of low-abundance intermediates.$^{29}$ In addition, top-down tandem MS fragmentation methodologies, available using FTMS, enable precise localization of these modifications on the polypeptide chain.$^{30–33}$ We have previously demonstrated the utility of FTMS in interrogating the nature and location of intermediates in the catalytic cycle of cysteine-dependent peroxiredoxins.$^{34}$

In the work presented here we show that FTMS can be utilized for pre-steady-state analysis of enzyme mechanisms. We have built an automated “online” rapid-quench microreactor which operates at low-millisecond temporal resolution. This device is interfaced directly with a commercial ESI Fourier transform ion cyclotron resonance (ESI-FTICR) MS instrument (Bruker Daltonics). Analogous to Wilson and Konermann, we have used the chymotrypsin-catalyzed hydrolysis of $\pi$-nitrophenyl acetate ($\pi$-NPA) as a model system.$^{35}$ Monitoring this reaction by FTICR MS, we were able to directly observe the appearance of the enzyme-bound acyl-intermediate and deduce pre-steady-state rate constants which are in agreement with previous MS- and optical-based studies. Furthermore, we have exploited the ultra-high resolving power and mass accuracy of FTICR, to analyze the quenched enzyme-bound acyl-intermediate by top-down fragmentation. Using electron capture dissociation we demonstrate that it is possible to locate the quenched transient acyl modification at position Ser195.

Our results highlight the advantages of using an “online” ESI quench-flow FTMS system, which can provide valuable insight into both kinetic and chemical mechanisms of enzymatic reactions.

EXPERIMENTAL SECTION

Materials. Chymotrypsin and p-NPA were purchased from Sigma Chemical Co. (St. Louis, MO). Methanol, water, and formic acid were purchased from Fischer Chemicals (Zurich, Switzerland) and were LC–MS or mass spectrometry grade. Prior to use, chymotrypsin was applied to a PD-Miditrap desalting column (GE Healthcare) and eluted with water.

Apparatus. MilliGAT pumps (Global FIA, Fox Island, WA) were used to deliver solutions with high precision. Nanomixers, fused-silica tubing, Teflon tubing, sleeves, and connectors were purchased from Upchurch Scientific (Oak Harbor, WA). Nanomixers were run using their low back pressure flow path purchased from Upchurch Scientific (Oak Harbor, WA). Nano-fused-silica tubing, Teflon tubing, sleeves, and connectors were used to deliver solutions with high precision. Nanomixers, fused-silica tubing, Teflon tubing, sleeves, and connectors were purchased from Upchurch Scientific (Oak Harbor, WA). Nano-fused-silica tubing was used.36 Using DataAnalysis software (Bruker Daltonics) each spectrum was background-subtracted. In order to account for the linear relationship between FTMS detector response and ion charge, these calculated areas were divided by their respective charge states. The resulting charge-normalized areas were combined to give the total area for both the apo and ES′ forms of the enzyme, and the relative ratios of these areas were used for quantitation. The results for each time point were an average of the processed data from the three acquisitions recorded. This lengthy data handling procedure was automated using specifically written software, produced in-house using the Labview visual programming platform (National Instruments; Austin, TX). Briefly, all three milliGAT pumps were controlled from the software via MicroLyx 4 microcontrollers/stepper drivers (Intelligent Motion Systems; Marlborough, CT) and FTICR MS data acquisition was controlled using a contact closure signal to trigger HyStar 3.4 software (Bruker Daltonics, Billerica, MA). A sample table in the controlling software allowed a series of reaction times to be specified, and MS data for each reaction time was collected in an automated fashion. Reactions times were achieved by controlling the flow rate of pumps A and B. After a specified equilibration time delay a trigger was sent to start MS acquisition.

ESI-MS Kinetic Data Collection and Processing. MS acquisitions consisted of 50 scans and were recorded in triplicate for each kinetic time point. This workflow resulted in an “experiment time” (the time taken to collect MS data for a single reaction time point) of approximately 5 min. In order to calculate the ratio of apochymotrypsin to acetylated chymotrypsin (ES) in each spectra, a variation of the “protein ion relative ratio” (PIRR) system was used.36 Using DataAnalysis software (Bruker Daltonics) each spectrum was background-subtracted. In order to account for all peaks within an isotope cluster, the data was then smoothed using a Gauss algorithm and a window of 0.2 m/z. Within this smoothed spectrum the areas under all 12 charge states, [M + 12H]\(^{2+}\) to [M + 24H]\(^{3+}\), were calculated for each species using DataAnalysis software. In order to account for the linear relationship between FTMS detector response and ion charge, these calculated areas were divided by their respective charge states. The resulting charge-normalized areas were combined to give the total area for both the apo and ES′ forms of the enzyme, and the relative ratios of these areas were used for quantitation. The results for each time point were an average of the processed data from the three acquisitions recorded. This lengthy data handling procedure was automated using specifically written software, produced in-house using the Labview visual programming platform (National Instruments; Austin, TX).

FTICR Mass Spectrometry. Mass spectrometry data was acquired on an Apex Ultra Qh-FTICR mass spectrometer equipped with a 12 T superconducting magnet and an electrospray ion source (Bruker Daltonics). Data acquisition was under the control...
of HyStar 3.4 software (Bruker Daltonics). Desolvated ions were transmitted to a 6 cm Infinity cell penning trap. Trapped ions were excited (frequency chirp 48–500 kHz at 100 steps of 25 μs) and detected between m/z 600 and 3000 for 0.5 s to yield a broadband 512K time-domain transient. Each spectrum was the sum of 50 mass analyses. The mass spectra were externally calibrated using ES tuning mix (Agilent) and analyzed using DataAnalysis software (Bruker Daltonics).

**Isotopic Fitting.** Isotope distributions of specific charge states were predicted using IsotopePattern software (Bruker Daltonics) from theoretical empirical formulas. These were overlaid upon the recorded experimental data as scatter plots, with the theoretical apex of each isotope peak designated by a circle.

**Top-Down Fragmentation.** Top-down fragmentation was performed on the I2T Qh-FTICR. First, a specific ion species was isolated using the mass-resolving quadrupole, and MS/MS was performed using collision-induced dissociation (CID) or electron capture dissociation (ECD). For CID, the collision voltage was typically set between 20 and 35 V. For ECD, 1.8 A was applied to the dispenser cathode filament (Heatwave Technologies), 20 V to the lens, 0.8 V to the bias, and a pulse of between 5 and 14 ms was employed. Fragmentation data was the sum of 250–750 acquisitions, and data analyses were performed using DataAnalysis (Bruker Daltonics). The SNAP 2.0 algorithm was used for automated peak picking; monoisotopic mass-to-charge ratios calculated using this process are highlighted in the text preceded by a prime symbol ('). The resulting top-down fragment mass lists were searched against the primary sequence of each polypeptide chain in mature chymotrypsin using BioTools 3.0 (Bruker Daltonics) and Prosight-PTM software packages. Mass error tolerances were set for all searches at 10 ppm.

