

Continuous and Segmented-Flow Microfluidics for Biomolecular Analysis

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degree of Doctor of Philosophy**

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Declaration of Originality

I declare that this thesis is my own work and has not been submitted in any form for another degree or diploma at any university. Information derived from the published and unpublished work of others has been acknowledged in the text and a list of references is given in the bibliography.

Fiona Pereira

For my Father, for all the inspiration

Abstract

Segmented flows in microfluidics have attracted much recent attention. Of particular interest is the use of nanolitre droplets as interface tools in microfluidics. A droplet interface connects techniques while leaving them sufficiently independent of each other; a task that difficult to perform within a continuous flow system.

The first part of this thesis is dedicated to sample preparation and analysis in continuous flow microfluidic systems. The polymerase chain reaction (PCR) is investigated, using electroosmotic flow to transport the sample across various temperature zones. Additionally, a novel sieving matrix for electrophoretic separation of dsDNA fragments and PCR amplicons on microchip and capillary is demonstrated.

The second part of this thesis focuses on interfacing segmented and continuous flows. Two novel interfaces are described and demonstrated. The first interface connects droplet flows and a chip-based electrophoresis device. Using this interface, samples or reactions performed in droplets can be directly transferred to a separation channel without suspending the separation. This allows multiple samples to be analysed in a single separation channel, without cross contamination between droplets. Consequently, PCR reactions and dsDNA calibration ladders can be prepared in droplet format and analysed in high throughput. The second interface links nano-liquid chromatography and MALDI mass spectrometry. Droplet fractionation post nano-LC separation is used to preserve resolution between separated bands. The droplets are subsequently delivered to a MALDI plate for mass spectrometric analysis by removing the continuous oil phase using a hydrophobic oleophilic membrane.

The tools developed here reduce manual intervention and provide a link between multiple analytical techniques involved in biomolecule analysis. These innovations will improve reproducibility and reduce cross-contamination between samples.

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List of Abbreviations

μ-CE	micro- Capillary Electrophoresis
μ-TAS	micro-Total Analysis Systems
2D	Two Dimension
3D	Three Dimension
A.C.	Alternating Current
ACN	Acetonitrile
ACTH	Adrenocorticotrophic hormone
AES	Atomic Emmision Spectroscopy
BHT	Butylated Hydroxy Toluene
bp	Base pair
BSA	Bovine Serum Albumin
CAD	Computer Aided Design
CCD	Charge Coupled Device
CE	Capillary Electrophoresis
CGE	Capillary Gel Electrophoresis
CIEF	Capillary Isoelectric Focussing
CTAB	Cetyl trimethylammonium bromide
CZE	Capillary Zone Electrophoresis
Da	Dalton
DAB	Diamino Butane
DAPI	4', 6-diamidino-2-phenylindole
dbpr	Dye Base Pair Ratio
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	deoxy Nucleotide Triphosphate
dsDNA	double-stranded Deoxyribonucleic Acid
EDL	Electric Double Layer
EDTA	Ethylene Diamine Tetraacetic Acid

EOF	Electroosmotic Flow
ESI	Electrospray Ionisation
FITC	Fluorescein Isothiocyanate
GST	General Separation Transform
HCCA	α -Cyano-4-hydroxycinnamic acid
HCl	Hydrochloric acid
HEC	Hydroxy Ethyl Cellulose
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	Human immunodeficiency virus
HPCE	High Performance Capillary Electrophoresis
HPMC	Hydroxy Propyl Methyl Cellulose
ID	Internal Diameter
ITP	Isotachopheresis
kDa	Kilo Dalton
kV	Kilo Volt
KOH	Potassium Hydroxide
LC	Liquid Chromatography
LFII	Label Free Intrinsic Imaging
LHS	Left Hand Side
LIF	Laser Induced Fluorescence
LOC	Lab-on-a-chip
LPA	Linear Polyacrylamide
MALDI	Matrix Assisted Laser Desorption Ionisation
MC	Methyl Cellulose
MDa	Mega Dalton
MECC	Micellar Electrokinetic Capillary Chromatography
MEKC	Micellar Electrokinetic Chromatography
MEMS	Micro Electromechanical Systems
MS	Mass Spectroscopy
MW	Molecular Weight
O.D.	Outer Diameter
PAGE	Polyacrylamide Gel Electrophoresis
PC	Poly Carbonate

PCR	Polymerase Chain Reaction
PDA	Photo Diode Array
PDMS	Poly Dimethyl Siloxane
PEB	Post Exposure Bake
PEG	Poly Ethylene Glycol
PEO	Poly Ethylene Oxide
PES	Poly Ether Sulfone
PID	Proportional–Integral–Derivative
PMMA	Poly (methyl methacrylate)
ppm	part per million
psi	pound per square inch
PTFE	Polytetrafluoroethylene
PVP	Poly Vinyl Pyrrolidone
qPCR	Quantitative Polymerase Chain Reaction
RHS	Right Hand Side
RNA	Ribonucleic Acid
RSD	Relative Standard Deviation
SDS	Sodium dodecyl sulfate
SELEX	Systematic Evolution of Ligands by Exponential Enrichment
SNP	Single Nucleotide Polymorphism
ssDNA	single-stranded Deoxyribonucleic Acid
STR	Single Tandem Repeats
TBE	Tris Borate EDTA
TEM-EDX	Transmission Electron Microscope & Energy Dispersive X-ray
TFA	Trifluoroacetic acid
UV	Ultra Violet

List of Symbols

μ	Mobility
μ_{app}	Apparent Mobility
μ_{e}	Electrophoretic Mobility

μ_{EOF}	Electroosmotic flow Mobility
σ^2	Variance
Ω	Ohms
$^{\circ}\text{C}$	Degrees Centigrade
$\text{\AA}$	Angstrom
T_m	Melting Temperature
ϵ	Permittivity
ζ	Zeta Potential
η	Viscosity
κ^{-1}	Debye Length
λ	Wavelength

List of Figures

Chapter I

Figure 1.1: Transcription and Translation. The amino acid sequence for every protein is stored in the cell in its DNA format and converted via transcription and translation when required. (a) Illustration of the transcription process, where a DNA template is read to its RNA format and transported outside the nucleus. (b) The translation process where the RNA generated in the nucleus is translated to a specific sequence of amino acids which can form a protein. The RNA is read by a cell organelle known as a ribosome, which catalyses the formation of the peptide bond between the appropriate amino acids.

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Chapter II

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Chapter III

Figure 3.1: Schematic of the basic steps of the polymerase chain reaction. In a single cycle the sample is exposed to three different temperatures. Initially, double stranded template DNA is separated into single strands at high temperature (95°C). Then at 55°C upstream and downstream primers anneal to the single stranded template DNA. The upstream and downstream primers are composed of sequences which are complementary to the region flanking the amplification sequence. Finally, extension occurs at 72°C when the polymerase binds to the primer –template complex and extends it by adding on deoxynucleotide triphosphates complementary to the template sequence. The process repeats itself, exponentially increasing the DNA concentration with every cycle. Typically, 20 to 30 cycles are performed. Image reproduced from reference [1].

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Figure 3.11: The effect of diode device on PCR solution. When loaded on to the diode device and exposed to an A.C. field the PCR solution turned green and a precipitate appeared. (a) A photograph of vials containing PCR solution exposed to A.C. field on the diode device compared to a standard untreated solution. The PCR solution depicted here is the PCR buffer diluted to one fifth it's in use concentration with or without magnesium chloride. Sample number corresponds to those listed in Tables 3.2 and 3.3. (b) An image of the diodes on the diode device after the PCR precipitate and solution was removed. The same electrode on all diodes in the device had disappeared. (c) An overlay of GST processed electropherograms obtained for the analysis of a standard PCR sample compared to PCR solution prepared with magnesium nitrate.

Figure 3.12: Analysis of the effect of salt and additives on the efficiency of PCR. (a) and (b) are overlays of GST processed electropherograms obtained from the analysis of PCR amplicons using capillary gel electrophoresis. All separations were performed using a 54 cm long capillary of effective length 40 cm, at 25°C and using a field strength of 203.7 V/cm. Separations were performed in the 1.75% PEO (5 MDa) sieving matrix. The standard PCR reaction mix was prepared as detailed in section 3.3.4, the reaction produces an amplicon fragment 323bp in length. (a) The effect of reducing the salt content of the PCR buffer. The PCR buffer was diluted to one half and one fourth its standard in-use concentration in deionised water or the PCR buffer was entirely replaced with deionised water. (b) The effect of additives such as Tween 20, Bovine serum albumin and fluorescent dye SYBR Green I (labelled SYBR).

Figure 3.13: Analysis of the effect polyvinyl pyrrolidone (PVP) and PDMS on the efficiency of PCR. The image is an overlay of GST processed electropherograms obtained from the analysis of PCR amplicons using capillary gel electrophoresis. PVP was added at a final concentration of 2% to the standard PCR mixture which was amplified in a standard micro-centrifuge vial and one coated in PDMS. The results were compared to a sample not containing PVP. The standard PCR reaction mix was prepared as detailed in section 3.3.4, the reaction produces an amplicon fragment 323bp in length. All separations were performed using a 54 cm long capillary of effective length 40 cm, at 25°C and using a field strength of 203.7 V/cm. Separations were performed in the 1.75% PEO (5 MDa) sieving matrix.

Figure 3.14: Analysis of the effect of A.C. field on the efficiency of PCR. The image is an overlay of GST processed electropherograms obtained from the analysis of PCR amplicons using capillary gel electrophoresis. All samples were prepared under the standard conditions as detailed in section 3.3.4, however the PCR buffer was diluted to one half its in-use concentration. The reaction produces an amplicon fragment 323bp in length. PCR mixture in 60 µL batches was exposed to A.C. voltage prior to amplification. Samples were exposed to A.C. voltage of peak to peak (V_{pp}) value 250 V, 350 V and 500 V. All separations were performed using a 54 cm long capillary of effective length 40 cm, at 25°C and using a field strength of 203.7 V/cm. Separations were performed in the 1.75% PEO (5 MDa) sieving matrix.

Figure 3.15: DNA amplification on PCR diode device. Overlay of GST processed electropherograms obtained from the analysis of PCR amplicons produced on the PCR diode device (green trace) and macroscale PCR thermo-cycler (blue trace) using capillary gel electrophoresis. Both samples were prepared under the standard conditions as detailed in section 3.3.4, however the PCR buffer was diluted to one half its in-use concentration. The reaction produces an amplicon fragment 323bp in length. The PCR results were compared to a 25bp dsDNA step ladder (red trace). All separations were performed using a 54 cm long capillary of effective length 40 cm, at 25°C and using a field strength of 203.7 V/cm. Separations were performed in the 1.75% PEO (5 MDa) sieving matrix.

Chapter IV

Figure 4.1: Microchip capillary electrophoresis platform. The aluminium MCE device holder was designed to be mounted on a microscope stage. There are 12 electrode ports surrounding the MCE device. High voltage connectors were screwed into appropriate ports; depending on the MCE device layout. Platinum electrodes were soldered to the high voltage connectors. A ledge was constructed (3mm wide and 3 mm down) from the top surface on the holder. In operation the MCE device is mounted on the ledge to off-set the change in focal length due to the thickness of the aluminium platform.

Figure 4.2: GST processed electropherograms of the 25bp ladder (3.6µg/ml in deionised water, Promega) and a 50bp fragment (10µg/ml in deionised water, Fermentas). (a) GST processed electropherogram of the 50bp fragment performed in 4% PEO (300kDa) in x1TBE. (b) GST processed electropherogram of the 25bp ladder performed in 4% PEO (300kDa) in x1TBE. (c) GST processed electropherogram of the 50bp fragment performed in 3% T LPA in x1 TBE. (d) GST processed electropherogram of the 25bp ladder performed in 3% T LPA in x1TBE. All four separations were performed using a PVA coated 34 cm long capillary of effective length 20 cm, at 25°C and using an electric field strength of 235 V/cm.

Figure 4.3: GST processed electropherograms of the EOF marker thiourea (100 µg/ml in deionised water). (a) Separations performed after the addition of 0.2, 0.4, 0.6 and 0.8% methyl cellulose, (b) Separations performed after the addition of 4% PEO (300 kDa), 5% PEO (300kDa) and 0.2% HPMC. All separations were performed using a 24 cm long capillary of effective length 10 cm, at 25°C and using an electric field strength of 333.3 V/cm. The low intensity, short duration peaks observed in all electropherograms are a result of bubbles and BHT particulates.

Figure 4.4: GST processed electropherograms and equiphase maps of the 25bp ladder (36µg/ml in deionised water, Promega). (a) Equiphase map of a separation performed without a 10% PVP pre-coat, (b) Equiphase map of a separation performed after the use of a 10% PVP pre-coat. The inset images in both (a) and (b) are expanded views of the equiphase map of the 25bp dsDNA ladder. The component bands of the ladder can be clearly seen and identified based on their characteristic slope. (c) GST processed electropherogram of a separation in (b). (d) GST processed electropherogram of the separation of ΦX174 RF DNA/HaeIII digest fragments under similar conditions to (b). All separations were performed using a 34 cm long capillary of effective length 20 cm, at 25°C and using an electric field strength of 353 V/cm.

Figure 4.5: Monitoring the effect of PVP on the resolution and mobility of the fragments of a 25bp dsDNA step ladder (Promega). (a) A log-log plot of mobility versus dsDNA fragment size. (b) A resolution versus fragment size plot.

Figure 4.6: Effect of polymer concentration and effective separation length on the mobility and resolution of a 25bp ladder (36 µg/ml in deionised water, Promega). (a) and (c) are log-log plots of mobility as a function of fragment size while (b) and (d) are plots of resolution versus fragment size. In (a) and (b) separations were performed in 4% PEO (300kDa) and in 5% PEO (300kDa). Both separations were performed using a 44 cm long capillary of effective length 30 cm, at 25°C and using an electric field

strength of 363.6 V/cm. In (c) and (d) separations were performed in 12 cm, 22 cm, 32 cm and 42 cm effective length capillaries. All four separations were performed using the 4% PEO (300kDa) sieving buffer. Additionally, both sets of analysis were performed at 25°C and using an electric field strength of 330 V/cm. For both experiments the capillaries were pre-coated with 10% PVP.

Figure 4.7: (a) Separation of a 25bp ladder from Promega (36 µg/ml in deionised water) (b) Separations of ΦX 174 RF DNA/*Hae*III digest fragments (500 ng/µl in deionised water, Invitrogen). (c) Separation of a 25bp ladder from Invitrogen. All separations were performed using a 24 cm long capillary of effective length 10 cm, at 25°C and using an electric field strength of 230 V/cm. In both cases the capillary was pre-coated with 10% PVP and the separation was performed in 4% PEO (300kDa).

Figure 4.8: Separation of a ΦX174 HAEIII digest fragments (50µg/ml in deionised water, Invitrogen) in filtered or re-crystallised PEO sieving matrices. (a) A log-log plot of mobility versus fragment size for the digest in each sieving matrix. (b) A resolution versus fragment size plot of the digest in each sieving matrix. The four sieving matrices tested included a 4% PEO (300 kDa) buffer which was not filtered or re-crystallized (■), 4% PEO (300 kDa) buffer which was filtered using a 5µm PES syringe filter but not re-crystallized (♦), 4% PEO (300 kDa) buffer which was not filtered but re-crystallized (▲) and 4% PEO (300 kDa) buffer which was filtered using a 5µm PES syringe filter and re-crystallized (▼) prior to use. All four separations were performed using a 54 cm long capillary of effective length 40 cm, at 25°C and using an electric field strength of 296.3 V/cm.

Figure 4.9: Separation of a 25bp dsDNA ladder (36µg/ml in deionised water, Promega) in the presence of 2% hexane in the separation matrix. (a) and (b) show the single pixel electropherogram (Pixel 250) and GST processed electropherogram respectively. While (c) details the Equiphase map from which the GST profile was extracted. (d) A plot of resolution versus fragment size. The sample was separated in a 4% PEO (300 kDa) buffer which was re-crystallized to remove BHT prior to use. 2% hexane was used as an alternative stabiliser to BHT. The separation was performed using a 54 cm long capillary of effective length 40 cm, coated with 2% PVP (360 kDa) at 25°C and using a field strength of 296.3 V/cm.