**DATA ANALYSIS**

Traditional rapid mixing continuous flow systems are operated under turbulent flow conditions, with constant mixing between fast and slow regions within the apparatus. However, turbulent flow cannot normally be achieved for miniaturized online ESI-MS experiments, and these systems operate in the laminar flow regime. The resulting parabolic velocity profiles associated with laminar flow act to distort the measured kinetics and were thought to prevent accurate data collection. However, recent computer simulations and experimental studies by Konermann and co-workers have shown that this “blurring” is reduced by the counteracting effects of molecular diffusion. This research group has developed a theoretical framework for performing kinetic calculations which takes into account laminar flow effects, and they have clearly demonstrated the feasibility of performing kinetic studies in this flow regime. Indeed, they successfully monitor the pre-steady-state kinetic of chymotrypsin under laminar flow conditions. To validate our quench-flow FTMS system, as this reaction has been extensively studied, using both MS- and optical-based detection techniques. The flow rates of pumps A and B were systematically altered to produce quenched reactions at various time points, and for each time point three ESI mass spectra were recorded (see the Experimental Section). The pump flow rates and MS acquisitions were controlled via custom software, which allowed kinetic data to be collected in a semiautomated manner. Using a fixed concentration of p-NPA, spectra for 15 different reaction time points could be collected in approximately 15 min. It is worth noting that the speed of data acquisition is governed by the scan speed of the mass spectrometer. The 500 ms acquisition speed utilized in FTICR MS is relatively slow, and by using a faster scanning instrument quench-flow data acquisition time could be dramatically decreased. Typical spectra, recorded using our quench-flow FTMS setup, are shown in Figure 2. Figure 2A shows the charge state distribution in a typical spectrum; species corresponding to the [M + 12H]2+ to [M + 24H]24+ charge states of chymotrypsin were observed, and the spectrum is consistent with that of a denatured protein. Analysis of the isotopic distribution of the [M + 20H]20+ charge state revealed that the mass of chymotrypsin used in this study was consistent with the δ-form of the enzyme (Figure 2, parts B and C top, theoretical empirical formula [C371H626N39O95S12]20+; red circles). The change in the abundance of the [M + 20H]20+ charge state of chymotrypsin observed after reacting the enzyme with 2.5

**RESULTS AND DISCUSSION**

**Pre-Steady-State Kinetic Analysis of p-NPA Hydrolysis by Chymotrypsin.** The chymotrypsin-catalyzed hydrolysis of p-NPA was chosen to validate our quench-flow FTMS system, as this reaction has been extensively studied, using both MS- and optical-based detection techniques. The flow rates of pumps A and B were systematically altered to produce quenched reactions at various time points, and for each time point three ESI mass spectra were recorded (see the Experimental Section). The pump flow rates and MS acquisitions were controlled via custom software, which allowed kinetic data to be collected in a semiautomated manner. Using a fixed concentration of p-NPA, spectra for 15 different reaction time points could be collected in approximately 15 min. It is worth noting that the speed of data acquisition is governed by the scan speed of the mass spectrometer. The 500 ms acquisition speed utilized in FTICR MS is relatively slow, and by using a faster scanning instrument quench-flow data acquisition time could be dramatically decreased. Typical spectra, recorded using our quench-flow FTMS setup, are shown in Figure 2. Figure 2A shows the charge state distribution in a typical spectrum; species corresponding to the [M + 12H]2+ to [M + 24H]24+ charge states of chymotrypsin were observed, and the spectrum is consistent with that of a denatured protein. Analysis of the isotopic distribution of the [M + 20H]20+ charge state revealed that the mass of chymotrypsin used in this study was consistent with the δ-form of the enzyme (Figure 2, parts B and C top, theoretical empirical formula [C371H626N39O95S12]20+; red circles). The change in the abundance of the [M + 20H]20+ charge state of chymotrypsin observed after reacting the enzyme with 2.5

**Pre-steady-state kinetic theory states that**

\[ k_{\text{obs}} = k_3 + k_2[S]/(K_d + [S]) \]  

where [S] is the concentration of substrate. Therefore, measurements of \( k_{\text{obs}} \) as a function of [S] allow the determination of \( K_d \), \( k_2 \), and \( k_3 \).

**Scheme 1**

\[
\begin{align*}
E + S & \rightarrow E-S \\
E-S & \rightarrow ES^+ \rightarrow E + P
\end{align*}
\]


mM p-NPA for 600 and 2500 ms is shown in Figure 2C. Over time it is clear that a second species, with a ∆mass of +42 Da, accumulates in the spectrum. This species has an isotope distribution consistent with δ′-chymotrypsin with the addition of a single acetyl group (Figure 2C bottom, theoretical empirical formula [C_{1120}H_{1782}N_{302}O_{353}S_{12}]^{20+}; blue circles), this is assigned as the pre-steady-state accumulation of the ES′ covalent intermediate. After 600 ms both the unmodified (apo) form of the enzyme and the covalent complex (ES′) are present, and by 2500 ms the ES′ complex has become the dominant species in the spectrum.

In order to convert the spectra recorded at each reaction time point into quantitative data suitable for kinetic analysis, a system based upon PIRR was used (see the Experimental Section). This analysis assumes that the ionization efficiency of the apo and ES′ forms of chymotrypsin are equal. For quantitative analysis of modified proteins (>10 kDa), previous reports have demonstrated that such an assumption is precise to ∼5%.42,43 Indeed, preliminary experiments demonstrated that the ionization efficiencies of apo- and ES′-chymotrypsin are similar to ∼4% (data not shown). In order to take into account this uncertainty during the calculation of the kinetic parameters, an intrinsic error of 5% was included with each value of $k_{obs}$.

Figure 3A shows the pre-steady-state buildup of the ES′ intermediate species over time for four different concentrations of p-NPA. The data was fitted to eq 3

$$[ES'](t) = C(1 - \exp(-k_{obs}t))$$  (3)

which provided an observed rate constant, $k_{obs}$, for each concentration of p-NPA. In total, kinetic data was collected for 10 different concentrations of the substrate. These $k_{obs}$ values were subsequently plotted as a function of substrate concentration and, on the basis of eq 2, this data allowed the determination of the kinetic constant of $K_d$, $k_2$, and $k_3$ (see Figure 3B). The values obtained for $K_d$, $k_2$, and $k_3$ are 1.6 ± 0.3 mM, 2.8 ± 0.2 s⁻¹, and 0.0 ± 0.2 s⁻¹, respectively. It is clear that the value of $k_3$ is too small to accurately determine using this approach, as found in the MS-based kinetic study performed by Wilson and Konermann.23 This is not wholly surprising, as $k_3$ must be much smaller than $k_2$ for pre-steady-state buildup of ES′. Thus, $k_3$ is the rate-determining step and can be approximated as $k_{cat}$, which can be calculated by kinetic analysis in the steady-state regime. The values of $k_2$ and $K_d$ reported here are in good

---

agreement with the values previously reported for $\delta'$-chymotrypsin by Wilson and Konermann of 1.7 ± 0.2 mM and 3.7 ± 0.3 s⁻¹ using an MS-based approach, and 1.6 ± 0.1 mM and 3.6 ± 0.2 s⁻¹ using optical detection.²³

The above results clearly demonstrate the feasibility of using this automated quench-flow FTMS approach for pre-steady-state enzyme kinetic analysis and add credence to the viability of previously published MS-based kinetic studies.¹⁸,²⁰,²¹,²³ To our knowledge, this work represents the first reported use of online quench-flow coupled with FTICR MS.

**Analysis of the ES' Complex by Top-Down Tandem Mass Spectrometry.** Interfacing a quench-flow microreactor with FTICR MS allows direct interrogation of covalently bound enzyme intermediates by a variety of top-down fragmentation techniques. Top-down fragmentation involves the intact mass measurement of a protein and dissociation of the intact protein to produce tandem mass spectrometry data. This can be used to determine the primary structure of the molecule and locate modified amino acids. The importance of high-resolution data sets and high mass accuracy for successful top-down analysis is well-documented, and FTICR mass analysers are unsurpassed in these respects.⁴⁴,⁴⁵

Successful, top-down fragmentation of the ES' complex would allow the nature of the quenched intermediate and the position of the catalytic residue in the polypeptide chain to be deduced, thus providing valuable insight into the chemical mechanism of enzymatic catalysis.

The “online” quench-flow MS apparatus used in this work prohibited derivatization of the quenched ES' complex before MS analysis. Therefore, reduction and alkylation of the quenched intermediate was not possible prior to MS analysis, and top-down fragmentation had to be conducted on the fully oxidized $\delta'$-chymotrypsin molecule. Traditionally, the presence of disulfide bonds is seen as a hindrance to tandem MS as the presence of intramolecular disulfide bridges can limit protein fragmentation and reduce the available sequence information.⁴⁶,⁴⁷ Indeed, our initial top-down experiments, using both CID and infrared multiphoton dissociation (IRMPD), resulted in no detectable cleavage of either the inter- or intramolecular disulfide linkages within chymotrypsin (data not shown). Consequently, after data analysis, the assigned fragments from these tandem MS experiments were limited to $b$-ions derived from the N-terminus of the B and C chains (data not shown). Not a single cleavage could be identified from within the region of the C chain containing the catalytic serine (Ser₁₉₃), which is situated within a loop formed by an intramolecular disulfide bond between Cys₂₀₁ and Cys₁₹₁ (see Figure 2B).

Since its introduction in 1998,⁴⁸ ECD has been recognized as a powerful technique for top-down tandem MS.⁴⁹,⁵⁰ ECD has garnered significant interest due to its tendency to retain post-translational and chemical modifications, resulting in more extensive sequence coverage when compared to other fragmentation techniques.⁵¹,⁵² Furthermore, with the development of activated ion (AI) ECD, the utility of this fragmentation technique has been extended to larger proteins, and top-down analysis of proteins up to 45 kDa has been reported.⁵³,⁵⁴ One other unique feature of ECD is that it can efficiently cleave disulfide bonds, presumably due to the S=S bond’s high hydrogen atom affinity.⁵⁵ Various groups have reported that ECD (or AI ECD) can be used to obtain sequence information of disulfide-rich peptides, for example, the...
It is interesting to note that, to obtain sequence coverage from a region of protein sequence within a disulfide bond, two cleavages must take place—cleavage of the disulfide bond and amide backbone cleavage.

The ES' complex of chymotrypsin and 3 mM ρ-NPA was generated by using the online rapid-quench microreactor attached to the 12 T FTICR MS, with the microreactor set to produce a quench reaction time of $t = 2000$ ms. At this reaction time the ES' complex was the dominant species in the mass spectrum and displayed an isotope distribution consistent with the $\delta^1$-isomer of ρ-NPA + $\text{C}_3\text{H}_6\text{O}$. Five charge states ($[\text{M} + 17\text{H}]^{17+}$ to $[\text{M} + 21\text{H}]^{21+}$) of the ES' species were systematically isolated and subjected to fragmentation using ECD. A typical ECD spectrum is shown in Figure 4A. Application of a collision voltage of between 5 and 10 V to the collision cell prior to ECD was found to produce more extensive fragmentation, presumably through an ion-activated process. All ES' charge states dissociated by ECD resulted in two dominant fragment ions. The first, a singly charged species, with monoisotopic $m/z$ 1253.6855 (Figure 4A, green diamond) was consistent with the A chain of chymotrypsin, formed by cleavage of the disulfide linkage between Cys1 and Cys122 and with addition of an H atom to the fragment (predicted isotope distribution $[\text{C}_{456}\text{H}_{718}\text{N}_{126}\text{O}_{141}\text{S}_7]^{6+}$; green circles). The second species displayed charge states of $[\text{M} + 4\text{H}]^{4+}$, $[\text{M} + 5\text{H}]^{5+}$, and $[\text{M} + 6\text{H}]^{6+}$ (Figure 4A, red diamonds), the $[\text{M} + 6\text{H}]^{6+}$ species displaying monoisotopic $m/z$ 1720.0040. Isotope modeling of this species was consistent with the acylated C chain of chymotrypsin formed by cleavage of the intermolecular disulfide between Cys136 and Cys201, and suggests that the species retained the two C chain intramolecular disulfide bridges, and occurs with addition of an H atom to the fragment (predicated isotope distribution $[\text{C}_{496}\text{H}_{718}\text{N}_{140}\text{O}_{141}\text{S}_6]^{5+}$; red circles). ECD-induced disulfide bond cleavage is thought to proceed via capture of a hydrogen atom, producing an even-electron reduced cysteine (Cys–SH) and an odd-electron cysteine radical (Cys–$\delta^1$S). It is interesting to note that the two major fragments observed in the ECD of chymotrypsin occurred with retention of the neutralized H atom, a phenomenon also observed by Zubarev et al.55

In addition to these two dominant species, extensive further fragmentation was observed for all the charge states studied (see Figure 4A, highlighted in blue; Figure 4B). Fragment mass lists were assembled used DataAnalysis (Bruker Daltonics), and these were searched using BioTools 3.0 (Bruker Daltonics) and Prosight-PTM software packages. After disulfide bond cleavage by ECD, one of the two cysteine residues increases in mass by 1 Da (the consequence of H atom capture). In order to take this into consideration during data analysis, the redox states of all 10 cysteine residues were designated as oxidized, and variable modifications of an additional H atom were specified for each. In total 177 fragments were assigned—5 derived from the A chain, 138 derived from the B Chain, and 34 derived from the C chain (see Figure 4C). Interestingly, many of these fragments are the result of two bond cleavages (one disulfide and one backbone amide cleavage) and, in some cases, three bond cleavages (two disulfide cleavages and a backbone amide cleavage).

tion of the C chain includes a series of both $\epsilon$ and $\zeta$ ions from the region between Cys191 and Cys201. Fragments containing the acetylated group allow the catalytic amino acid to be pinpointed to a five amino acid containing region (192MGDSG196), and the unacylated $\epsilon_{10}$ ion suggests that the modification is limited to the dipeptide (195SG196).