Figure 4.11: Sieving matrix effect on dsDNA velocity and resolution. (a) and (b) are mobility versus fragment size plots while the (c) and (d) are resolution versus fragment size plots. Both sets illustrate the effect of changing sieving matrix composition on the migration behaviour and resolution between dsDNA fragments. (a) and (c) show the effect of increasing polyethylene oxide (5 MDa) concentration from 0.75 % to 1.75% in the sieving matrix. (b) and (d) compare three different sieving matrices, 1.75% PEO (5MDa), 1.75% PEO (5MDa) with 5% glycerol and a mixture of several polymer lengths of PEO. A 100bp dsDNA size ladder at µg/ml was employed to demonstrate these changes. All the buffers were prepared in x1 TBE (pH 8.3). Separations were performed using a 54 cm long capillary of effective length 40 cm, at 25°C and using a field strength of 222.2 V/cm.

Figure 4.10: Separation of dsDNA ladders in a 1.75% PEO (5 MDa) buffer. (a) 25bp dsDNA ladder (36µg/ml in deionised water, Promega). (b) 50bp dsDNA ladder (25µg/ml in deionised water, Roche). (c) DNA marker VIII dsDNA ladder (25µg/ml in deionised water, Roche). (d) pBR322 dsDNA ladder (10µg/ml in deionised water). All four separations were performed using a 54 cm long capillary of effective length 40 cm, at 25°C and using an electric field strength of 222.2 V/cm. The separation performed on a 2% agarose gel provided by the manufacturer is also shown in each panel.

Figure 4.12: Overlay of GST processed electropherograms illustrating the effect of increasing concentration of SYBR Green I on electrophoretic migration of dsDNA fragments. Separation of 50bp dsDNA size ladder (0.34µg/µl in deionised water, Promega) treated SYBR Green I with dye to base pair ratios of 163.8, 327.7, 1638, 3277, 16380 and 32770 compared to an unlabelled sample. All separations were performed using a 54cm long capillary of effective length 40cm, at 25°C and using a field strength of 203.7 V/cm. Separations were performed in the 1.75% PEO (5 MDa) sieving matrix.

Figure 4.13: Plots illustrating the effect of increasing concentration of SYBR Green I on the mobility of dsDNA fragments. (a) and (b) are mobility versus fragment size plots of 25bp and 50bp dsDNA size ladders respectively, comparing increasing dye to base pair ratios to an unlabelled sample. The mobility versus dye to base pair ratio plot in (c) illustrates the effect of increasing levels of SYBR Green I on the mobility of individual fragments. (d) Plot of percentage RSD as a function of fragments size for both ladders. All separations were performed using a 54 cm long capillary of effective length 40 cm, at 25°C and using a field strength of 203.7 V/cm. Separations were performed in the 1.75% PEO (5 MDa) sieving matrix.

Figure 4.14: Plots illustrating the effect of increasing concentration of SYBR Green I on the mobility of PCR amplicons. (a) The electrophoretic profile of the 323bp amplicon with increasing concentrations of SYBR Green compared to an unlabelled sample and a 50 bp dsDNA ladder analysed under similar conditions. (b) The calibration curve generated from the 50bp dsDNA ladder. This was used to determine the fragment size from the mobility values of each amplicon. (c) A plot of mobility versus fragment size illustrating the increase in estimated fragment size with increase in SG I concentration. All separations were performed using a 54 cm long capillary of effective length 40 cm, at 25°C and using a field strength of 203.7 V/cm. Separations were performed in the 1.75% PEO (5 MDa) sieving matrix.

Figure 4.15: Injection and separation of dsDNA fragments on a microchip electrophoresis device fabricated in PDMS. (a) A schematic of the device with cross piece injector. The total length of the separation channel (from reservoir A to B) was 6 cm while its effective length was 5.5 cm. (b), (c), (d) and (e) illustrate the injection and separation mechanism using a 100 bp dsDNA size ladder labelled with SYBR Green I. Under an applied field of 200 V/cm. The negatively charged DNA is drawn down from the sample reservoir to the sample waste reservoir. The sample is prevented from diffusing into the separation channel using ‘pinching’ voltages (see text). The sample plug formed at the intersection is injected into the separation channel under an applied field. A discrete sample plug is maintained by applying pull back voltages at the sample and sample waste reservoirs (c) and (d). Separation between fragments occurs almost immediately. Sieving matrix employed was a 2.5% PEO prepared in 0.1x TBE.

Figure 4.16: Injection mechanism on a dual channel cross-injector (upper panel) and double-T injector (lower panel) using a FITC.

Figure 4.17: Electrophoretic separation of a 25 bp size ladder (Promega Corporation). (a) and (b) detail the separation of all 12 fragments of the 25bp dsDNA size ladder on the PEREGRINE CE instrument and PDMS microchip respectively. The 12 fragments extend from 25 bp to 300 bp in 25 bp increments. The microchip separation was performed in a 2.5% PEO (5MDa) in 0.1% TBE sieving matrix while the CE separation was performed in a 1.75% PEO (5MDa) in x1 TBE sieving matrix. (c) The separation of the 25 bp ladder at two points on the microchip, separated by a distance of 700 μm . The migration time values of the ladder peaks from these two points were used to determine the mobility of each band. (d) A plot of mobility versus fragment size for the microchip separation of the 25bp ladder.

Figure 4.18: Electrophoretic separation of Roche Marker VIII. (a) and (b) detail the separation of 13 fragments of the restriction digest ladder on the PEREGRINE CE instrument and PDMS microchip respectively. The 16 fragments extend from 67 bp to 1114 bp and table lists fragment sizes and corresponding peak numbers. The microchip separation was performed in a 2.5% PEO (5MDa) in 0.1% TBE sieving matrix. (c) A plot of mobility versus fragment size for the microchip separation.

Figure 4.19: Electrophoretic separation of a 50 bp size ladder (Promega Corporation) in sieving matrices with and without glycerol. (a) and (b) detail the separation of dsDNA size ladder on a PDMS microchip using a 2.5% PEO (5MDa) in 0.1% TBE and 2.5% PEO (5MDa) with 5% glycerol in 0.1% TBE sieving matrix respectively. The 16 fragments extend from 50 bp to 800 bp in 50 bp increments. (c) A plot of mobility versus fragment size for the microchip separation of the 50bp ladder in both sieving matrices. (d) A plot of resolution as a function of fragment size for the microchip separation of the 50bp ladder in both sieving matrices.

Figure 4.20: The calculated distribution of electric potential at the cross region during a pinched injection. (a) Potential distribution during loading. The voltages applied at sample (S), sample waste (SW), buffer (B) and buffer waste (BW) reservoirs were 990V, 0 V, 880 V and 1100 V respectively. (b) Potential difference during dispensing and separation. The voltages applied at sample (S), sample waste (SW), buffer (B) and buffer waste (BW) reservoirs were 880V, 880 V, 1100 V and 0 V respectively. Images reproduced from reference [77].

Figure 4.21: Electrophoretic separation of a 50 bp size ladder (Promega Corporation) spiked with 323 bp PCR amplicon on a PDMS microchip. Sample was prepared with x50 SYBR Green I concentration. (a) and (b) detail the migration of the amplicon fragment after the 300 bp of the dsDNA size ladder. The PCR amplicon co-elutes with several fragments, thus obscuring them. The microchip separation was performed in a 2.5% PEO (5MDa) in 0.1% TBE sieving matrix under an applied field of 183.3 V/cm.

Figure 4.22: A three dimensional plot describing the effect of separation length (in centimetres) and electric field (in Volts/centimetre) on resolution. Image reproduced from reference [83].

Figure 4.23: PDMS microchip with inserted bare fused silica capillary of 100 μm internal diameter and 375 μm outer diameter. (a) and (b) illustrate the pinched injection of a 100 $\mu\text{g}/\text{ml}$ FITC solution using a cross piece injector. Post injection the sample plug migrates down the separation channel and into the inserted capillary as shown in (c). (d) The sample band migrating within the capillary outside the PDMS device.

Chapter V

Figure 5.1: Droplet interfaces to CE separation microdevices. (a) Droplet interface for sample injection reproduced from reference [35]. The interface consisted of a droplet generation region and a separation channel which were separated by an immiscible partition at the point of injection. (c) K-shaped droplet interface for sample injection reproduced from reference [36]. The device consisted of three regions segmented flow channel, a V-shaped cross-flow channel and a separation channel. (e) EOF driven droplet interface for analyte fractionation reproduced from reference [38]. The interface consisted of a sample injection region, a separation channel and a droplet-generation region. (b), (d) and (f) A mixture of FITC labelled amino acids separated on the devices shown in panels (a), (c) and (e) respectively. The inset in (f) shows the droplet-formation induced band oscillation on the D/L-Glu peak.

Figure 5.2: Oil absorbing materials examined for use in the droplet-MCE interface and LC-MALDI interface device (Chapter 6). (a) A super-hydrophobic and super-oleophilic mesh film developed for the separation of oil from water. Image reproduced from reference [39]. (b) Zorboid foam[®], used in swimming pools as a scum remover; this is an oil absorbing hydrophobic foam. (c) The oil absorbing capability of the PTFE membrane employed in this work. An aqueous solution of red dye mixed with FC-40 oil is pipetted on to the film; the oil is absorbed by the film following which the aqueous droplet is pipetted off.

Figure 5.3: Schematics of droplet microchip interface devices. (a) and (b) detail the top view of the interface with chip-based and capillary based separation channel respectively (c) The side-on view of the droplet interface device at the intersection of the droplet delivery tubing and the separation channel. The aqueous droplets carried by the oil are delivered to the intersection between the open electrophoresis microchannel and the droplet delivery tubing. The oil flows out of the tubing and is absorbed by the PTFE membrane. The aqueous droplets arrive at the intersection and jump into the hydrophilic separation channel.

Figure 5.4: The effect of Duxback on the buffer loading process in the glass bottom droplet to CE interface device. Red food dye was added to the buffer to enable visual monitoring of the loading

process. The edge of the glass slides and the droplet delivery tubing are highlighted with the aid of dotted lines in both images. (a) In the absence of Duxback the buffer leaks into the separation channel. (b) The buffer moves directly into the separation channel when the edges of the slides are coated with Duxback.

Figure 5.5: Loading of CZE running buffer into the droplet to CE interface. The device has two PDMS bottom layers, a single separation channel ($100\ \mu\text{m} \times 100\ \mu\text{m}$) top layer, with a 3 mm gap at the interface. The gap is situated near the inlet buffer reservoir and enables the droplet delivery tubing to access the separation channel. (a) The open section of the separation channel prior to buffer loading. A red dye is used to monitor buffer loading, and the buffer is loaded from the outlet end of the separation channel. (b) and (c) show the buffer approaching and crossing of the open section of the separation channel. (d) An expanded view of the set up, with the separation channel now completely filled with red dye. All components of the device are labelled. (f) Provides a clear view of the placement of the droplet delivery tubing under the separation channel while (e) shows the device once the inlet reservoir has been filled.

Figure 5.6: Modified droplet microchip electrophoresis device. The PDMS channel is replaced with a bare fused silica capillary. The main component of this device and the stage used for both PDMS and capillary device are labelled and listed. (a) The PDMS device used to connect the droplet delivery tubing to the capillary. The PDMS device is filled with $\times 0.1$ TBE buffer and mounted on the PTFE film. A detection window is burnt in to the capillary from the point of insertion prior to conditioning and loading the capillary with separation buffer. The outlet end of the capillary is placed in a micro-centrifuge tube which operates as the buffer waste reservoir. (b) The dual electrode microchip stage designed for the interface separations. The droplet delivery tubing is mounted on to the PTFE membrane (highlighted in red). The PDMS capillary device is then placed on top of the droplet delivery device such that the mouth of the tubing lies under the separation channel. This is held in place using the acrylic top plate, which has a circular opening to allow access to the inlet reservoir. The electrodes are inserted into the reservoirs at either end and a conductivity check is conducted. If successful the device is ready for use.

Figure 5.7: Overall experimental workflow for droplet to CE interface device. The PDMS interface device and capillary are prepared, loaded with separation matrix and then connected. Following which the entire device is placed above the droplet delivery tubing and a voltage is applied across the length of the separation channel. A stable voltage and current read-out is an indicator of good connectivity and absence of bubbles in the separation channel. Droplets, prepared earlier and stored in tubing, are connected to the droplet delivery tubing and pumped towards the interface device. Droplet injection is monitored at the interface to ensure that injection into the separation channel occurs efficiently at the set oil flow rate. For separation of complex mixtures with wide molecular weight dynamic range, droplet injection should be controlled to allow separation between components of a single droplet before the next is injected. This will prevent co-elution of bands from neighbouring droplets. Finally, detection is carried out several centimeters from the point of injection.

Figure 5.8: The traditional cross piece injector format microchip electrophoresis device. (a) A schematic of the cross piece device while (b) shows the typical pinching injection used to deliver a discrete sample plug in to the separation channel. See Chapter 4 for more details.

Figure 5.9: Sample injection using droplet CE interface device. (a) A top down schematic of the interface device. (b), (c), (d) and (e) show the delivery, jumping and movement of a FITC loaded aqueous droplet into a PDMS separation channel. The droplet arrives at the intersection, the carrier phase is absorbed into the PTFE film while droplet remains suspended beneath the channel. Further oil flow lifts the droplet connecting it to the liquid in the separation. The charged analyte and surrounding water is dragged into the channel under the applied field. In the absence of the field the droplet jump is pushed towards the inlet reservoir due to the pressure difference between the inlet and outlet reservoir. (f) The plot recording droplet jumping over time.

Figure 5.10: Current trace for channels with gaps between 0 mm and 12 mm. (a) The current trace obtained when there is a gap of 6 mm and 12 mm and for no gap (0 mm). (b) The RSD value for current versus the gap size. The variation observed with an open section up to 3 mm in length is similar to that observed in a completely sealed channel.

Figure 5.11: Effect of air bubbles on the droplet CE interface. (a) Illustrates the passage of a air bubble across the droplet interface. The emerging air bubble does not enter the separation channel but dissipates into the surrounding atmosphere once the oil containing is absorbed in to the PTFE membrane. (b) The voltage and current plots taken during the expulsion of the air bubble; both voltage and current values remain stable.

Figure 5.12: Images (a) to (d) illustrating the effect of large droplets on the injection. The droplets feed into the continuous flow electrophoretic channel and fractionate into several small plugs. These injections are often insufficiently spaced to allow all the components of each sample plug to resolve without overlap. Injections 1, 2 and 3 detailed in images (b), (c) and (d) respectively are separated by 0.5 seconds each.

Figure 5.13: Free solution separation of fluorescent dyes, Eosin Y and FITC using a single channel droplet interface device. (a) The electrophoretic profile obtained for seven consecutive multiple injections. (b) Mobility versus injection number plot for the Eosin Y and FITC. Separation was performed in x0.1 TBE under an applied voltage of 266.66 V/cm.

Figure 5.14: Free solution separation of fluorescent dyes, Eosin Y and FITC using a dual channel droplet interface device. (a) A fluorescent image of the separated bands on both channels. (b) An overlay of the electrophoretic profiles obtained from both channels. (c) Mobility versus injection number plot for the Eosin Y and FITC on both channels. Separation was performed in x0.1 TBE under an applied voltage of 436.36 V/cm.

Figure 5.15: Gel separation of a mixture of FITC and Eosin Y on the droplet CE interface device, using a 2.5% PEO (5MDa) in x0.1 TBE sieving matrix. (a) and (c) show the multiple injection of the mixture detected at 4 cm and 1.5 cm from the injection point respectively. (b) A closer view of two consecutive injections, each set consists of 7 peaks. (d) The CE separation of each dye in 1.75% PEO (5MDa) in x1TBE sieving matrix using UV absorbance detection. Separations were carried out under a field strength of ~ 253.9 V/cm on the droplet CE interface device, and ~ 203 V/cm on the CE instrument.