**CONCLUSION**

We have developed a semiautomated quench-flow microreactor which is compatible with ESI mass spectrometry for kinetics measurements. Reaction time is governed by flow rate through a fixed volume capillary, and software written in-house is used to control the device and for synchronized MS data acquisition. We have interfaced the microreactor with a 12T FTICR MS in order to monitor the accumulation of a transient covalent acyl enzyme intermediate over time and have demonstrated that this system can produce accurate pre-steady-state kinetic data.

By using the FTMS instrument as the detector, we were able to utilize the top-down fragmentation capabilities of the instrument to interrogate the quenched enzyme intermediate. Using ECD we demonstrated the power of this approach by locating the transient modification to a five amino acid section within the 250 amino acid enzyme. It is easy to envisage how the top-down experimental approach used here could be employed to locate key residues involved in the catalytic cycles of a variety of enzymes.

This study highlights the power of using FTICR in enzyme analysis. The approach described above can yield valuable information into both the kinetic mechanism of enzyme mechanisms, providing kinetic parameters using unlabeled substrates, as well as significant insight into the chemical mechanism of enzyme catalysis, by the identification of the nature and location of enzyme-bound intermediates.

**ACKNOWLEDGMENT**

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Short communication

Top-down protein sequencing by CID and ECD using desorption electrospray ionisation (DESI) and high-field FTICR mass spectrometry

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Desorption electrospray ionisation (DESI) is one of the most exciting developments in mass spectrometry in recent years. It allows the ionisation of solid phase samples deposited on almost any surface and at ambient temperature and pressure. DESI was invented in the labs of Professor R. Cooks at Purdue University [1] with subsequent development and improvement by a number of groups around the world, including our own. Many groups have investigated the use of DESI for analysis of small inorganic molecules [2] and although some research has been conducted into analysis of biomolecules this has, for the most part, been confined to lipids and peptides [3–5]. The possibility of analysing biomolecules ranging in molecular weight from hundreds to tens of thousands of Daltons and at biologically important temperatures and pressures means that DESI is potentially a very important and powerful technique for analysing proteins. This analysis could be simple measurement of the molecular mass or by using MS/MS techniques, elucidation of the amino acid sequence and identification of sites of post-translational modifications. Recent publications using ambient pressure ionisation techniques include; DART [6], ASAP [7], MALDESI [8–10], LAESI [11] and IR-LADESI [12] and each of these techniques allows ionisation at ambient pressure with little or no sample preparation.

As described in this paper, we have designed, built and tested a versatile DESI source for top-down protein analysis using a 12 T Bruker Daltonics Apex Qe FTICR mass spectrometer. This has enabled direct analysis of intact proteins to be performed with minimal sample preparation. In a previous study, Takats et al. [13] reported a top-down DESI FTICR analysis of a synthetic polypeptide containing 36 residues. Our work presented here on myoglobin builds on the work by Basile and co-workers [14] and demonstrates that it is possible to produce a DESI signal from an intact protein with sufficient magnitude over an extended period of time (~10 min) such that top-down sequencing by DESI FTICR can be performed on proteins with a molecular weight of at least 12 kDa. We report single acquisition DESI FTICR mass spectra for both myoglobin and cytochrome-c that correspond to picomole sample consumption. In addition, for cytochrome-c we present top-down DESI FTICR mass spectra obtained using both collision induced dissociation (CID) and electron capture dissociation (ECD).

1. Experimental

1.1. Reagents

The materials used in this work, protein standards (which were used as model samples) and consumables required for sample preparation, such as solvents and acids, are listed below.

Apo-myoglobin and cytochrome-c (Cat# 10K7026 and 065K7001) were purchased from Sigma–Aldrich (St. Louis, MI, USA), formic acid (Cat# 94318) was purchased from Fluka. LC-MS grade methanol, water and acetonitrile (Cat# M/4062/17, W/0112/15 and A/0638/17) were purchased from Fisher Scientific.
1.2. Mass spectrometry

In order to record DESI FTICR mass spectra of the intact proteins, myoglobin and cytochrome-c were dissolved at a concentration of 1 mg/mL in H$_2$O:MeOH (1:1). 50 µL of these solutions were applied to the etched side of a glass microscope slide, which was used as the DESI target plate. The slide was then incubated at room temperature to evaporate the solvent.

The tandem mass spectra presented for cytochrome-c required an increased sample concentration. In this case the protein (2 mg/mL) was dissolved in H$_2$O:MeOH (1:1) and 200 µL of the solution was applied to target plate and the solvent allowed to evaporate.

The etched glass microscope slides were mounted on the XY stage of the DESI source using a spring loaded clamp. Further details of the DESI source are given below. The critical geometrical DESI parameters, as defined by Takats et al. [15] were determined empirically, each being co-varied until the maximum signal was obtained. A solution of methanol, water and formic acid (1:1:0.001) was used as the spray solution, 3.5 kV was applied to the spray needle and approximately 2.5 L/min N$_2$ was used as the nebulising gas.

The geometrical source parameters used to obtain tandem mass spectra were identical to those used to record spectra for the intact proteins. The mass resolving quadrupole was set to transmit only those ions that corresponded to the [M+15H]$_{15}^+$ charge state of cytochrome-c (m/z 824), and the sample target plate was rastered beneath the DESI probe. In order to acquire the ECD spectrum, the electron source (Heatwave Technologies) in the FTICR cell was conditioned by applying 1.8 A overnight to the cathode filament. To record the spectrum the ECD lens was set to 30 V, and a pulse length of 6 ms and ECD bias of 1.2 V was employed. For the CID spectrum the collision voltage was set between 20 and 35 V.

Fragmentation spectra were acquired at a FID size of 512 kword and were the sum of 200 acquisitions. Data analysis was performed using Bruker Daltonics DataAnalysis 3.4 software. The SNAP 2.0 algorithm was used to generate fragment mass lists and the resultant top-down fragment mass lists were searched against Prosight PTM-2.0 [16].