Figure 5.16: Comparison of different results obtained using standard techniques like capillary electrophoresis and Slab gel electrophoresis with those obtained on the microchip format. Separation of Roche marker VIII dsDNA restriction fragments (Hoffman La Roche, $0.034 \mu\text{g}/\mu\text{l}$) labelled with SYBR Green I ($50 \mu\text{g}/\text{ml}$). Droplets of the labelled dsDNA step ladder were generated using the robot and injected in to the separation channel at a flow rate of $0.05 \mu\text{l}$ per minute. (a) The electrophoretic profile obtained in a bare fused silica capillary of total length 5.5 cm using the droplet interface device. Total length of the separation channel from inlet to outlet reservoir was 6.3 cm. Detection was carried out ~ 3.2 cm from the point of injection. (b) The separation of the same sample on a standard PDMS microchip. Total length of the separation channel on-chip was 6 cm and the effective length was 4.5 cm. (c) The capillary separation of the same unlabelled ladder in a capillary of total length 54cm and effective length 42 cm. Separations were carried out under a field strength of ~ 253.9 V/cm on droplet CE interface device, ~ 166.6 V/cm on microchip and ~ 203 V/cm on the CE instrument. Both chip separations analysis were carried out in a 2.5% PEO in x0.1 TBE sieving matrix. The CE instrument separation was carried out in a 1.75% PEO (5MDa) in x1 TBE. Both capillaries were pre-coated with 10% PVP prior to loading with the sieving matrix. (d) The separation of the same ladder in 1.8% agarose as provided by the manufacturer (Roche). The band molecular weights and assigned peak numbers are also provided. (e) The ratio of peak height or fragment concentration to fragment size.

Figure 5.17: Log $-\log$ plots of mobility versus fragment size for droplet interface electrophoresis. (a) Results for Marker VIII (Roche). (b) The curve obtained for 3 consecutive injections of the 50 bp dsDNA step ladder. Both chip separations were carried out in a 2.5% PEO in x0.1 TBE sieving matrix and the capillaries employed were pre-coated with 10% PVP prior to loading with the sieving matrix. The Roche ladder and 50bp dsDNA step ladder were separated under a field strength of 253.9 V/cm and 246 V/cm respectively.

Figure 5.18: Multiple injection and separation of 50 bp dsDNA ladder (Promega, 0.034 $\mu\text{g}/\mu\text{l}$) labelled with SYBR Green I (50 $\mu\text{g}/\text{ml}$). Droplets of the labelled dsDNA step ladder were generated using the robot and injected in to the separation channel at a flow rate of 0.02 μl per minute. (a) The electrophoretic profile obtained for the multiple injection of the sample in to a bare fused silica capillary of total length 1 cm using the droplet interface device. Total length of the separation channel from inlet to outlet reservoir was 6.5 cm. Detection was carried out ~ 1 cm from the point of injection. (c) The capillary separation of the same unlabelled ladder in a capillary of total length 54cm and effective length 42 cm. Separation was carried out under a field strength of ~ 246 V/cm on droplet CE interface device and ~ 203 V/cm on the CE instrument. For both capillary separations analysis was carried out in a 2.5% PEO in x0.1 TBE sieving matrix and the capillary was pre-coated with 10% PVP prior to loading with the sieving matrix. (b) The separation of the same ladder in 2% agarose as provided by the manufacturer (Promega). The band molecular weights and assigned peak numbers are also provided.

Figure 5.19: Co-elution of peaks due to matrix degradation. Multiple injections lead to stretching of matrix pores, consequently there is a loss in resolution between the shorter DNA fragments.

Figure 5.20: (a) Separation profile of four consecutive droplet injections containing sample with different ratios of Fluorescein and Eosin Y. Samples were prepared as detailed in the text. Change in sample concentration is reflected in the signal obtained. (b) The plot of peak amplitude versus droplet number. Separation was performed in 2.5 % PEO (5MDa) in x0.1 TBE sieving matrix with a total channel length of 6.5 cm and effective length of 0.5 cm. The inserted capillary was conditioned with 1M HCl and 10% PVP prior to loading the sieving matrix.

Chapter VI

Figure 6.1: Schematic of the Nano-LC MALDI-MS droplet interface device. The top panel details the interface device which was fabricated in PDMS. Water-in-oil droplets were generated from oil and aqueous solutions using the T-junction microchip having two aqueous inlets, one oil inlet and an outlet. The LC effluent was transferred in to the device via a silica capillary which was inserted into the side of the PDMS device using a specially designed channel. The second aqueous inlet was used to deliver MALDI matrix. FC-40 oil was used as the carrier phase. The LC effluent and MALDI matrix meet at the T-junction and are then pumped into the oil stream where they are broken into droplets due to the shear force of the oil. The S-bends in the device are used to induce rapid mixing between the LC-effluent and the MALDI matrix. The lower panel illustrates the components forming the deposition probe. These include the 200 μm internal diameter Teflon tubing and the oleophilic PTFE membrane. Droplets generated in the interface device are transported to the MALDI target using the Teflon tubing. The tubing is wrapped with the PTFE membrane which extracts the oil leaving the aqueous droplet suspended at the tip of the tubing. This droplet is then spotted on to the MALDI target via contact deposition.

Figure 6.2: Peptide calibration ladder analysed prior to MALDI mass spectrometry analysis. The sample contained 5 peptides listed in the text above and was prepared in 0.1% TFA. Each peak is labelled with the expected mass value.

Figure 6.3: Droplet malformation in PDMS microdevice. The left and right panels are separated in time with the right showing the progress of droplet formation 2 seconds after the left. The mix of MALDI matrix and protein solution showed better wettability compared to the oil phase, generating oil in water droplets at the T-junction of the microdevice. The oil droplets migrate towards the outlet driven by the flow of sample and matrix. On entering the Teflon tubing inserted at the outlet the condition reverses to 'normal' with the formation of water in oil droplets. Within the Teflon tubing the oil phase showed greater wettability compared to the mixture of matrix and sample leading to the reversal.

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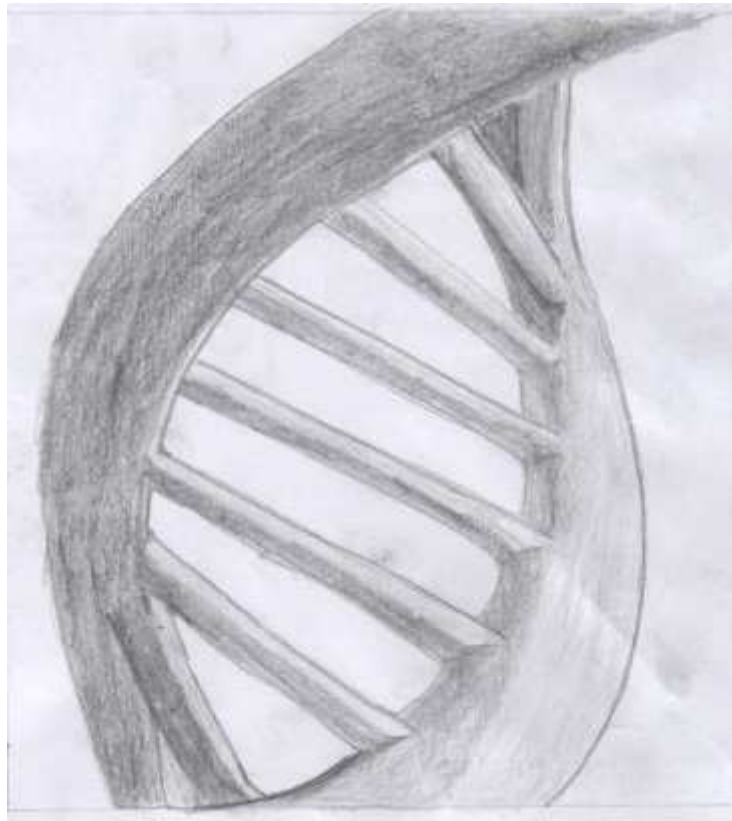
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Chapter I: Introduction



“It is a capital mistake to theorise before one has data. Insensibly one begins to twist facts to suit theories instead of theories to suit facts.”

Sherlock Holmes

1.1 Biology at the Microscale

In 1665 Robert Hooke published the *Micrographia* [1] in which he coined the word “cell” to describe the basic unit of life. This book also contains illustrations of his observations using a microscope. Since then man’s fascination with life on the microscale has not waned. In fact, experimentation at this scale has led to many important discoveries. Louis Pasteur discovered the cause of fermentation, developed pasteurisation and a vaccination for Rabies by examining samples under a microscope. Robert Koch, in his publication the *Aetiology of traumatic infective diseases* [2], described the use of several newly developed microscopic lenses including an oil-immersion lens. With these additions to his microscopy arsenal he discovered bacteria causing septicaemia, anthrax and gangrene. Consequently, biology and its development has been inextricably linked to analysis at the micro-scale. In recent years, research has shifted from simple observation to manipulation of disease causing agents and cellular responses to them. The accessibility of microscale tools has enabled efficient analysis of lower sample volumes in an automated fashion and at high speed. Disease mechanisms can now be traced and examined down to the level of proteins and DNA.

1.2 From DNA to Proteins

Proteins and DNA have been the subject of intense study for most of the last century. These biomolecules, often described as biopolymers because of the repeating nature of their structure, are involved directly or indirectly in almost every aspect of cell structure and function. Moreover, pathogenic processes cause alterations to the normal pattern of behaviour of these molecules, leading to changes in their structure, function or interaction with other molecules. The fields of genomics and proteomics have evolved to establish ‘normal’ conditions for DNA and protein respectively. They also identify and understand the changes to biomolecules brought about by disease. This information guides therapeutic development and is extremely valuable.

One of the canons of molecular biology describes the mechanism of information transfer between proteins and DNA. It was first articulated by Francis Crick in 1958 [3] and re-iterated in another paper published in *Nature* in 1970 [4]. The canon is known as the central dogma of molecular biology and explains the unidirectional flow of information from DNA to RNA to protein.

Information is stored in a sequential manner within the structure of DNA which is then transcribed to messenger RNA within the nucleus of the cell (Figure 1.1a). Transcription occurs under the aegis of several enzymes which help to unwind (Helicases) and transcribe (RNA Polymerase) the helix in the 5' to 3' direction. The information stored within the RNA sequence is translated to amino acids which are the building blocks of proteins. The translation and tethering of the amino acids to form a protein is carried out by the cellular organelle called the ribosome (Figure 1.1b). During translation, messenger RNA (mRNA) produced via transcription is decoded by the ribosome to produce a specific amino acid chain that will later fold into an active protein.

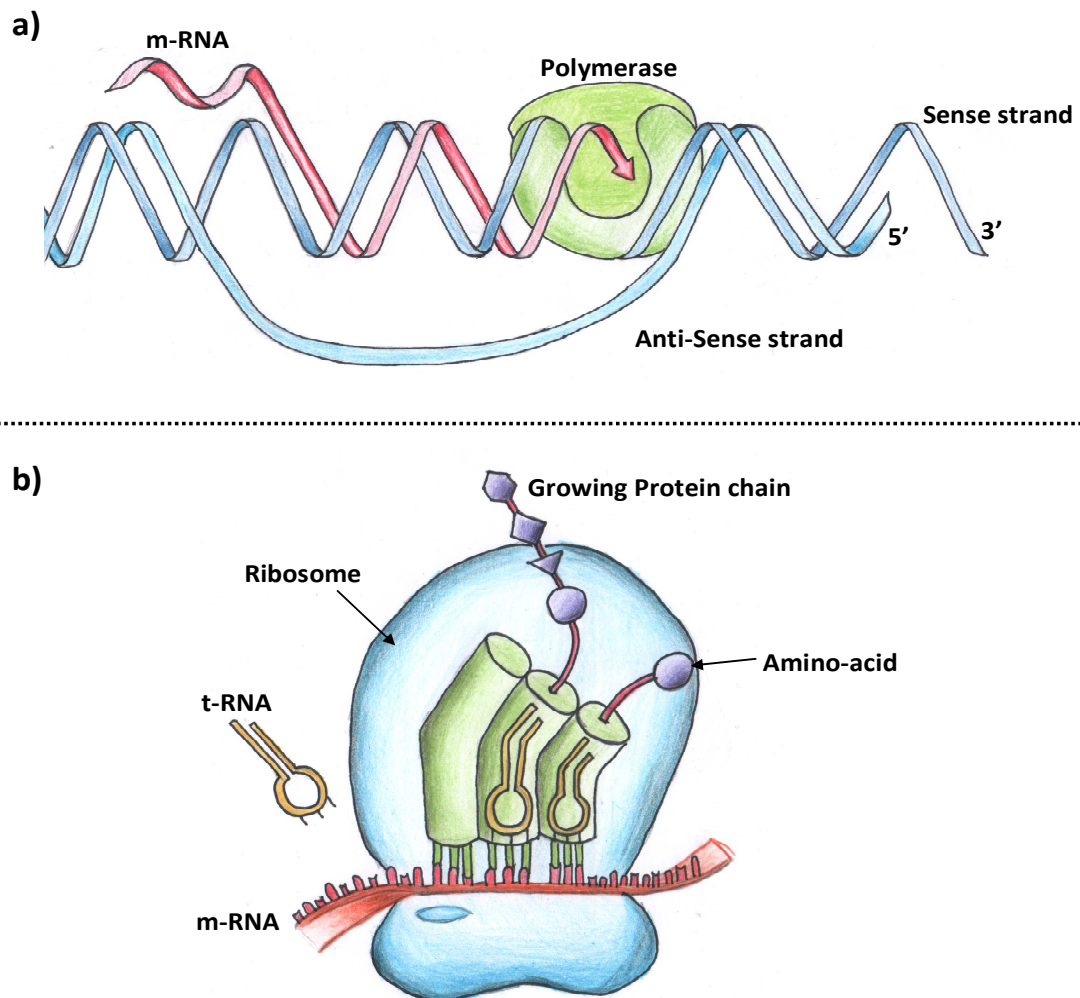


Figure 1.1: Transcription and Translation. The amino acid sequence for every protein is stored in the cell in its DNA format and converted via transcription and translation when required. (a) Illustration of the transcription process, where a DNA template is read to its RNA format and transported outside the nucleus. (b) The translation process where the RNA generated in the nucleus is translated to a specific sequence of amino acids which can form a protein. The RNA is read by a cell organelle known as a ribosome, which catalyses the formation of the peptide bond between the appropriate amino acids.

1.2.1 Deoxyribonucleic Acid

There are two major types of nucleic acid, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). DNA is mainly found in the cell nucleus but also occurs in the mitochondria and chloroplasts of all eukaryotic cells. In 1869, Friedrich Miescher found that the nucleus of a cell contained a mixture of compounds that he labelled nuclein [5]. Over the next 100 years advances in understanding the structure and relationship of nucleic acid to other cellular components were made by several scientists. In 1902, Archibald Garrod described the human disease alcaptonuria as having a Mendelian recessive trait, therefore associating a disease with a mutated gene. In the same year Walter Sutton and Theodor Boveri proposed the chromosome theory of inheritance, linking genes to chromosomes. T. Morgan and C. Bridges in 1910 and 1916 provided physical evidence proving the theory that genes were located on chromosomes. In 1944, O. Avery, C. McLeod and M. McCarty identified DNA as the material that genes were constructed from [6] and almost ten years later, in 1953, J. Watson and F. Crick with the help of R. Franklin and M. Wilkins determined the structure of DNA to be a double helix [7, 8].

The diameter of a typical human cell is 10 μm while the length of DNA if isolated from a single human chromosome is approximately 3 cm long. Therefore, in order to fit within a cell DNA is stored in a highly compact and organised structure called a chromosome. A schematic of the packing of DNA into a chromosome is illustrated in Figure 1.2 and is described in several reviews [9-11]. Briefly, linear DNA is wrapped around a protein core forming a bead-like body known as a nucleosome. Approximately 146 nucleotide pairs wrap around the each bead and linker DNA (about 60 base pairs (bp) long) connects adjacent nucleosomes. The protein core is formed of a group of basic proteins known as Histones. The fibre formed from a length of DNA wrapped around several protein complexes has a bead-like appearance and is known as chromatin fibre. This fibre is packed in on itself forming a thicker chromatin fibre about 30 nm in diameter. Further packing leads to a solenoid shaped ultra-structure 300 nm in diameter and then a super-solenoid structure 700 nm wide. The super-solenoids are further condensed to take the shape of the widely recognised chromosome. Super-coiling permits the extensive genetic information required to maintain and reproduce cellular life to be stored and accessed within each cell [6].