2. Results and discussion

2.1. DESI source design and optimisation

Autodesk Inventor (Autodesk Inc., CA) was used to design a versatile DESI source to interface with the Bruker Daltonics Apex Qe 12 T FTICR mass spectrometer. Modelling the source design permitted rapid design iteration and fault finding. Fig. 1 shows the final design fitted to the mass spectrometer.

The stepper controlled XY stage was sourced from a Bruker Daltonics Ultraflex MALDI instrument and implemented as a structural component of the DESI frame. Software control of the XY sample stage was achieved using Enhanced Machine Controller 2 to generate step and direction inputs to the motor control driver boards.

As previously mentioned DESI has critical geometrical parameters which greatly influence the ionisation efficiency. To provide a high degree of reproducibility a solid frame was used to hold both the ESI sprayer and sample stage. The spray needle was mounted in an insulated XYZ Θ micromanipulator equipped with vernier scales for accurate measurement of angles and distances. To allow the XY sample stage to raster below the mass spectrometer source inlet a hybrid glass-brass capillary with a stainless steel extension was employed. This consisted of a conventional glass-capillary modified to include a solid brass end-cap and a 1/16 in. outer

Fig. 1. DESI source mounted on Bruker Daltonics 12 T Apex Qe FTICR mass spectrometer. Insert shows a close-up view of the DESI spray needle, target plate and capillary inlet extension.

Fig. 2. Single scan DESI FTICR mass spectra for (A) myoglobin and (B) cytochrome-c from an etched glass substrates. Inserts show an expanded view of the most abundant charge states.
diameter—0.020 in. inner diameter stainless insert (Upchurch). The stainless insert was recessed 3 mm into the glass and extended 80 mm out through the brass end-cap.

The critical geometrical DESI source parameters, as described by Cooks et al. [1] were co-varied empirically until the largest ion-abundance was found. Nebulising gas pressure, electrospray voltage and solvent flow rate were kept constant at 3.5 kV, 100 psi and 3 μL/min respectively. The insert in Fig. 1 shows a close-up view of the DESI source, with the correct positioning of the critical geometrical parameters for the incident and collection angles, and distances. Interestingly the ion-abundance increased with the distance from the spray-tip to the mass spectrometer inlet, this may be due to the increase in time for desolvation of the nebulised droplets.

2.2. Intact protein analysis using DESI FTICR-MS

Samples were prepared and the DESI source and instrument parameters were set up as described above.

Fig. 2 shows the intact mass spectra obtained for myoglobin (16.9 kDa) and cytochrome-c (12.4 kDa) under mild denaturing conditions. These spectra correspond to a single-acquisition. The insert mass spectra show an expansion of the most abundant charge states, [M+14H]14+ and [M+13H]13+ for myoglobin and cytochrome-c respectively. These spectra have a FWHM resolution of 57,000 at 611 m/z (cytochrome-c) and 148,000 at 774 m/z (myoglobin), thus demonstrating the intrinsically high resolving power that can be achieved by DESI FTICR mass spectrometry.

Knowing the concentration and volume of sample deposited on the DESI target plates, together with the area over which the sample wets (ca. 500 mm2) and the DESI spot size (ca. 1 mm2) we calculate that these single-acquisition spectra correspond to approximately 5 pmol of protein sample consumed. A typical ESI experiment by comparison, using typical experimental values (10μM protein sprayed at 3 μL/min) would require a total sample amount of 0.5 pmol, a factor of 10 less.

2.3. Top-down analysis using DESI FTICR-MS

Figs. 3 and 4 show the ECD and CID tandem mass spectra for cytochrome-c obtained by isolating the [M+15H]15+ charge state in the mass resolving quadrupole of the FTICR mass spectrometer. Both the ECD and CID spectrum represent an accumulation of 200 acquisitions. Peak assignments were made using the SNAP 2.0 algorithm. In addition, the fragmentation maps obtained by searching Prosight PTM-2.0 [16−19] are shown. The software was able to match 43 (30%) of the 140 ECD peaks picked by the SNAP 2.0 algorithm with an expectation of 2.45 × 10−8. The algorithm returned 377 peaks from the CID analysis of which 50 (13%) were matched to the theoretical fragmentation map in the database, yielding an expectation of 1.42 × 10−5.

Once again we can estimate the amount of sample consumed, with the assumption that during the time required for 200 acquisitions the DESI probe is rastered over a maximum sample area of 200 mm2. This would correspond to approximately 10 nmol of sample consumed. Again by comparison with ESI (10 μM protein

Fig. 3. (A) DESI ECD FTICR mass spectrum obtained for the isolated [M+15H]15+ charge state of cytochrome-c with assigned fragments labelled. (B) Prosight PTM-2.0 fragment map.
sprayed at 3 μL/min and with typically 20 acquisitions required). DESI MSMS requires 10 pmol, approximately 10^3 more sample. As others have shown [20] we find that the quality of mass spectra obtained using DESI is hugely dependent on the source parameters, particularly the respective geometrical orientation and position of the sprayer, sample stage and MS-inlet.

In this work we have shown that top-down sequencing by DESI ECD FTICR mass spectrometry of intact proteins at least a large as 12 kDa is feasible. Clearly, the addition of air amplifiers and/or flared capillaries, which have been described in the literature, to improve the ion collection efficiency from the DESI plume may well extend the mass range and sensitivity of this technique further.

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References

Anodic Ta₂O₅ for CMOS compatible low voltage electrowetting-on-dielectric device fabrication


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High-K dielectric
Post-process

A B S T R A C T

This paper reports a CMOS compatible fabrication procedure that enables electrowetting-on-dielectric (EWOD) technology to be post-processed on foundry CMOS technology. With driving voltages less than 15 V it is believed to be the lowest reported driving voltage for any material system compatible with post-processing on completed integrated circuits wafers. The process architecture uses anodically grown tantalum pentoxide as a pinhole free high dielectric constant insulator with an overlying 16 nm layer of Teflon-AF®, which provides the hydrophobic surface for droplets manipulation. This stack provides a very robust dielectric, which maintains a sufficiently high capacitance per unit area for effective operation at a reduced voltage (15 V) which is more compatible with standard CMOS technology. The paper demonstrates that the sputtered tantalum layer used for the electrodes and the formation of the insulating dielectric can readily be integrated with both aluminium and copper interconnect used in foundry CMOS.

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1. Introduction

In recent years lab-on-a-chip and bio-MEMS systems, which can manipulate and analyse biological fluidic samples in micro- and nano-litre scales, have emerged as a solution for automating repetitive laboratory tasks [1,2]. Digital microfluidic devices based on technologies such as dielectrophoresis (DEP), electrowetting-on-dielectrics (EWOD) and surface acoustic waves (SAW) provide a potentially reconfigurable method of obtaining a bio-MEMS system [2,3], enabling different manipulations and transport routes to be programmed using the same device. Of these, EWOD technology is an attractive option that has a low power consumption making it well suited for the design and manufacture of microfluidic systems [2]. EWOD uses surface tension as a driving force, which can be controlled by applying a suitable voltage to an array of electrodes covered by a two layer dielectric.