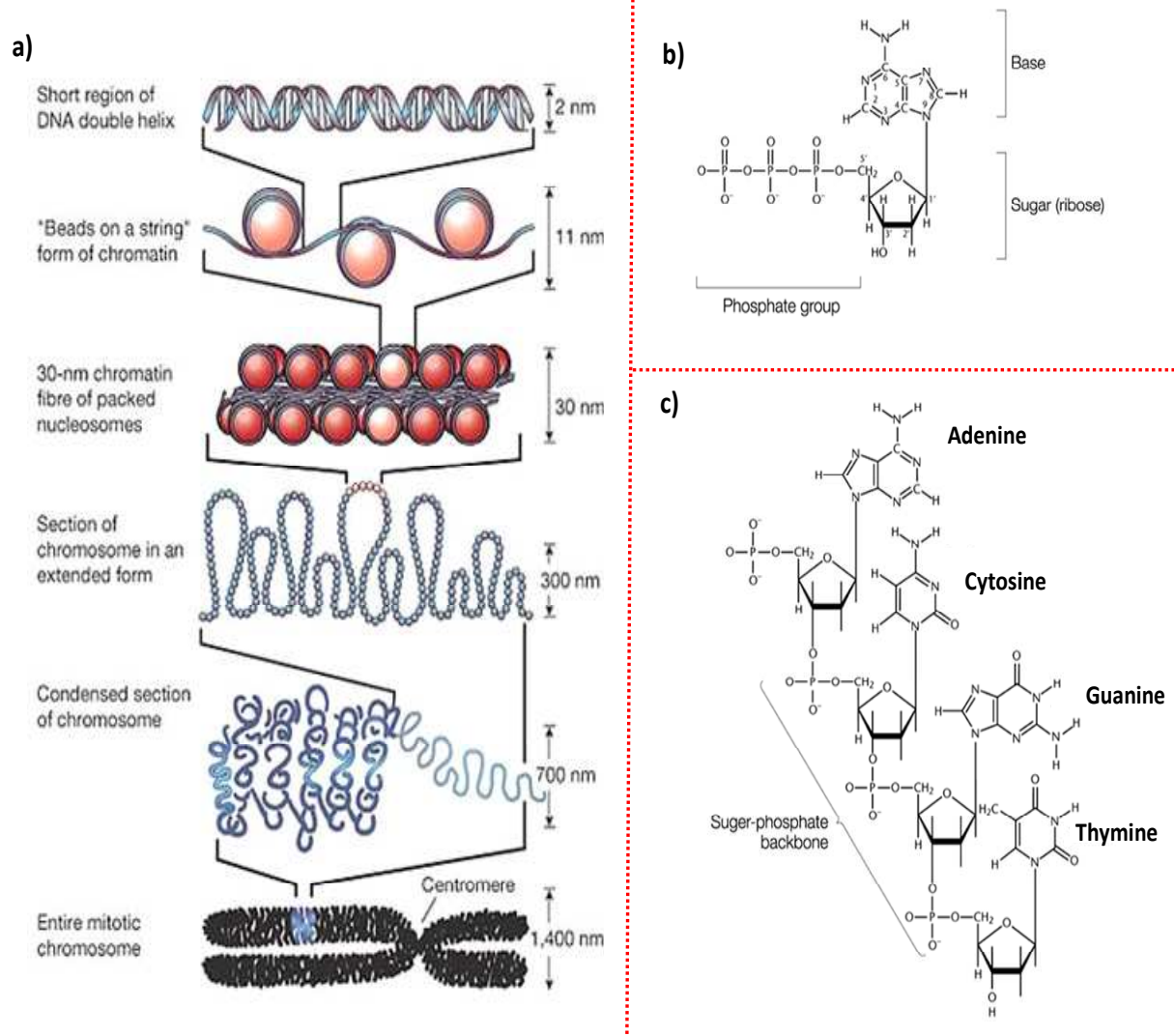


Figure 1.2: The structure of DNA from chromosome to double helix. (a) A schematic of the levels of packing and coiling applied to the DNA helix to store it within the nucleus of the cell. The DNA helix itself is constructed from nucleotides. (b) The chemical structure of the basic components of a nucleotide; a phosphate group, a sugar group and a nitrogenous base. Nucleotides are linked via a phosphodiester bond formed between the phosphate group and sugar group of adjacent nucleotides as shown in (c). Images (b) and (c) were reproduced from reference [12] while image (a) was reproduced from reference [13].

A DNA helix is composed of several thousand nucleotides linked together to form a polynucleotide chain. A nucleotide is composed of a nitrogenous base, a five-carbon sugar (either ribose or 2'-deoxyribose), and between one and three phosphate groups (Figure 1.2b)[14]. There are two types of nitrogenous bases, purines and pyrimidines. Pyrimidines are heterocyclic aromatic organic compounds. The two pyrimidine derivatives found in DNA are thymine and cytosine (Figure 1.2c). A purine consists of pyrimidine ring fused to an imidazole ring. The nucleobases adenine and guanine are the purine derivatives found in DNA (Figure 1.2c). Adjacent nucleotides

are connected by phosphodiester bonds. These are two covalent ester linkages formed by a phosphate group to link the 3' carbon atom of one five-carbon sugar to the 5' carbon atom of an adjacent sugar. During DNA replication or amplification, strand extension occurs in the 5'-3' direction, therefore phosphodiester bonds are also formed in this direction.

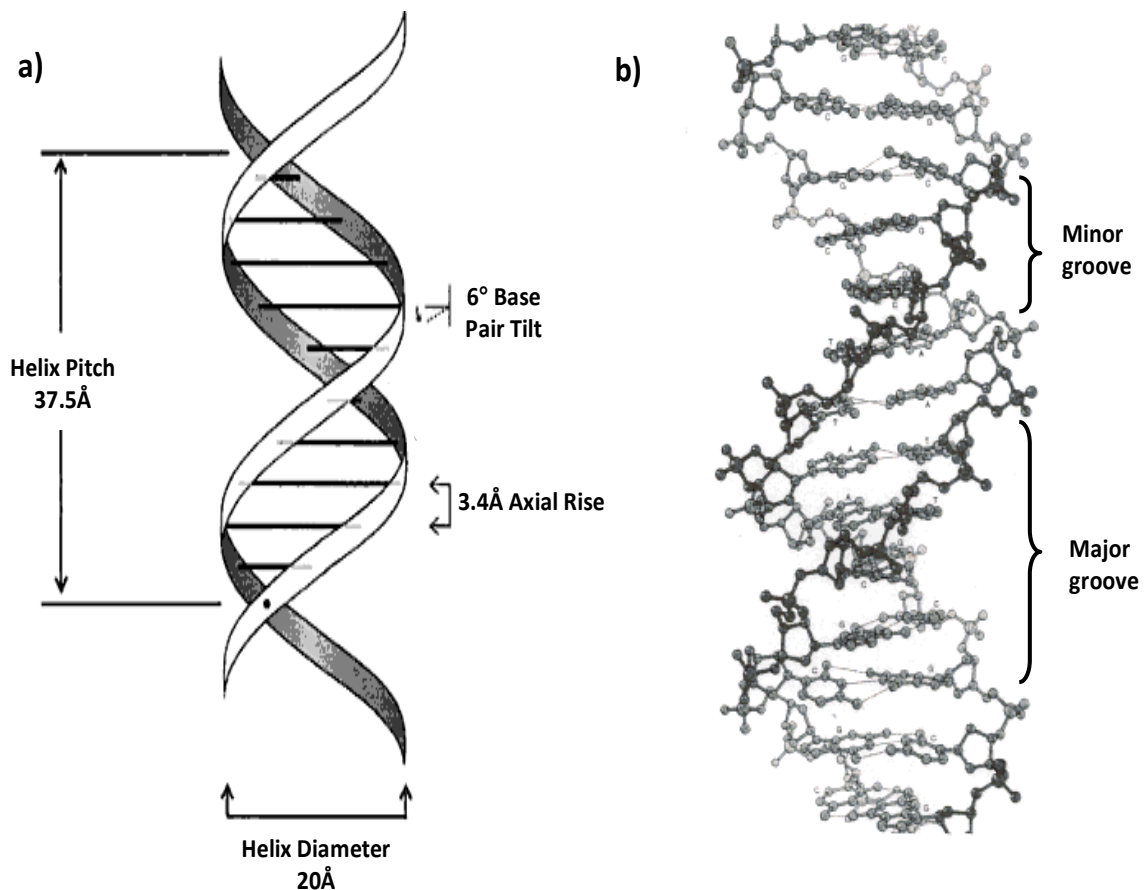


Figure 1.3: The structure of B-form double helix. (a) A schematic version of B-form DNA, detailing its helical pitch, helix diameter, axial rise, base pair tilt and rotation per residue. (b) A “ball and stick” representation of a B-form DNA helix. This format illustrates the orientation of individual components and the space they require to form a helix. The major and minor grooves which are characteristic of a B-form helix are highlighted. Images reproduced from reference [12].

Nitrogenous bases of the two strands are linked through hydrogen bonds between the oxygen and nitrogen atoms of adjacent bases. Since there is a defined distance between the two strands, the helix diameter is 20 Å and only specific base pairs can fit. A purine always pairs with a pyrimidine. Adenine pairs with Thymine via two hydrogen bonds and Guanine pairs with Cytosine via three

hydrogen bonds. Consequently, the two strands are complementary [7, 15, 16]. That is, if the base sequence of one strand is known the sequence of its partner can be deduced based on the specific pairing of the bases. In addition, to accommodate such base pairing the two polynucleotide strands have to run anti-parallel to each other (Figure 1.3a).

In nature the majority of DNA helices are right-handed. Several forms of right-handed DNA helices have been discovered and described, including, A, B, C, D and T forms [17, 18]. Helices are formed to protect the hydrophobic nitrogenous bases from the aqueous environment they are found in. The water soluble sugar and phosphate backbone surrounds the bases reducing access of water molecules. The double helical structure proposed by Watson and Crick describes the B-form helix [7, 15]. This is the most common form and dominant features include the major and minor grooves formed by its structure (Figure 1.3b). The major groove is 22 Å wide while the minor groove 12 Å wide. Other features include an axial rise (distance between adjacent planar bases) of about 3.4 Å per base and an average of 10.5 base pairs per helical turn. The rotation per residue which is the angle between adjacent base pairs is 34.3° in solution and the helix pitch which is the total length of one complete helical turn is 35.7 Å.

1.2.2 Why DNA Lends Itself to Electrophoretic Analysis

A common technique used to size single or double stranded DNA is gel electrophoresis. DNA can be denatured from its native double stranded helical structure using heat or chemicals to two single strands. Both forms of DNA can be transported under an applied field and separated using sieving matrices which offer a resistance to migration that is proportional to the length of the fragment. The charge acquired by DNA in solution is due to the presence of phosphate groups within its structure. The phosphate molecule has three hydroxyl groups and its pK_a in solution varies depending on solution pH. pH controls the number of hydroxyl groups that are deprotonated and hence the overall charge of the molecule. In DNA, phosphate groups are linked to sugar groups via a phosphodiester bond which engages two out of three hydroxyl groups. Therefore in solution, phosphate groups forming part of the helix backbone have a single deprotonated hydroxyl group and a pK_a of 2.1. Accordingly, they are negatively charged in solution at pHs above 3. As each nucleotide has one phosphate group, in solution it will have a single negative charge associated with it. Accordingly, DNA will have a constant mass-to-charge ratio, regardless of length and under an applied field will migrate towards the cathode. This characteristic

also prevents its analysis using capillary zone electrophoresis. The similar overall charge of DNA molecules of different length means that the molecules will migrate as a single band in the absence of a sieving matrix. Any small differences in mobility observed (in the absence of gel) may occur due to stoichiometric differences between the molecules. The addition of a gel permits a separation based exclusively on the molecular weight (length) of the DNA molecule.

The nitrogenous bases of DNA and RNA have an absorbance maximum at 260 nm. This enables their detection using ultra-violet (UV) spectroscopy. The extinction coefficient of single stranded DNA (ssDNA) is higher than double stranded DNA (dsDNA). This increase in optical density of a material is termed as hyperchromicity [19]. Denaturing a dsDNA fragment to its ssDNA form exposes the nitrogenous bases which are typically packed into the centre of the helix in dsDNA. Therefore, based on the Beer-Lambert Law, for the same concentration of dsDNA and ssDNA, a higher value of absorbance should be recorded for the ssDNA sample. UV absorbance has been successfully applied to the detection of both single and double stranded DNA primarily due to simplicity of the system employed. However, fluorescence detection has been established over the years as more sensitive technique [20]. Laser induced fluorescence (LIF) has enabled dsDNA detection in CE at 10^{-12} to 10^{-15} M [21] while the general limit of detection using direct absorbance is between 10^{-5} to 10^{-6} M [22, 23]. Fluorescence detection of DNA is facilitated by labelling the molecule with a fluorescent probe [24]. The fluorescent probe may be covalently attached to the terminal ends of the DNA fragment or reversibly linked through intercalation and groove binding [25]. Dye attachment to the major or minor groove of dsDNA is stabilised by hydrophobic, electrostatic and hydrogen bonding interactions. Intercalative binding occurs when a planar, polycyclic, aromatic moiety slides between adjacent DNA bases pairs. Intercalating dyes such as ethidium bromide, propidium iodide, SYBR Green I, SYBR Gold, Hoechst 33342 and 4'-6-diamidino-2-phenylindole (DAPI) are extensively used in DNA labelling [26-28].

The intercalative process, first described by Lerman in 1961 [29], affects the helical structure of B-form DNA by increasing its axial rise by 3.4Å and decreasing the rotation per residue. This has the effect of extending and unwinding the DNA helix and occurs as a consequence of accommodating the intercalator between the base pairs. The increase in length is directly proportional to the amount of dye bound. Additionally, electrostatic interactions between intercalator and adjacent base pairs via π - π stacking and dipole-dipole interactions stiffen the DNA helix [30]. Intercalation occurs via a two step process. The cationic intercalator when added to a solution containing DNA

is attracted to the anionic phosphate groups of the helix. Following which the aromatic rings of the intercalator are attracted to and slide into the less polar environment created by the hydrophobic nitrogen bases. Consequently, intercalating dyes are specifically attracted to the helical structure of DNA; however their concentration distribution across a DNA sample containing differing fragment length is random. Importantly, intercalation can affect DNA electrophoresis since it neutralises some of the anionic phosphate group altering the overall charge and therefore the charge-to-mass ratio. This affects both DNA fragment migration and injection [31, 32]. Based on **equations 1.7 and 1.15**, the dye-induced change in overall charge of the molecule will affect its mobility and consequently the concentration of the molecule injected. Additionally, the added dye increases fragment weight and can adversely affect DNA sizing.

1.3 Electrophoresis

Electrophoresis is defined as the differential migration of charged species under an applied electric field. This separation format was first demonstrated by Tiselius in 1937 [33], for the free solution separation of protein mixtures in U-shaped glass tubes. This format was superseded in the 1950s by zone electrophoresis which relied on a solid support, typically paper, for the separation of molecules. Electrophoresis now encompasses three main formats: slab gel electrophoresis [34, 35], capillary electrophoresis [36, 37] and microfabricated electrophoresis [38, 39]. The fundamental principles governing electrophoretic separation remain the same across all three formats. However, there are design-specific variations in both separation protocols and matrix development.

1.3.1 Capillary Electrophoresis

Capillary Electrophoresis (CE) is an efficient separation method that is widely used in biochemical and pharmaceutical research. Its application in these fields stems from its speed, high sample throughput, ease of automation, high separation efficiency, high precision and low sample volume. The first recorded demonstration of this technique was in 1967 [40], when Hjerten demonstrated a free solution electrophoretic separation using a capillary of 3 mm internal diameter. Work conducted by Mikkers, Everaerts and Virtanen [41-45], in the 1970s, verified that narrow bore capillaries reduced problems associated with convection. They successfully performed free solution separations in 200 μm diameter Teflon tubing without the aid of a solid support. In 1981

[46] and 1983 [47], Jorgenson and Lukacs published a series of papers which triggered the explosion in research and development in CE. They were the first to demonstrate the use of even narrower capillaries (75 μm internal diameter) which they used to separate a mixture of amino acids in glass capillary. There are two main differences between capillary electrophoresis and classical zone electrophoresis described by Tiselius. CE is accomplished in narrow bore capillaries and does not require a solid support to act as an anti-convective medium. These differences confer several advantages on the daughter technique. The narrow bore capillaries enable better heat dissipation which in turn allows application of higher voltages across the separation channel. The higher voltages reduce analysis time and improve component resolution and separation efficiency. The optical properties of the capillary also permit on-column, real time detection of sample bands.

Capillary electrophoresis has evolved into a family of techniques [36, 48] including capillary gel electrophoresis (CGE) and capillary zone electrophoresis (CZE) which were both translated from traditional formats. CZE also known as free solution capillary electrophoresis is the simplest form of electrophoresis and is used to separate cations, anions and neutral molecules. The separation is typically driven by an electrically induced bulk flow of solution within the capillary known as the electroosmotic flow (EOF). CGE is used to separate analytes based on their molecular weight and is generally applied to the analysis of proteins and DNA [37, 49, 50]. Another member of the CE family is capillary iso-electric focusing (cIEF)[51]. Here species (most notably proteins) are separated based on their pI values. By using an ampholyte solution, a pH gradient can be created across the length of the capillary. Under an applied field proteins will migrate to the pH zone at which they exhibit an overall neutral charge. Following focussing they can be mobilised across the detector by applying a pressure difference between capillary ends. Micellar electrokinetic chromatography (MEKC) also known as micellar electrokinetic capillary chromatography (MECC) employs ionic surfactants in the separation medium to afford phase separation [52-54]. Separation buffers are prepared with ionic surfactants above their critical micellar concentrations, leading to the formation of micelles with hydrophobic interiors. The sample components partition into the formed micelles depending on their hydrophobicity and are then mobilised past the detector. Consequently, CE enables separation of samples based on a variety of intrinsic characteristics that include molecular weight, surface charge, isoelectric point and hydrophobicity.

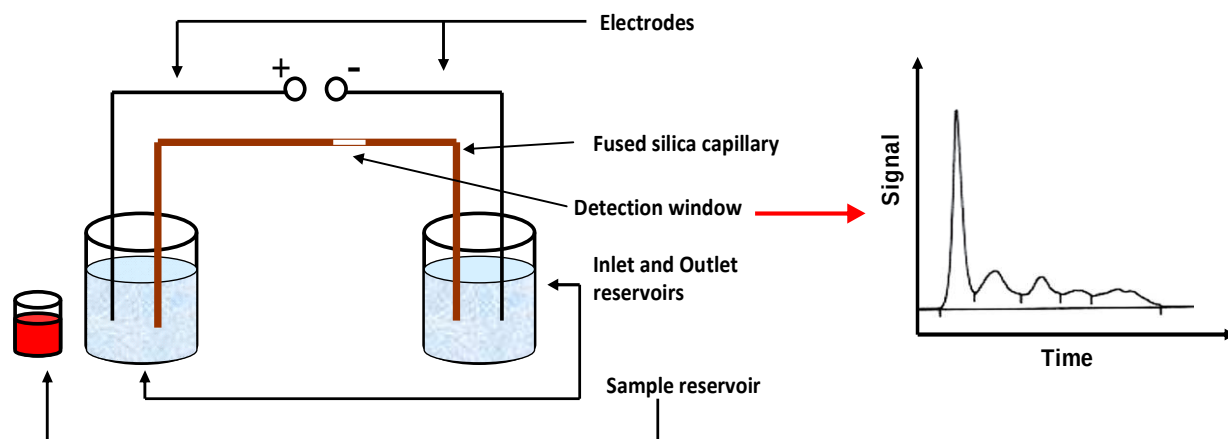


Figure 1.4: Basic schematic of a capillary electrophoresis instrument. Components include a fused silica capillary bridging two buffer reservoirs, electrodes to apply an electric field across the capillary and an appropriate detection system. The digital signal obtained is generally displayed in the form of an electropherogram.

A schematic of a CE system is shown in Figure 1.4 highlighting its basic components. A narrow bore glass capillary, typically between 20 and 200 μm internal diameter, is used to bridge two buffer reservoirs. Prior to separation the capillary is filled with sieving matrix following which the sample is injected into the capillary using a pressure differential or by applying a voltage across the capillary. The capillary end is then replaced in a buffer reservoir and a field applied across it. This induces migration towards the outlet and detector. The sample migrates through the capillary and separates into its constituent components. On traversing the detection window each component generates a signal. This signal is converted to a trace often called an electropherogram. The separated components of a sample, seen as peaks on an electropherogram, are characterised by their mobility (μ , $\text{cm}^2 \text{V}^{-1} \text{s}^{-1}$). Mobility is defined as the velocity (v) of a charged particle when placed under an applied electric field (E).

$$\mu = \frac{v}{E} \quad (1.1)$$

Often the recorded (apparent) mobility of an analyte is not its inherent mobility value. The inherent electrophoretic mobility (μ_e) of the analyte, which is based solely upon its overall surface charge and movement induced by the electric field, can be determined from its apparent mobility (μ_{app}) using the formula

Chapter I

$$\mu_e = \mu_{app} \pm \mu_{EOF} \quad (1.2)$$

Here μ_{EOF} is the electroosmotic mobility. Therefore depending on the charge of the analyte the mobility of the EOF can reduce or enhance its intrinsic mobility. The apparent mobility (μ_{app}) is typically determined from experimental data by calculating the velocity of the peak and the applied field using

$$\mu_{app} = \frac{L_s L_c}{Vt} \quad (1.3)$$

where L_s is the separation length of the capillary, L_c is the total length of the capillary, t is the peak migration time (in seconds) and V is the separation voltage. The electroosmotic mobility can be determined using the same equation and a neutral analyte which will migrate at a similar velocity to the EOF. Here μ_{app} is equal to μ_{EOF} and due to its neutral character under the applied separation parameters μ_e has a zero value. Inherent mobility depends on the balance of two forces acting on the charged particle, the electrical force favours forward motion while the frictional force acts against motion. The electric force (F_E) which depends on the charge of the particle (q) and the strength and direction of the applied electric field (E ; in V/cm) is given by

$$F_E = qE. \quad (1.4)$$

The second is an opposing frictional force (F_F) defined by

$$F_F = 6\pi\eta rv. \quad (1.5)$$

Here η is the viscosity of the medium (run buffer), r is Stokes' radius of the molecule and v is the ionic velocity. At equilibrium these forces are balanced ($F_E = F_F$) and the electrophoretic velocity (v) of the charged particle is given as

$$v = \frac{qE}{6r\pi\eta}. \quad (1.6)$$

Using **equation 1.1 and 1.6**, mobility can also be defined as

$$\mu = \frac{q}{6r\pi\eta}. \quad (1.7)$$

Consequently the electrophoretic mobility of an ion is dependent on its overall charge and size. The size of the charged particle of molecule is based on its molecular weight, three-dimensional structure and degree of solvation (hydration layer surrounding the molecule).

1.3.2 Electroosmotic Flow

In addition to electrophoresis, the other fundamental process occurring within the capillary under an applied field is electroosmotic flow. This is an electrically-induced bulk flow of solution, and in most modes of CE it is the driving force enabling the separation of positively charged, negatively charged and neutral molecules simultaneously. Silanol groups (SiOH) pKa 3.4, present on the internal surface of the capillary dissociate when treated with alkaline reagents to give the surface a negative charge (SiO⁻). Cations in the separation medium are attracted to the surface forming two layers collectively known as the electrical double layer (EDL) (Figure 1.5b). The voltage drop across the EDL is known as the zeta potential (ζ). Cations closest to the wall are immobile, even under an applied field and form the Stern layer. Further from the capillary surface a compact mobile layer forms with substantial cationic character, this diffuse layer is known as Gouy-Chapman layer. Beyond this layer the solution becomes electrically neutral. When a voltage is applied across the length of the capillary the diffuse cationic layer moves towards the cathode. As the cations are solvated by water, they drag the surrounding water molecules with them, inducing a bulk flow of solution.

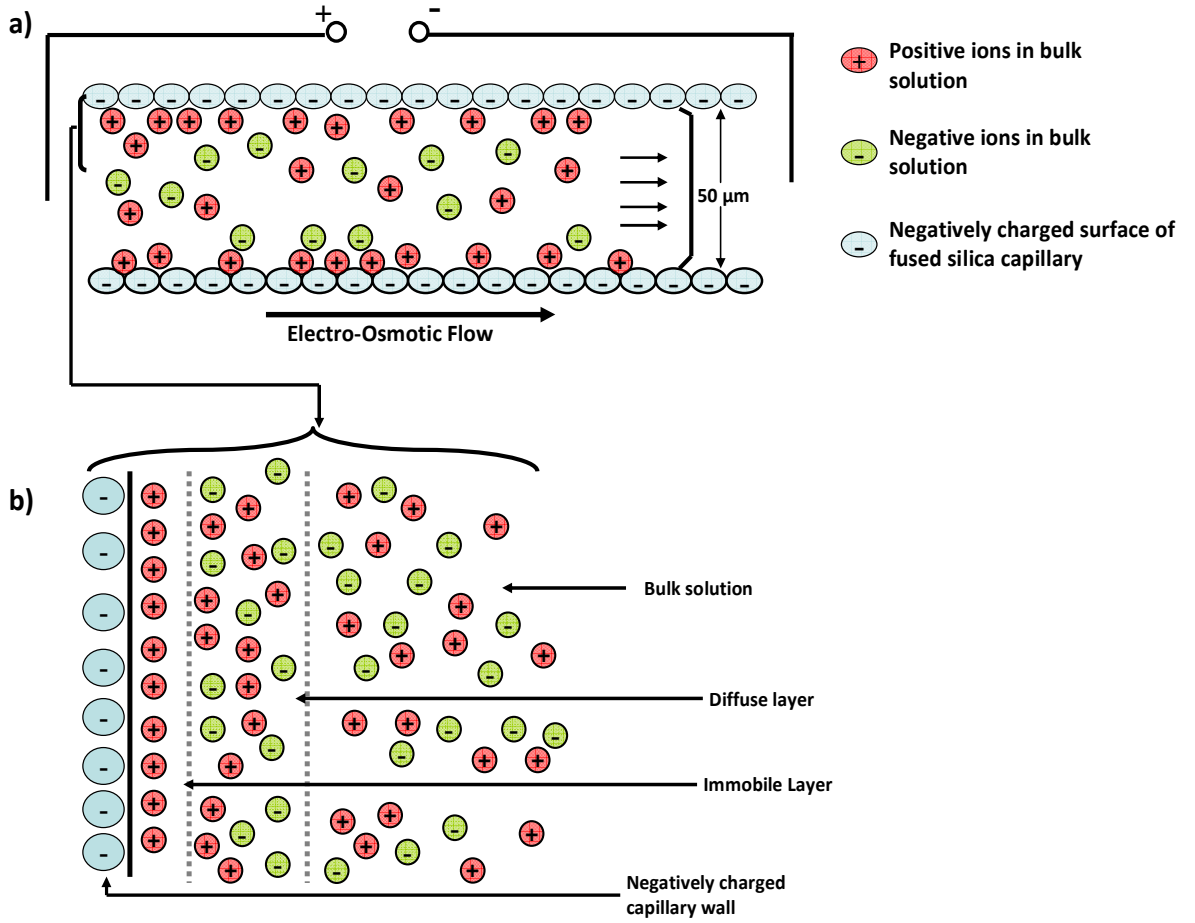


Figure 1.5: Schematic of the internal surface of a fused silica capillary depicting the formation of the electrical double layer. (a) Under an applied field the positive ions near the internal surface of the capillary move towards the cathode driving the entire solution in a similar direction. EOF generated in this way shows a flat flow profile. (b) A closer view of the molecular interaction occurring near the surface of the capillary. Once treated with 1M NaOH most of the SiOH groups are hydrolysed to SiO⁻ groups giving the surface a negative charge. Cations in the separation buffer migrate to surface to help balance the excess charge, once at the surface these ions form an immobile layer which does not move even under an applied field. A little further away from the wall a diffuse layer develops which is mainly cationic in nature. It is this layer that moves under an applied field generating an electroosmotic flow.

The thickness of the EDL is known as the Debye length (κ^{-1}), and is a function of the ionic strength (I) of the separation medium ($\kappa^{-1} = 0.304\sqrt{I}$). Although, the Debye length is typically only 100 Å thick the effect of its motion is transmitted across the diameter of the capillary. The velocity (v_{eof}) of the field induced flow was defined by Smoluchowski in 1903 as,

$$v_{eof} = \frac{\epsilon \zeta}{\eta} E \quad (1.8)$$

Where ε and η are the dielectric constant and viscosity of the buffer and ζ is the zeta potential at the solid-liquid interface. As indicated by **equation 1.8**, the EOF velocity is directly proportional to the applied field and inversely proportional to the viscosity of the buffer. Consequently, high separation speeds can be obtained by using high voltages and low viscosity separation media. As zeta potential values are not easily established, the strength of the EOF can be monitored by using a neutral marker (**equation 1.3**).

Capillaries can be treated with 1M sodium hydroxide (NaOH) prior to use and loaded with high pH buffers to induce strong electroosmotic flows. The alkaline environment ensures the surface silanol groups are completely ionised, generating a dense EDL and strong zeta potential. In uncoated bare fused silica capillaries, EOF always moves towards the cathode (Figure 1.5a) and the direction of the field is referred to as “standard polarity”. Besides the applied voltage, buffer pH and viscosity, several other factors influence the strength and direction of the EOF [55]. These include ambient temperature[56], buffer ionic strength, presence of organic modifiers[57], surfactants [58, 59] and polymers in the separation medium and the surface chemistry of the separation channel. All of these parameters are under direct experimental control except for the surface chemistry of the separation channel. Controlling and maintaining the surface chemistry of a separation channel is crucial when employing materials other than glass. This is demonstrated by the extensive research into controlling the surface chemistry of polydimethylsiloxane (PDMS)[60], the elastomer used to construct microfluidic separation channels[61].

1.3.3 EOF on Polydimethylsiloxane Surfaces

Polydimethylsiloxane (PDMS) is an elastomeric polymer used routinely in the fabrication of microfluidic devices[62]. The techniques used to fabricate PDMS devices are described in detail in chapter 2. However, its application to microfluidic electrophoretic separation devices along with other polymeric materials is surprising. EOF necessitates the formation of an EDL which in turn requires a surface charge density. These materials do not contain any charged or ionisable groups in their monomer units. Nevertheless, Ocuvirk and co-workers [63] demonstrated the presence of EOF in native PDMS microchannels and other authors have separated proteins[64], peptides and small molecules employing an EOF to drive them past the detector. In addition, Wheeler *et al.* [65] proved that EOF was not influenced by components in the curing agent and PDMS monomer.

The origin of the EOF on native PDMS surfaces is not clearly understood. One explanation offered by Bao *et al.* [66] suggests that cations and anions from the separation buffer adhere to the surface of PDMS generating EOF. Whatever the origins may be, one widely accepted fact is the inconsistency of EOF velocity over the length of the separation channel and over the period of the separation.

There are three general approaches to modifying PDMS surface chemistry, these include dynamic coatings with a cationic, anionic and non-ionic surfactants, adsorbed polymer coatings and a static permanent coating where ionisable groups are covalently linked to monomers on the PDMS surface[67]. Of the three, dynamic coatings are easiest to apply. Another method commonly applied to modify PDMS surface chemistry is treatment with an oxygen plasma [68]. This process is generally used to increase the wettability of the hydrophobic PDMS surface. Here the $-\text{O}-\text{Si}(\text{CH}_3)_2-$ repeat units of the PDMS material develop silanol groups ($-\text{OH}$) in the presence of an O_2 plasma with the loss of the methyl groups. Silanol groups are polar in nature and make the PDMS surface hydrophilic. In the presence of a high pH separation buffer these groups provide a strong EOF. However, over time the oxygen treated PDMS recovers its hydrophobicity due to the diffusion of un-oxidised low molar mass PDMS oligomers to the surface[69]. Hydrophobic recovery may also occur due to reorientation of hydrophilic groups from the surface into the bulk PDMS, therefore hydroxyl groups are replaced by methyl groups on the surface of the PDMS. Polymer substrates like PDMS may provide many advantages from the manufacturing point of view; however it is important to ensure that they also provide optimal performance from an operational standpoint. In microfluidic devices surface-to-volume ratios are larger and understanding the surface chemistry of the materials employed is critical to the success of the separation.

1.3.4 Separation Efficiency and Resolution

The performance of an electrophoretic separation is often characterised in terms of efficiency and resolution between analyte peaks. Efficiency is defined in terms of the theoretical plate number (N), which is a ratio between separative forces driving the partition of the sample components and dissipative forces which are responsible for band broadening and component mixing (Figure 1.6a).