A key parameter in EWOD technology is the driving voltage \( V_D \). Earlier work on electrowetting arrays required driving voltages in the range 80–100 V [4]. More recently with a more judicious choice of materials, processes and dielectric thickness, the voltage required to manipulate droplets has been reduced below 15 V [4]. However, the temperatures required for the deposition of one of these dielectric layers is well in excess of 450 °C [5], making the process incompatible with CMOS post-processing. Being able to construct EWOD structures on top of CMOS technology is attractive because it facilitates large EWOD electrode array with on-chip control and sensing. This paper reports a process architecture that matches the driving voltage of [4] while requiring process temperatures considerably less than 450 °C.

2. Background

2.1. Electrowetting-on-dielectrics

The technology of the electrocapillary phenomenon that is used has been extensively described elsewhere [1], and will only be discussed briefly. For an EWOD system, the Young–Lippmann equation predicts the contact angle (defining the surface wettability as described in [1]). The change from \( \theta(0) \) to \( \theta(V) \) for a droplet in terms of the applied voltage \( V \), the relative dielectric constant \( \varepsilon_r \), the liquid–gas surface tension \( \gamma_{lg} \) and the thickness \( t \) of the dielectric is given by

\[
\cos \theta(V) = \frac{\varepsilon_r}{2\gamma_{lg} t} V^2 + \cos \theta(0)
\]

(1)
Eq. (1) identifies the important role played by the dielectric covering the electrodes in determining the driving voltage $V_D$ required to modify the contact angle by the $40^\circ$ for droplet manipulation. On a Teflon-AF® surface (commonly used EWOD hydrophobic coating), this contact angle change required is from 120° to 80°, where the Eq. (1) can then be re-written as

$$V_D^2 = \frac{0.67 \times 2_{v_H}^2}{\epsilon_{v_0}}$$

(2)

From Eq. (2), it is clear that in order to reduce the droplet driving voltage $V_D$, a dielectric with a high permittivity is required. This is in addition to the requirement for this layer to be totally impervious to the liquid that forms the droplet being manipulated. Failure to meet this latter criterion leads to electrolytic action at the electrode causing the device to cease functioning. Hence, a robust pinhole free dielectric with a sufficiently high breakdown voltage that also acts as barrier to the liquid is essential for any EWOD device. Finally the dielectric also has to display a hydrophobic surface, which is not typically available with materials meeting the above specifications.

As a result EWOD dielectric's typically consist of two layers; the insulating dielectric discussed above which is covered by a thin hydrophobic surface layer such as Teflon-AF®. Table 1 compares the characteristics of some of the dielectrics which have been proposed for EWOD technology.

### 2.2. Large EWOD arrays and multi-level metallisation

In recent years, there has been an increased number of demonstrations of bioassays executed concurrently on a digital microfluidics-based biochip [2,6]. Furthermore, it is clear that system integration and application complexity are expected to increase steadily.

One of the advantages of a digital microfluidic system based on EWOD technology is the ability to reconfigure the system. This means that the different manipulations required can be achieved on the same electrode array by simply modifying the control software. Examples of reconfigurable digital microfluidic systems based on EWOD technology and DEP technology have been reported for sample analysis that use reagent mixing [2,7–10].

Large electrode arrays have the potential to greatly increase the reconfiguration possibilities, including:

- Greater system flexibility: increases the defect tolerance of the system, allowing increased flexibility in route selection [2,6]. More functional units, such as droplet mixers, consisting of different numbers of electrodes [2,6].

- Higher sample processing throughput: more droplet samples can be processed simultaneously.

- Finer control of droplet volume: enables the system to have a higher resolution of droplet volumes.

Several examples showing defect tolerant design and spontaneous multiple droplet manipulations on electrode arrays can be found in the literature [2,6].

Obviously in a passive EWOD system, there is no internal control circuitry available, and each driving electrode in the device must be individually addressed from a contact pad via interconnect. While the interconnect for single and double rows of electrodes can be simply implemented using a single level of metallisation, the same is not the case for arrays with electrode counts of $3 \times 3$ or greater. The interconnects from the inner electrodes in the $M \times N$ array to the exterior control circuit must run between the electrode gaps. This approach is demonstrated in Fig. 1 which shows a single-level-metallisation micro-heater array using a passive single-level-metal addressing mechanism.

In EWOD devices the interconnects are, in effect, just small electrodes and can also suffer from unwanted wetting of non-electrode areas (Fig. 2). This wetting phenomenon will potentially affect droplet manipulation, especially when many tracks are routed between electrodes as can be observed in Fig. 1. Even when the interconnect is held at ground potential, the electrode gaps may be unacceptably wide when several tracks pass between electrodes.

Hence, for EWOD arrays equal to or larger than $3 \times 3$ electrodes, multi-level metallisation is required to avoid parasitic effect of interconnects by burying them beneath the EWOD functional electrodes.

---

### Table 1

Comparison of EWOD dielectric properties [4,13]

<table>
<thead>
<tr>
<th>Dielectric Constant</th>
<th>Dielectric Strength (MV/cm)</th>
<th>Process Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BST</td>
<td>&gt;30&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10</td>
</tr>
<tr>
<td>LPCVD</td>
<td>3.8</td>
<td>10</td>
</tr>
<tr>
<td>SiO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>6–8</td>
<td>10</td>
</tr>
<tr>
<td>LPCVD</td>
<td>3.8</td>
<td>8</td>
</tr>
<tr>
<td>Si&lt;sub&gt;3&lt;/sub&gt;N&lt;sub&gt;4&lt;/sub&gt;</td>
<td>6–9</td>
<td>6</td>
</tr>
<tr>
<td>Parylene-C</td>
<td>3.1</td>
<td>2.7</td>
</tr>
<tr>
<td>Anodic Sili</td>
<td>8–23&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6</td>
</tr>
<tr>
<td>Ta&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;5&lt;/sub&gt;</td>
<td></td>
<td></td>
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</tbody>
</table>

<sup>a</sup> >100 after annealing at higher temperature.

<sup>b</sup> Increases with thickness (>25 when thicker than 200 nm).

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![Fig. 1. Ninety six element micro-heater array, an example of a single-level-metallisation large array having interconnect tracks running between elements/electrodes.](image1)

![Fig. 2. Wetting phenomenon on interconnects in a single metallisation EWOD device.](image2)
2.3. Large EWOD arrays on a CMOS backplane

For arrays of EWOD electrodes, the realistic number of electrodes for passive systems is also limited by packaging considerations. For example, a $30 \times 30$ array would require a 900 pin package which is not particularly practical.