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The application of theoretical plates (N) as a descriptor of CE efficiency was first introduced by Giddings in 1969 [70] as

$$N = \frac{L_s^2}{\sigma_{tot}^2}. \quad (1.9)$$

Where σ_{tot}^2 is the total variance of the width-integrated concentration distribution of the analyte band (Figure 1.6b) induced by dissipative forces. The number of plates required (N_{req}) for complete separation of two sample components is defined as

$$N_{req} = 16 \frac{1}{\left(\Delta v / \bar{v} \right)^2}, \quad (1.10)$$

here Δv is the difference in the peak migration velocities and $\bar{v}$ is the average peak velocity. Other related terms include plate height (H) and peak capacity (n_p). Peak capacity can be defined as the maximum number of peaks that could be observed if the entire separation length of the capillary were filled with consecutive peaks. Each peak should be baseline resolved from any adjacent peaks. The plate height characterises the dissipation induced by the sample plug per unit separation length [71].

Several factors influence the peak width variance which in turn determines the theoretical plate number and efficiency of the separation. Peak width is generally defined in terms of variance (σ^2) (Figure 1.6a and b) of the concentration distribution of the analyte under the peak. Factors contributing to peak width variance include diffusion (σ_D^2), initial peak width (σ_{inj}^2), detector width (σ_{det}^2), Joule heating and temperature variations ($\sigma_{\Delta t}^2$), surface adsorption (σ_{ads}^2) and turn geometry (σ_{geo}^2). The total variance (σ_{tot}^2) [72] is assumed to be the sum of the all variances contributing to dispersion and is defined as

$$\sigma_{tot}^2 = \sigma_D^2 + \sigma_{inj}^2 + \sigma_{det}^2 + \sigma_{\Delta t}^2 + \sigma_{ads}^2 + \sigma_{geo}^2 \quad (1.11)$$

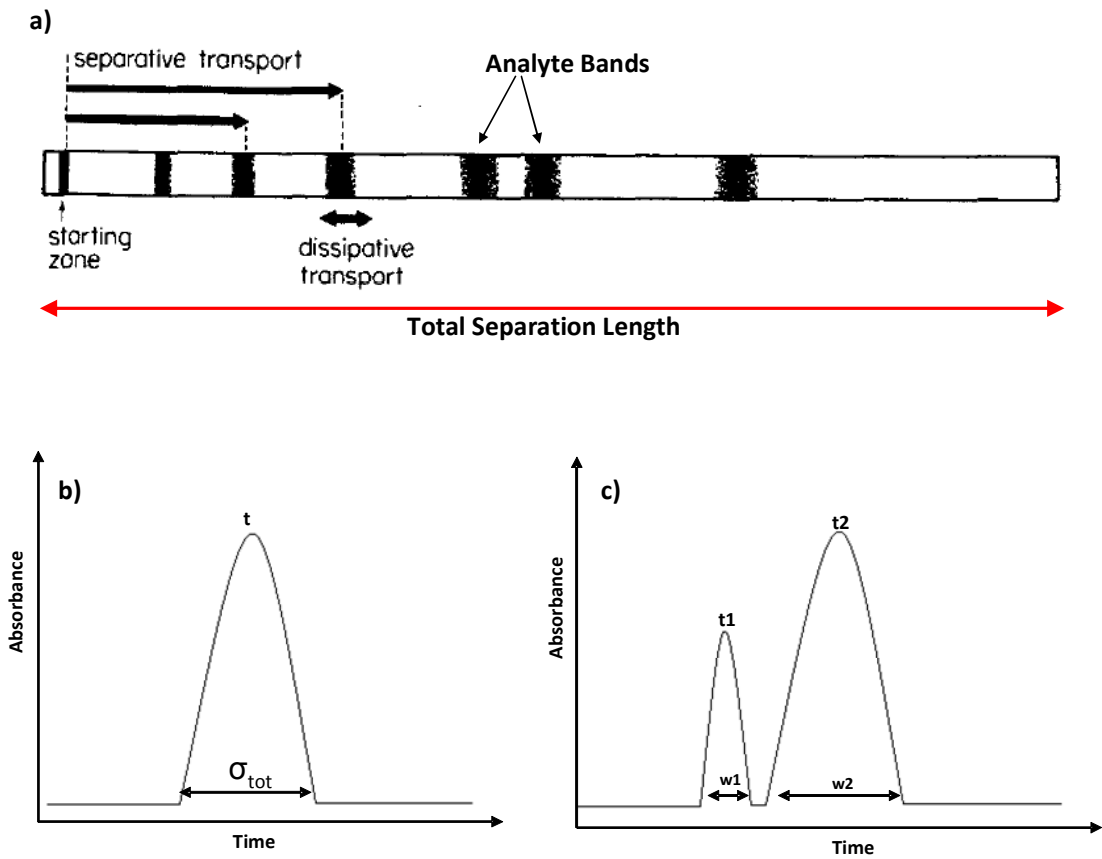


Figure 1.6: (a) Schematic of the effects of separative and dissipative transport on the width of an analyte band. Starting from the point of injection (starting zone) sample components resolve from each other as they move down the separation column towards the detector, this action is driven by the separative force. Simultaneously, dissipative forces act to increase the width of each analyte band reducing peak capacity, efficiency and resolution. Image reproduced from reference. (b) and (c) are plots of absorbance versus time illustrating σ_{tot} and peak components used to determine resolution respectively.

Separation processes generate concentration gradients between the analyte and the surrounding environment. These gradients vary between analytes depending on their concentration within the sample plug. Additionally, the gradients are typically out of equilibrium as the narrower the separating analyte band the steeper the concentration gradient and the greater the propensity of the gradient to dissipate [73]. Consequently, analyte band dissipation is driven by the tendency of entropy to maximise dilution. Entropy encourages this action through a variety of mechanisms as demonstrated in **equation 1.11**; however entropy most commonly exercises itself through the process of diffusion. The effect of molecular diffusion on peak width variance may be defined using

$$\sigma_D^2 = 2Dt \quad (1.12)$$

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Here D is the diffusion coefficient ($\text{m}^2 \text{s}^{-1}$) and t is the migration time of the analyte. Accordingly, the theoretical plate number (N) can be defined in terms of diffusion using

$$N = \frac{L_s^2}{\sigma_D^2}, \quad (1.13)$$

for a effective length of L_s . Therefore higher diffusion coefficient values lead to an increase in peak width variance and consequently a decrease in theoretical plate number and separation efficiency. Also t can be defined as

$$t = \frac{L_s}{v}. \quad (1.14)$$

Thus,

$$t = \frac{L_s L_C}{\mu V}. \quad (1.15)$$

Inserting **equation 1.12** and **1.15** into **equation 1.13** yields a new expression for N , that is

$$N = \frac{\mu V}{2D} \frac{L_s}{L_C}. \quad (1.16)$$

Here μ is mobility, V is applied voltage, D is the diffusion coefficient, L_s is the effective length and L_C is the total capillary length. **Equation 1.16** only provides an estimate of the efficiency of the separation as it does not take into account other factors contributing to peak broadening during separation (equation 1.11).

The resolution (R) is defined in terms of the peak width and peak time as shown in Figure 1.6c. Resolution values in this work were determined using **equation 1.17**, using the migration time difference ($t_2 - t_1$) between the peaks and w_1 and w_2 are the width at the base of each peak.

$$R = \frac{2(t_2 - t_1)}{w_1 + w_2} \quad (1.17)$$

1.3.5 Sample Preparation and Injection

As with any analysis technique sample preparation is often complex and causes significant variability in analytical results. Ideally, sample manipulation prior to CE analysis should be as simple as possible and objective oriented. Typical objectives for sample preparation of biological fluids are the removal or isolation of macromolecular components (specifically high abundance proteins[74]), the removal of salts, concentration of the analyte of interest and matching the sample buffer to the CE separation buffer. Automation of this procedure is driven by the need to make the process fast, safe and less prone to manual error. Methods for sample preparation include protein precipitation[75, 76], liquid-liquid extraction[77], solid-phase extraction[78] and membrane methods such as dialysis and ultracentrifugation[79]. While advances have been made in the automation of sample preparation for CE, it still remains the step which ultimately limits analytical throughput.

Designing and optimising an electrophoretic separation can be visualised using a Venn diagram. The first distinction (or set) can be made based on the mode of capillary electrophoresis best suited to the analysis. The second distinction depends on the nature of the sample which may include proteins, DNA, small molecules, peptides or RNA. Another distinction can be made depending on the source of the sample. Often proteins and DNA are isolated from a complex biological mixture prior to analysis. These extraction procedures may alter the nature of the biomolecule under analysis and the solution it is suspended in. A fourth set may define the objective of analysis. If sensitivity is paramount, samples can be for example prepared in low conductivity sample buffers to allow for field amplified capillary stacking[80], that will pre-concentrate the analytical sample. Or else fluorescent probes can be covalently linked to the sample prior to analysis to increase sensitivity. The intersection of all sets represents the minimum information needed to design an efficient sample preparation protocol.

Post sample preparation the sample is loaded into a sample vial or well-plate and then injected into the capillary. In CE, sample injection is typically carried out using one of two forms of injection, electrokinetic or hydrodynamic injection. A controlled and narrow sample plug into the capillary is critical for efficient analysis as it influences the initial peak width (σ_{inj}^2). The σ_{inj}^2 can be determined using

$$\sigma_{inj}^2 = \frac{l_{inj}^2}{12} \quad (1.18)$$

where l_{inj}^p is the initial plug width. Consequently larger sample plugs will contribute significantly to the increase in peak width variance (equations 1.18 and 1.11) and result in a decrease in theoretical plate number. Otsuka and Terabe [81] determined the maximum injection plug length (l_{inj}) and volume (V_{inj}), using a capillary of 50 μm internal diameter, for various separation lengths (L), plate numbers (N) and plate heights (H). The values they obtained are listed in Table 1.1, which demonstrate that obtaining higher theoretical plate numbers is contingent on small l_{inj} and V_{inj} values.

Parameter	N								
	100 000			500 000			1 000 000		
L (mm)	150	250	500	150	250	500	150	250	500
H (μm)	1.50	2.50	5.00	0.30	0.50	1.00	0.15	0.25	0.50
5% increase:									
l_{inj} (l_{det}) (mm)	0.37	0.62	1.24	0.17	0.28	0.56	0.12	0.20	0.39
V_{inj} (nl)	0.73	1.22	2.43	0.33	0.55	1.09	0.23	0.38	0.77
10% increase:									
l_{inj} (l_{det}) (mm)	0.53	0.89	1.77	0.24	0.40	0.79	0.17	0.28	0.56
V_{inj} (nl)	1.04	1.75	3.48	0.47	0.78	1.55	0.33	0.55	1.10

Table 1.1: Calculated maximum injection plug length and injection volume for a specific separation length. Table reproduced from reference [81].

The most common form of sample injection employs an electric field to move charged analyte molecules into the separation channel. This form of injection is easily applied on CE instruments and on chip-based electrophoresis devices. It is known as an electrokinetic injection. The number of moles (m) of analyte transferred into the capillary using this form of injection can be determined using

$$m = \mu_{app} \left(E \frac{\kappa_b}{\kappa_s} \right) t r^2 C \pi . \quad (1.19)$$

Where t is the injection time, C is the concentration of the analyte in the sample, r is the radius of the capillary, κ_b and κ_s are the conductivity values of separation and sample buffer respectively. From the above equation it is evident that the amount of analyte injected using an electrokinetic

injection is dependent on its mobility. Mobility is dependent on the charge, size and shape of the analyte (**equation 1.7**). Consequently, this format of injection is biased and the sample plug formed within the capillary does not accurately reflect the content of the original sample [82]. The amount of sample entering the capillary is also dependent on the ratio of separation and sample buffer conductivities. Field amplified sample stacking utilises this fact to pre-concentrate the sample. To minimise such biasing samples are often prepared in samples buffers whose conductivities match the separation buffer.

A hydrodynamic injection is performed by inducing a pressure difference between the two ends of the capillary. The volume (Vol) of sample injected is determined using the Poiseuille equation:

$$Vol = \frac{\Delta P d^4 t \pi}{128 \eta L_t} . \quad (1.20)$$

Here ΔP is the difference in pressure applied between the ends of the capillary, d is the internal diameter of the capillary, t is the injection time, L_t is the total length of the capillary and η is the sample viscosity. Therefore the main factors controlling the volume injected are the sample viscosity and applied pressure difference. Hydrodynamic injections allow quantitative analysis as a defined and reproducible sample volume is injected if identical injection protocols are applied.

1.4 Microfluidic Systems

The advent of microelectronic circuitry, in the 1950s led to the ‘Information Revolution’, which dramatically improved performance, functionality, and reliability, while reducing the cost and size of electronic devices [83-85]. More recently, the concept of miniaturization was applied to mechanical devices and promised similar benefits to the mechanical world as integrated circuit technology has given to the electrical world. While complex electronics provide the ‘brains’ for today’s advanced systems, micromechanical devices can provide the “the eyes and ears, hands and feet” which interface with the outside world. The term Micro Electro Mechanical Systems (MEMS), was coined in the 1980’s to describe new, sophisticated mechanical systems such as micro electric motors, resonators, gears, etc. [85]. Today, the term MEMS is used to refer to any

microscopic device with a mechanical function, which can be fabricated in a batch process. Microfluidics is a cross-disciplinary science that involves the design, manufacture and assembly of devices and processes that handle liquid volumes on the order of microlitres to picolitres (10^{-6} to 10^{-12} litres). The devices themselves have dimensions ranging from millimetres down to micrometres. A combination of microfluidics and MEMS emerged at the beginning of the 1980s and was used in the development of inkjet print heads, DNA chips, lab-on-a-chip technology, micro-propulsion, and micro-thermal technologies [84-86].

Miniaturisation of analytical tools in the life sciences is driven by a variety of motivations. These include rapid response times, low sample and reagent consumption, higher information content, and full or partial integration of sequential sample preparation and analysis steps [87-89]. Additionally, the behaviour of fluids at the microscale differs significantly from their 'macrofluidic' behaviour. Factors such as surface tension, energy dissipation, and fluidic resistance start to dominate [90]. For example, at larger scales fluids mix convectively and inertia asserts itself over viscosity. However, at the microscale fluids do not mix, indeed two fluid streams will flow in parallel down a microchannel (laminar flow) and mixing will occur only due to diffusion. Hence, microfluidic hardware requires construction and design methods that differ from macroscale hardware. It is not generally possible to scale down conventional devices and expect them to work in a microfluidic environment [84, 85].

1.4.1 DNA Analysis and Integration

As mentioned above, miniaturisation of bioanalytical techniques offers many advantages. The development of the micro total analysis system (μ -TAS) was first proposed in the early 1990s by Manz to describe the integration of whole processes on a planar, chip-based substrate. Since then the idea has blossomed and become a key component of biological and chemical analysis [91-94], point-of-care testing, clinical and forensic analysis [91, 95, 96], molecular diagnostics and medical diagnostics[97].

Biological assays currently involve a series of connected procedures where the sample is prepared and transferred manually between each procedure. This factor alone introduces several problems including sample loss, human error in sample preparation and inter-laboratory variations in preparation, results and analysis. Additionally, the instrumentation required for these procedures

are problematic in terms of footprint, cost and maintenance. Implementing a μ -TAS device circumvents these problems and improves the quality of an assay in terms of reproducibility, quantification and sample tracking. For example, a macroscale automated process for preparing, purifying, sequencing and analysing DNA samples was developed by the Joint Genome Institute. The process is initiated by a transformation step where cells are transformed with a pUC19 sequencing vector containing a ~ 1 kbp insert. Post transformation the cells are plated and grown for 18 hours. Following which they are picked using automated colony pickers and then amplified. Post amplification a sequencing reaction is performed using Sanger dideoxy dye-terminator chemistry. Samples are then purified from template and free dye-terminators using magnetic beads and analysed using capillary array electrophoresis. Figure 1.7 illustrates a flowchart of the process, detailing the total time for each process, instrumentation used and their approximate size. The estimated total time for complete analysis is 3 days using a fully functioning laboratory with the appropriate equipment. In a review in 2003, Paegel *et al.* [98] determined that using a single *Escherichia coli* cell and similar chemistry as described above, 15 nL of ready-to-inject product copies could be prepared at a concentration 10 μ M. Consequently, the biology and chemistry of these processes would not pose a bottleneck to the development of a μ -TAS device. Additionally, the injection plug on a CE system is less than 1 nL and even lower on a chip-based CE system, therefore sufficient volume can be generated from a single cell for multiple analysis. Furthermore, the authors find that each process employed in the conventional analysis has been executed using lower reagent volumes and smaller time scales on chip-based devices. They anticipate the design of an integrated sequencing lab-on-a-chip device (shown schematically in Figure 1.7), where an individual subclone is sorted from a transformed culture and then amplified. The amplicon is then purified and provides the template for Sanger sequencing. The extension products obtained from this process are isolated, purified and concentrated following which they are analysed using electrophoresis. Such a device would reduce analysis times by an order of magnitude and reduce reagent consumption by several orders of magnitude. Moreover the integration of these processes would eliminate manual sample transfer and related errors and increase throughput, reducing the overall cost of sequence analysis.

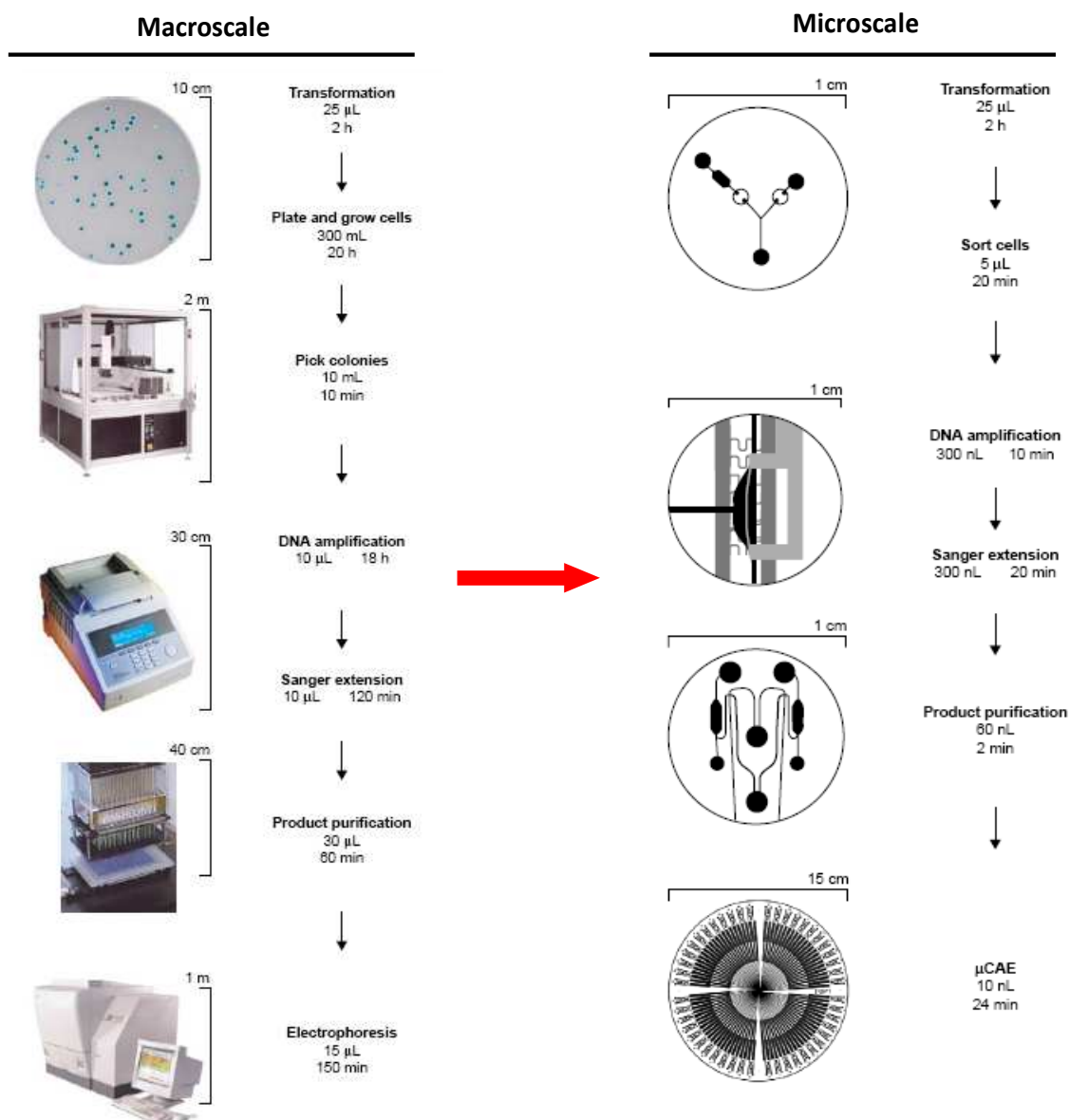


Figure 1.7: Flowcharts of conventional macroscale and integrated lab-on-a-chip device for DNA sample processing, sequencing and electrophoretic analysis. The images highlight the size, volume and time scales for each process performed at the macro- and micro-scale. Images reproduced from reference[98].

Genetic assays are excellent candidates for miniaturisation. Using essentially the same procedure (design) the genetic content of any organism can be probed. Consequently, the same μ -TAS device design may be used for samples of different origin without redesign. The human genome consists of approximately 25,000 genes [99, 100], all of which can be tested for mutations. Furthermore, there are at least 400 diseases which are diagnosable by molecular analysis of nucleic acids. Miniaturisation of these assays would reduce the cost of genetic information retrieval, making the

possibility of point-of-care systems real. Aside from clinical applications, genetic tests may also be applied to the detection of pathogens[101, 102], forensics[103, 104], agriculture[105] and farming.

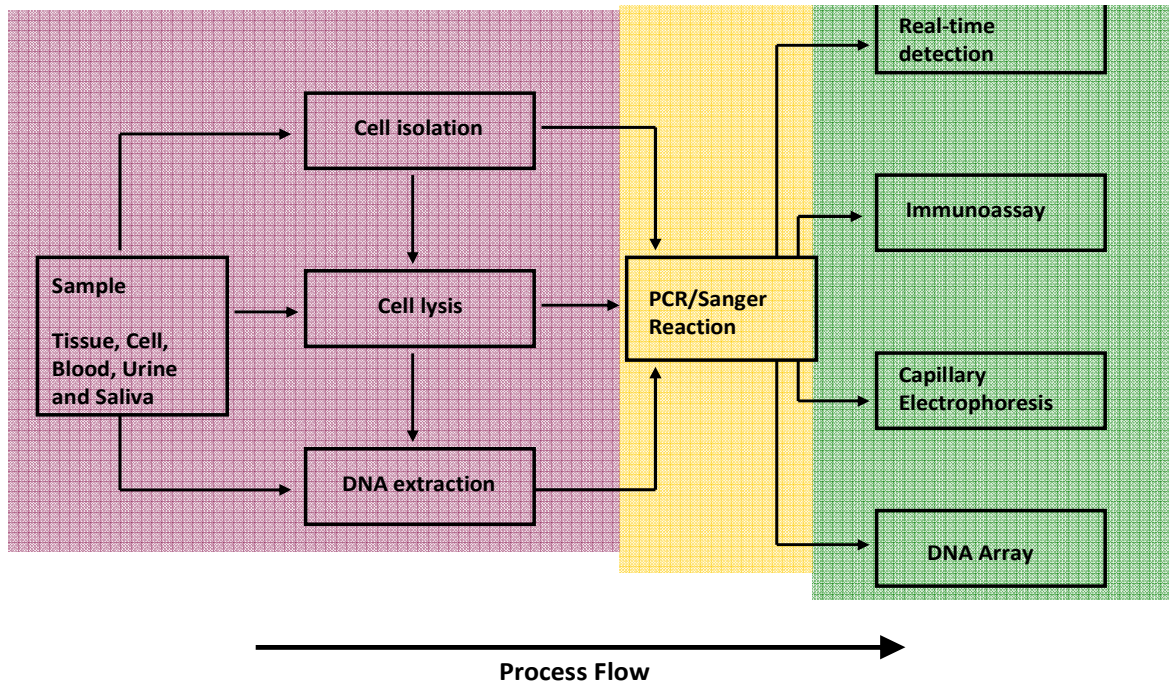


Figure 1.8: Connected processes involved in genetic analysis.

In terms of a μ -TAS device, the process flow of a genetic assay may be split into three major groups: sample preparation, DNA amplification via PCR or Sanger sequencing and product analysis [106]. Figure 1.8 illustrates the three major groups, processes within each group and their relationship with each other. Biological samples may be obtained in the form of tissue, blood, saliva, urine and skin cells. These samples are treated to extract the DNA and purify it prior to amplification. Amplification is usually performed using the polymerase chain reaction (PCR) which may be followed by a Sanger sequencing reaction. Post amplification several detection methods can be employed independently, sequentially or in-tandem. Various research groups have developed individual components of this process flow. For example, devices for cell sorting and isolation, based on size using filters and charge using electro-dynamic assemblies, have been described [107, 108]. Microfluidic devices for DNA extraction from isolated single cells have also been described. Solid phase extraction is the most common method employed on-chip for DNA or RNA extraction [109-111]. Here a solid phase with high affinity for DNA or RNA and low

affinity for proteins, typically in the form of beads or membranes, is appropriately positioned within the microdevice. The sample is washed over the material which selectively binds nucleic acid. The captured nucleic acid is released from the beads using buffers with different polarity. An alternative approach is isotachophoresis (ITP). This is a commonly used process where the sample is purified and pre-concentrated during sample injection. ITP focuses the sample between a 'leading' electrolyte and a 'trailing' electrolyte. Focusing occurs due to differences in ion mobility between the sample and the leading and trailing electrolytes. In 2006, Jung and co-workers [112] demonstrated a 2×10^6 fold increase in signal obtained from a ITP CE analysis of Alexa Fluor 488. The authors achieved a limit of detection of 100 fM for the same sample at a signal to noise value of 11.

After the DNA is extracted and purified, it is typically amplified before it is analysed. There are two basic formats for microchip DNA amplification which include stationary chamber PCR devices and continuous flow PCR devices. The former is the simplest chip-based PCR format, and was first demonstrated by Northrup and co-workers in 1993 [113] and Wilding *et al.* in 1994 [114]. In the continuous flow format the PCR mixture is moved through channel traversing heating blocks held at the three appropriate temperatures. The initial working version of this device was described by Kopp *et al.* [115] in 1998. A variation of the continuous flow format employs water-in-oil droplets, where the parent reaction mix is fractionated into droplets separated by an oil phase and then amplified. Typically the droplets are transported through serpentine channels traversing the required temperatures for DNA amplification. Compared to conventional systems both formats of chip-based PCR have smaller thermal masses which allows significantly higher temperature ramping rates, thereby increasing the rate of heat transfer between the temperature controller and the PCR reaction mix. This reduces the total amplification time from hours to minutes. Additionally these formats allow parallel amplification with minimal sample cross-talk and facile integration into a more complex μ TAS.

Detection and identification of amplified products may be carried out in several ways and depends on the objective of the genetic assay. The simplest method applied to quantify amplified DNA is real-time PCR. Here an intercalating fluorescent dye is added to the PCR reaction mix and the formation of the reaction products is monitored via dye binding. In 1998, Northrup and co-workers [116] developed the first miniaturised analytical thermal cycling instrument (MATCI) with real-time fluorescent detection. Using this device they demonstrated the amplification and

detection of Hantavirus, *Borrelia burgdorferi* (lyme disease causing spirochete bacteria), HIV RNA isolate and human β -actin target from human genomic DNA. Real time detection was achieved by monitoring the increase in fluorescent signal from two-dye labelled probes or ethidium bromide. DNA hybridisation is another common molecular biology detection technique that has been successfully miniaturised. Typically a library of gene sequences are immobilised on a surface in a specific sequence. A PCR amplicon may be identified by its specific binding to a known immobilised complementary sequence. Liu *et al.* [117] demonstrated on-chip PCR with hybridisation detection of both *Escherichia coli* and *Enterococcus faecalis*. Other groups have demonstrated hybridisation-based devices for the detection of mutations in the HIV genome and for genotyping of Chinese medicinal plants [118]. However, the most commonly applied method for detection and identification of amplified DNA fragments is electrophoresis.

1.4.1.1 Microfluidics and DNA Electrophoresis

Chip-based electrophoresis employing a sieving matrix can be applied to DNA analysis permitting fragment sizing, genotyping, DNA sequencing and mutation detection. The first chip-based CE device was demonstrated by Harrison *et al.* in 1992 [119] using CZE to separate fluorescent dyes Calcein and Fluorescein. The authors obtained theoretical plate numbers similar to those obtained for open tubular electrophoresis separations, indicating that even at this initial stage μ -CE could be optimised to deliver efficiencies obtained using conventional systems. They also demonstrated the effect of increasing injection plug length and applied voltage on the efficiency of the separation. The first chip-based CE separation of anti-sense oligonucleotides was demonstrated in 1994 by Effenhauser and co workers [120]. The authors separated fluorescently labelled single stranded DNA (ssDNA) on a PDMS device with an effective separation length of 3.8 cm under an applied field of 2300 V/cm. In the same year, Woolley *et al.* [121] demonstrated the complete analysis of the Φ x174 HaeIII DNA restriction digest (dsDNA ladder) in 120 seconds on a glass device. Single and double stranded DNA size ladders are used to generate polymer length calibration curves which are then applied to sizing DNA samples of unknown length. However, analysis of the calibration ladder and unknown sample on the same chip may lead to cross-contamination between the samples and obscure co-eluting peaks. Independent analysis of each sample on different devices typically leads to mobility differences induced by differing surface chemistries and small variations in channel length and applied voltage. Consequently, separations cannot be compared and the percentage error in fragment sizing will increase. An alternative approach is to

use a capillary array device; here the separation of sample and ladder can be performed simultaneously under comparable conditions. In 1997, Woolley and co-workers [122] described a high throughput μ -CE device with 12 parallel channels (Figure 1.9a). Simultaneous separation of pBR322 MspI dsDNA fragments in all twelve lanes was demonstrated (Figure 1.9b) in less than 160 seconds. Two years later, Shi *et al.* [123] demonstrated the separation of 96 samples using a 96-channel chip (Figure 1.9c). The channels were arrayed about the centre of the device and interrogated using a radial confocal laser induced scanning (LIF) detection scheme.