Gong et al. partly address the packaging problem by using printed circuit board (PCB) technology together with land grid array (LGA) sockets [7]. The advantage of this solution is its low-cost and system flexibility (i.e., scalable). However, this does not solve the practical aspect of the interconnect problem entirely since a $32 \times 32$ digital microfluidic array used in [10] requires over 1000 pins. This is at the practical limit of a passive electrode drive system and any size bigger really requires an active controlling backplane.

CMOS technology has been widely used for row-column addressing of large numbers elements, of which the largest application is related to memory devices. Others examples include CMOS imaging chips [11] and micro displays [12]. Addressing arrays using this approach is obviously ideally suited to the realisation of EWOD arrays with a large matrix of electrodes. In addition the underlying CMOS also makes it possible to provide the electrodes with additional capabilities such as sensing (e.g., pH, temperature, light, voltage, etc) and actuation (e.g., temperature control).

Obviously a clear advantage of using on-chip addressing for large two-dimensional arrays is the significant reduction in the number of bond pads which also simplifies the packaging. A dielectrophoresis (DEP) system with a $32 \times 32$ array of individually addressable electrodes using a CMOS solution has already been demonstrated [10]. Manipulating droplets having more than a 100 fold volume range, it shows a scalable architecture of digital microfluidic systems based on CMOS technology [10].

In another commercialised prototype system, more than 600,000 electrodes have been embedded and separately controlled to create more than 100,000 DEP cages for droplet manipulation. Similar success can be achieved with active EWOD electrode arrays (i.e., integrated CMOS backplane electrode control), if appropriate post-processing technology is available.

As part of this work an EWOD/CMOS chip has been fabricated and tested [13] (Fig. 3). This used conventional foundry processes and materials, with the EWOD post-processing involving the deposition of appropriate dielectric and surface treatment layers.

2.4. Low voltage EWOD processes

Previously reported low voltage EWOD fabrication processes have all used dielectrics deposited at high temperature (e.g., 700 °C MOCVD for barium strontium titanate [4], thermal oxidation [14], 700 °C annealed BZN (Bi$_2$O$_3$–ZnO–Nb$_2$O$_5$) [15]). None of these are compatible with CMOS technology incorporating aluminium (or copper) interconnect as deposition temperatures in excess of 450 °C are required.

In this work the CMOS foundry process that was used as the backplane of the EWOD device employed aluminium as its interconnect. Hence, the electrode metallisation and the passivation layer were determined by the foundry. For typical foundry processes the passivation is a relatively thick dielectric layer (0.5–1.0 μm) of silicon dioxide or nitride (or oxinitride), neither of which possesses a very high dielectric constant.

For an EWOD implementation using foundry passivation as the insulating dielectric a comparatively high operating voltage in the region of 70 V is required to drive droplets. This is as a direct consequence of the low dielectric constant of the passivation materials. The resulting high drive voltage requirement drove the selection of a 100 V CMOS foundry process for the demonstration EWOD backplane.

Having fabricated and post-processed a high voltage CMOS EWOD device the challenge was to identify and demonstrate an improved material system that was fully compatible with EWOD, while at the same time being suitable for integration with a lower voltage CMOS technology. The material system selected is based upon a tantalum pentoxide insulating layer with a high $\varepsilon_r$ dielectric constant (in our case around 19 at a thickness of 95 nm), which can be grown pinhole-free. This is covered by a uniform and thin (approx. 10 nm thick) overlying layer of teflon to provide the required hydrophobic surface. This material system, which involves no high temperature process (except for sputtering tantalum), simply consists of Ta/Ta$_2$O$_5$/teflon layers, which is compatible with standard foundry CMOS IC technology with aluminium interconnect.

3. Multi-level metallisation EWOD array

Multi-level metallisation EWOD arrays using chromium and chromium/platinum on glass and silicon substrates have previously been fabricated for reconfigurable multi-functional microfluidic systems [16,6].

Aluminium multilayer interconnect structures have been widely employed in standard CMOS circuitry fabrication, while a single layer of aluminium has been demonstrated as a passive EWOD electrode material in [13]. Hence, a two-level aluminium metallisation process for $M \times N$ EWOD electrode array fabrication is clearly feasible. Similar multi-level aluminium metallisation structures are commonly employed in standard CMOS integrated circuits devices.

The process flow for fabricating a two-level metallisation EWOD electrode array is detailed below and illustrated in Fig. 4:

(a) The bottom aluminium layer is sputtered on an SiO$_2$ insulated silicon substrate.
(b) It is then patterned to form the bond pads and the interconnects, thicknesses ranges between 0.1 and 1 μm.
(c) After patterning, 1 μm of PECVD SiO$_2$ is deposited as the inter-metal dielectric. Vias are then open by patterning the PECVD SiO$_2$ and the second aluminium layer is then sputtered to form the electrodes an dielectrical connection to the bottom aluminium layer.
(d) After the patterning the second aluminium layer, the insulating dielectric 500 nm parylene-C (room temperature vapour deposition), and 50 nm Teflon-AF/C210 hydrophobic layers (spin-coating) were finally deposited.

(e) After dicing the microscope slide sized chip can then be probed or packaged in the same manner as for single metal systems [13].

Using the same bond pad layout and chip size discussed in [13], a 5 × 8 EWOD electrode array was designed. The bottom aluminium interconnect lines were 10 µm wide. The top metal electrodes were 1 mm × 1 mm interdigitated square electrodes with 100 µm wide gaps in between. The electrodes were covered with a 500 nm layer of parylene-C covered by 50 nm of Teflon-AF/C210. This required a driving voltage of 60 V and Fig. 5 shows droplet manipulation on a 5 × 8 EWOD electrode array.

By using the same post-process steps on a custom designed CMOS backplane chip, droplet movement was achieved with a drive voltage of 60 V as shown in Fig. 6.

4. Tantalum–aluminium structures for low voltage EWOD–CMOS systems

As has been previously mentioned a low voltage EWOD electrode array based upon a high-K tantalum pentoxide insulating layer has been developed. This involves no high temperature process and simply consists of Ta/Ta2O5/Teflon-AF/C210 or CYTOP/C210 layers. This is compatible with standard foundry CMOS IC technology with conventional aluminium interconnect. This section will focus on the fabrication of EWOD systems based on tantalum–aluminium structures.

4.1. Structure design and fabrication

To demonstrate the Ta2O5–Teflon-AF/C210 dielectric system, the top aluminium layer in Fig. 4 was replaced by sputtered tantalum which was patterned using the same mask.

There are a number of options for etching tantalum. It can be etched in fluorine-containing plasmas such as CF4, SF6, and CF3Cl with CH3F, sometimes mixed with O2 [17,18]. The drawback is that these processes will potentially attack any underlying PECVD SiO2 layer, which may be problematic if the tantalum etching is not uniform. By using SiCl4 mixed with NF3 plasma, Shimada et al. obtained an etch selectivity greater than 80:1 between tantalum and SiO2 (10:1 in absence of NF3) [19].

An alternative is XeF2 dry etching. This is commonly used for silicon etch release in MEMS fabrication, especially post-CMOS etch release due to its high selectivity with other materials (greater than 1000:1 for silicon to SiO2 and aluminium) [20]. XeF2 rapidly etches tantalum and no aluminium or SiO2 attack was observed when using a Memstar® tool. The only potential issue is the degree of undercut, as shown in Fig. 7 with an average value of

![Fig. 4. Process flow for a two-level metal EWOD electrode array with aluminium as the interconnect and electrode material.](image)

Fig. 5. (a) Photograph of a moving droplet on a two-level metallisation (aluminium) 5 × 8 EWOD electrode array with a driving voltage of 60 V. This is a two-plate EWOD device with a 440 µm droplet height. (b) Layout of the 5 × 8 EWOD electrode array.

![Fig. 6. Droplet movement (three frames, left to right) on a post-processed EWOD electrode array controlled by a CMOS backplane.](image)

500 nm layer of parylene-C covered by 50 nm of Teflon-AF/C210. This required a driving voltage of 60 V and Fig. 5 shows droplet manipulation on a 5 × 8 EWOD electrode array.

![Fig. 7. Microscope photos showing a higher etching selectivity of tantalum to SiO2 in XeF2 gas (right) than in SiCl4 plasma (left). Isotropically etched tantalum patterns in XeF2 gas (right) have a smaller feature size than those anisotropically etched in SiCl4 plasma (left).](image)
3.0 µm on each side being measured when etching 0.45 µm thick tantalum. As the gap between EWOD electrodes in this case is 30 µm, this undercut rate is acceptable and if need be, could be accounted for by a bias in the mask.

After patterning, the tantalum electrodes are anodised with a gel form citric acid solution to form the tantalum pentoxide insulating layer [21]. In this work the applied voltage was 50 V and for the electrode array shown in Fig. 5 the current compliance was 2 mA. A thin Teflon-AF® layer is then deposited using a standard spin coater on the oxidised tantalum electrodes. The surface roughness of the anodic Ta2O5 has been measured using AFM to have a mean roughness Ra between 0.4 and 0.6 nm (peak-to-valley value down to few nanometers) with the Teflon-AF® layer thickness uniformity across the wafer within 10%.

The resulting two-level metal EWOD array has aluminium as the bottom metal with tantalum as the top metal electrode. The 50 V anodisation voltage used in this process resulted in 95 nm of Ta2O5. This was followed by a 0.3% Teflon-AF® solution (diluted in Fluorinert solvent FC-75) being spin coated at 2000 rpm for 50 s, giving 16 nm of Teflon-AF®.

4.2. Experiment and results

4.2.1. Low voltage EWOD manipulation on large arrays

A common two-plate configuration EWOD chip [22] has been used in the experiments to evaluate the Ta2O5/Teflon-AF® system. A conductive indium tin oxide (ITO) covered glass plate coated with 20 nm Teflon-AF® was placed above the 5 x 8 electrode array previously presented. Spacers were used to set the distance between the plates and the height of droplets. In this case the spacers were 258 microns. The above combination of dielectric layers between the plates and hence the height of droplets. In this case, each unit electrode was 1 x 1 mm in size and the gap 80 µm. More volume choices are obviously available as the electrode size reduces and the number of electrodes increases.

4.2.2. Droplet size manipulation using low voltage

Fig. 9 shows a digital droplet dispense system which uses a glass fibre capillary attached through a plastic ferrule as a liquid input into a two-plate EWOD system. This Ta2O5 low voltage EWOD system is based on a tantalum electrode array with aluminium interconnects and uses a 15 V DC drive voltage. The liquid input is pressurised through the fibre capillary from a syringe which fills up the reservoir attached to the EWOD electrodes. The liquid is then extruded and cut into droplets of by manipulating the electrodes in the EWOD array in a similar manner to that described in [22,13].

Fig. 9 shows the creation of droplets with volumes of 80 nL, 160 nL and 320 nL by switching on 1, 2 or 4 electrodes simultaneously. The volume is defined by the size of a single electrode and the gap between the two EWOD plates. In this case, each unit electrode was 1 x 1 mm in size and the gap 80 µm. More volume choices are obviously available as the electrode size reduces and the number of electrodes increases.

5. Conclusion and future work

This paper has described what is believed to be the first fully CMOS compatible EWOD system that can drive liquid droplets using voltages less than 20 V. The method of producing a thin uniform film of high permittivity dielectric by the anodisation of tantalum, together with a reliable method of spinning thin uniform teflon films, are the key to achieving the required low operating potential. The resulting system, with its robust and pinhole free anodised Ta2O5 provides a high dielectric constant and an impervious barrier to the liquids being transported, which is not always the case with deposited dielectrics.

The integration of CMOS and EWOD technologies helps solve the issues relate to increasing the EWOD electrode array scales. The benefits have already been shown in other CMOS integrated digital microfluidic systems such as a DEP system [1] and other advantages such as possible on-chip sensing can also be obtained by integrating sensing circuits with the EWOD systems.

In addition an EWOD post-process foundry CMOS chip has been processed and droplet movement demonstrated. The next step is to significantly increase the number of electrodes so that it becomes possible to implement a programmable electrode array size and start integrating further functionality into the electrodes. Fig. 10 shows part of a prototype design that provides an example of both these elements. It consists of an EWOD array with 200 µm x 100 µm electrodes integrated with SPADs (single photon avalanche diodes) for light detection and this gives one example of the direction digital microfluidics will be moving in the future.

Fig. 8. Three frames (left to right) showing a moving droplet on a two-level metal EWOD chip coated with 95 nm Ta2O5 and 16 nm Teflon-AF® (the outlines of the droplet have been enhanced for clarity).

Fig. 9. Moving droplets (black boxed) with different volumes (a) 80 nL, (b) 160 nL and (c) 320 nL, dispensed from a liquid input capillary fibre (on the right of each figure).

Fig. 10. A prototype design of an EWOD array with electrodes integrated with SPAD (single photon avalanche diodes) [23].
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Best Regards,

Kurt Petersdorff  
Commercial Director  
Samsung LCD Netherlands R&D Cente
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Science Park, Milton Road, Cambridge CB4 0WF, UK
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Thank you for your request.

For your records the following options have been submitted.

Name: Adam A. Stokes  
Address: Whitesides Group Box 70, Department of Chemistry and Chemical Biology  
Harvard University, 12 Oxford Street, Cambridge, MA 01238  
Tel: 617-319-6133  
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Email: stokes@fasmail.harvard.edu

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Additional Comments: Aaron Wheeler has given full backing to this request

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