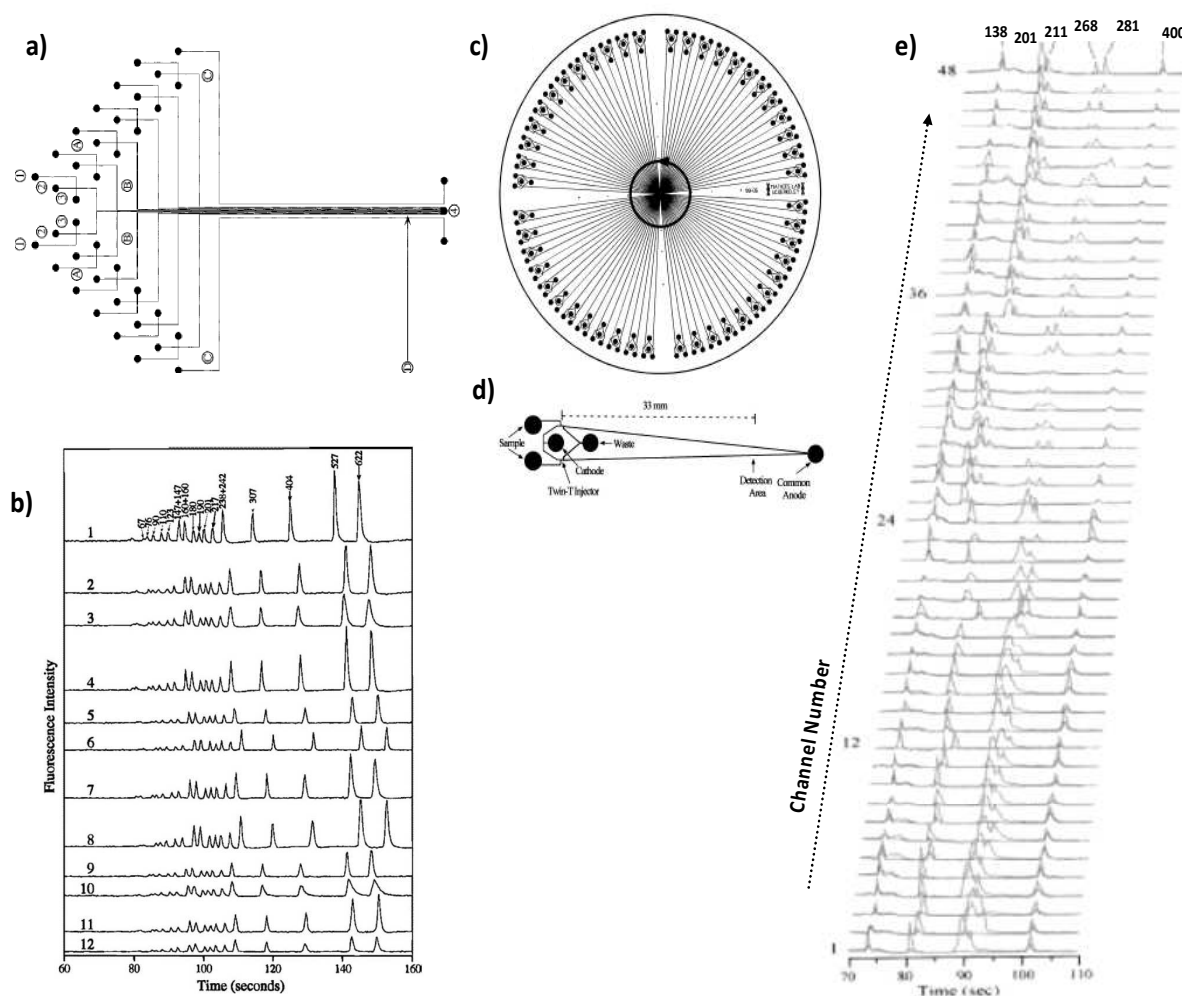


Figure 1.9: Micro capillary array electrophoresis. (a) and (c) are images of the photomasks used to pattern the 12 channel and 96 radial capillary array devices respectively. (b) An overlay of electropherograms obtained from the 12 channel array device, illustrating the separation of pBR322 MspI dsDNA fragments (d) Enlarged view of the two separation channels forming a single spine of the radial array depicted in (c). The separation set up consists of a twin injector and a common cathode, anode and sample waste for both separation channels. The design was adapted from reference. (e) Electrophoretic separations for homozygote (channel 1-24) and heterozygote (channel 25 to 48) samples separated in two quadrants of the radial array device. The peaks in the 48th channel have been labelled. Images (a) and (b) have been reproduced from reference [122] and images (c), (d) and (e) have been reproduced from references [124].

DNA sequencing, genotyping and mutation detection are genetic analyses that generally require single base resolution. Microchip DNA sequencing was first achieved by Woolley and Mathies in 1995 [39], when they achieved single base resolution for 150-200 bases using four colour sequencing. The separation was completed in less than 15 minutes using a 3.5 cm long separation channel and a polyacrylamide sieving matrix. Base calling accuracy was improved by increasing channel length. Over 99% accuracy was observed for 500 bases separated in 20 minutes using a 7 cm long separation channel. An average of 505 bases were called in 27 minutes from samples derived from human chromosome 17, using a 11.5 cm long separation channel. Consequently, to obtain results comparable to those obtained employing conventional systems a simple reduction in initial injection plug size will not be sufficient and an increase in the total channel length is unavoidable. Chip-based genotyping enables rapid identification of alleles and was applied to the allelic profiling assay by Schmalzing *et al.* in 1997 [125]. They demonstrated the separation of the CTTv Kit (Promega Corporation) consisting of short tandem repeat (STR) loci CSF1P0, TH0X, TH01 and vWA. The analysis was completed in 2 minutes using a simple cross-injector device with a 2.6 cm long separation channel.

Post sequencing the human genome, analysis of gene variants (mutations) in the human population for the disease diagnosis, prognosis and management is the natural next step. The most common type of variation is the single nucleotide polymorphism (SNP), where the expected nitrogenous base is replaced with another altering the read. Therefore, during the translation process an alternative amino acid may be added to the growing protein chain causing misfolding, chain termination or changes to the shape and charge of an active site. All three may severely affect protein function and lead to disease. The presence of a SNP can lead to single-strand conformation polymorphism (SSCP) which has knock on effects on its electrophoretic migration. This mutation-induced alteration of mobility was used to demonstrate and compare on-chip SNP detection to conventional CE analysis by Tian *et al.* in 2000 [126]. Electrophoretic analysis of the wild type and mutant (185delAG, 5382insC and 6174delT) alleles by both formats show obvious differences in separation profiles obtained. Additionally, the chip-based separation was completed in 120 seconds, four times faster than the CE analysis and 100 times faster the traditional slab gel analysis. In the same year, using the radial 96 capillary array electrophoresis device in Figure 1.9d and e, a SNP typing assay was developed by Medintz *et al.* [124], enabling the genotyping of the S65C polymorphism associated with hereditary haemochromatosis. DNA amplification via PCR and electrophoretic separation were accomplished on the same device. The homozygous and

heterozygous samples were separated using a 0.9% w/v hydroxyethyl cellulose sieving matrix in channels of effective separation length 3.3 cm. The simultaneous separation across all 96 microfabricated channels was completed in less than two minutes, generating a large amount of statistical data for the assay in a short time. Accordingly, single channel and multichannel chip-based separation devices have been designed and demonstrated for conventional and complex analysis of DNA. Translating electrophoresis from the conventional slab-gel and capillary electrophoresis formats to a chip-based format has numerous advantages, including low sample and reagent consumption, portability and design flexibility. Furthermore, miniaturised CE (μ -CE) achieves high-speed, high-efficiency and high-throughput. The improved heat dissipation enables application of higher field strengths which increases the speed of the separation and separation efficiency. The increased field results in a higher efficiency per unit length of the separation channel. High throughput is realised by fabrication of massively parallel separation devices mimicking the 96 capillary array format developed for macroscale instruments. It should be noted that these improvements mirror those cited for translation from slab-gel to capillary electrophoresis.

1.3.1.2 Integration

On-chip integration of pre-PCR and post-PCR processes has been demonstrated. Several groups have shown the isolation of genomic DNA from whole blood using filters [127], immunomagnetic beads [128] and silica pillars [129] followed by on-chip PCR. However, due to the complexity of the purification protocols involved and the problems associated with low volume liquid transfer between processes, on-chip sample preparation and its integration has not been comprehensively studied using continuous flow microfluidics.

In a recent review on integrated microfluidic devices Manz *et al.* [106] opine “The requirement of μ -TAS in the context of nucleic acid analysis is not a re-packaging, scaling down exercise, but more an assertive step towards the bridging of sampled cells to a quantitative calculation of disease or condition-related nucleic-acid sequences.” Quantitative results presented so far are based on a known concentration of starting material. Most on-chip nucleic acid analyses of real world samples are qualitative. Quantification based on intercalating fluorescent dyes requires prior knowledge of nucleic acid starting concentrations. Excess dye will affect processes downstream to PCR, leading to anomalous identification. Furthermore, current microscale devices rely on external macroscale

infrastructure. Imaging systems, power supplies and pumps are some of the macroscale equipment used regularly to drive experiments conducted on microdevices. Consequently, the implied advantage of portability for miniaturised devices has not really been achieved. Additionally, multiple sample transfer from one process to another without cross-contamination, sample dilution or sample degradation on a single channel device has not yet been demonstrated. Multiple channel or capillary array systems may increase throughput but simultaneously increase complexity of the device. Furthermore the supporting infrastructure to load and run these highly parallel devices is costly and multifaceted.

In 2010 and 2011 two research groups presented microfluidic systems developed for STR analysis of sample obtained from buccal swabs. Both groups performed DNA purification, amplification and electrophoretic analysis on-chip. Although the objective of each system remained similar they differed in one fundamental aspect – integration. The automated instrument described by Hurth *et al.* [130] is an integrated system linking the entire DNA analysis workflow, going from “sample to profile” without human intervention. Cells from a buccal swab were lysed, purified, amplified and separated in approximately three and half hours. DNA from 16 STR loci, fragment sizes between 30- 800 bp were separated on a glass microchip with an effective separation length of 11 cm (Figure 1.10a) using a 3.5 to 4% solution of hydroxyethylcellulose (250 kDa) with 7.1 M urea sieving matrix. Their results show that all 16 markers were resolved with an average resolution of 1.8 bp across the 30-800 bp size range. In comparison, the microfluidic system developed by Reedy *et al.*[131] employs a modular approach, where individual processes are performed on a microfluidic device, however each device is separate (Figure 1.10bi, ii and iii). The chip-based CE was performed using the traditional cross-piece injector format with an effective separation length of 6.5 cm (Figure 1.10biii). Separations were performed in a 4% (w/v) solution of linear polyacrylamide copolymerised with dihexylacrylamide including 7 M urea. The authors employed an AmpF ℓ STR[®] MiniFiler[™] PCR Amplification kit to amplify DNA from a buccal swab. The kit aids in amplification of 8 core STR loci and amelogenin, enabling identification and gender determination. The conventional analysis performed on an ABI 310 Genetic Analyser and the chip-based separation obtained by Reedy and co-workers is illustrated in Figure 1.10c and d. All 9 loci were detected in less than 5 minutes on their chip-based device. The entire analysis, including purification using micro-solid phase extraction, amplification employing non-contact infra-red mediated heating followed by chip-based CE fragment analysis was completed in two and half hours.

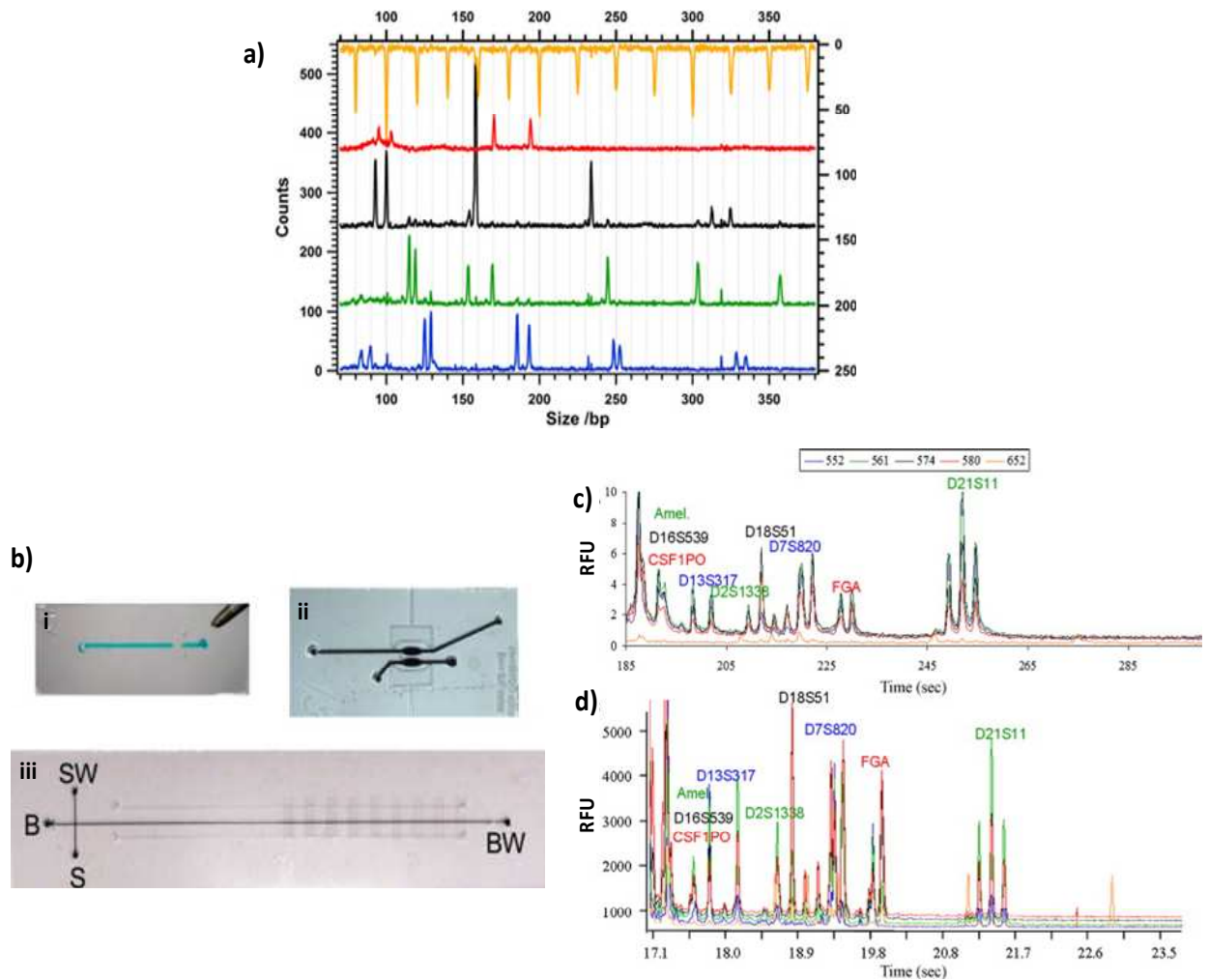


Figure 1.10: Chip-based STR analysis using an integrated and modular approach. (a) The STR separation profile from 16 loci produced from analysis on the integrated microfluidic DNA analysis system. Image reproduced from reference [130] (b) Chip-based devices used in the modular approach to STR analysis. (i) The solid phase extraction (SPE) device, (ii) the polymerase chain reaction device and (iii) the μ -CE device with a total separation length of 7.5 cm. The μ -CE device included a cross-piece injector, where sample is loaded into the sample reservoir (S). A sample plug is formed at the intersection of the two perpendicular channels, when the sample is drawn under an applied field towards the sample waste reservoir (SW). A potential drop is applied across the buffer B and buffer waste (BW) reservoirs inducing the sample plug to migrate into the separation channel and migrate towards the detector. (c) and (d) are the electropherograms obtained after DNA purification and amplification using microchip SPE and microchip infra-red MiniFiler™ PCR followed by separation using μ -CE (device in b(iii)) and ABI 310 Genetic Analyser respectively. Images (b), (c) and (d) are reproduced from reference [131].

The premise behind the modular approach, employed by Reedy and co-workers is to allow the user (here the forensic scientist) to make critical decisions on the direction of analysis depending on sample quality. The analytical route on an integrated device is fixed and out of the hands of the scientist whereas the modular microfluidic approach enables user intervention but realises the

advantages of a miniaturised device. However, user intervention brings with it the associated manual/human error and the possibility of sample contamination. Integration of consecutive continuous flow processes using either approach has some disadvantages. Furthermore, both systems use glass μ -CE devices with cross-piece injectors. This traditional injection format and its flaws have been well documented, but is still an integral part of state-of-the-art microfluidic devices. In the race to build ever more complex devices, research and development on its fundamental building blocks seems to have been left by the way side.

1.5 Droplets

Multiple sample analysis in continuous flow or single phase devices is complicated because of cross-contamination and dilution of samples. To circumvent these problems highly parallel devices can be employed using a “one sample one microchannel” approach. This is analogous to other array systems and necessitates complicated fluid handling. Additionally, as described above integration of continuous flow systems using single phase continuous flows is problematic. Droplet-based microfluidics addresses these problems by creating discrete sample volumes using immiscible phases. Typically, an aqueous phase is encapsulated within an oil phase (identified as water-in-oil droplets [132]) however several authors have demonstrated oil-in-water and air-liquid [133, 134] droplet systems. Droplet systems enable compartmentalisation of samples without increasing the size or complexity of the device promoting high-throughput analysis. In 2007, Kobayashi *et al.* [135] generated monodisperse oil-in-water droplets with nanometer to micrometer diameters at rates of up to 20,000 droplets per second. The authors achieved a 9% co-efficient of variation in droplet size. Over the last 6 years several groups have employed microdroplets to perform PCR [136-138] and enzyme assays [139, 140], generate nanoparticles [141, 142], crystallise proteins [143], encapsulate and incubate cells [139, 144-146] and perform chemical reactions. Nanolitre and microlitre droplets generated this way have a higher surface to volume ratio compared to continuous flow microfluidic devices. This reduces heat and mass transfer times and the diffusion distance for encapsulated molecules enabling faster reaction times. Each droplet serves as an isolated compartment and can be individually addressed and indexed. Therefore, multiple analyses may be performed without increasing device size or complexity. The droplet format makes the possibility of high-throughput screening, device integration and multiple sample analysis on lab-on-a-chip devices feasible.

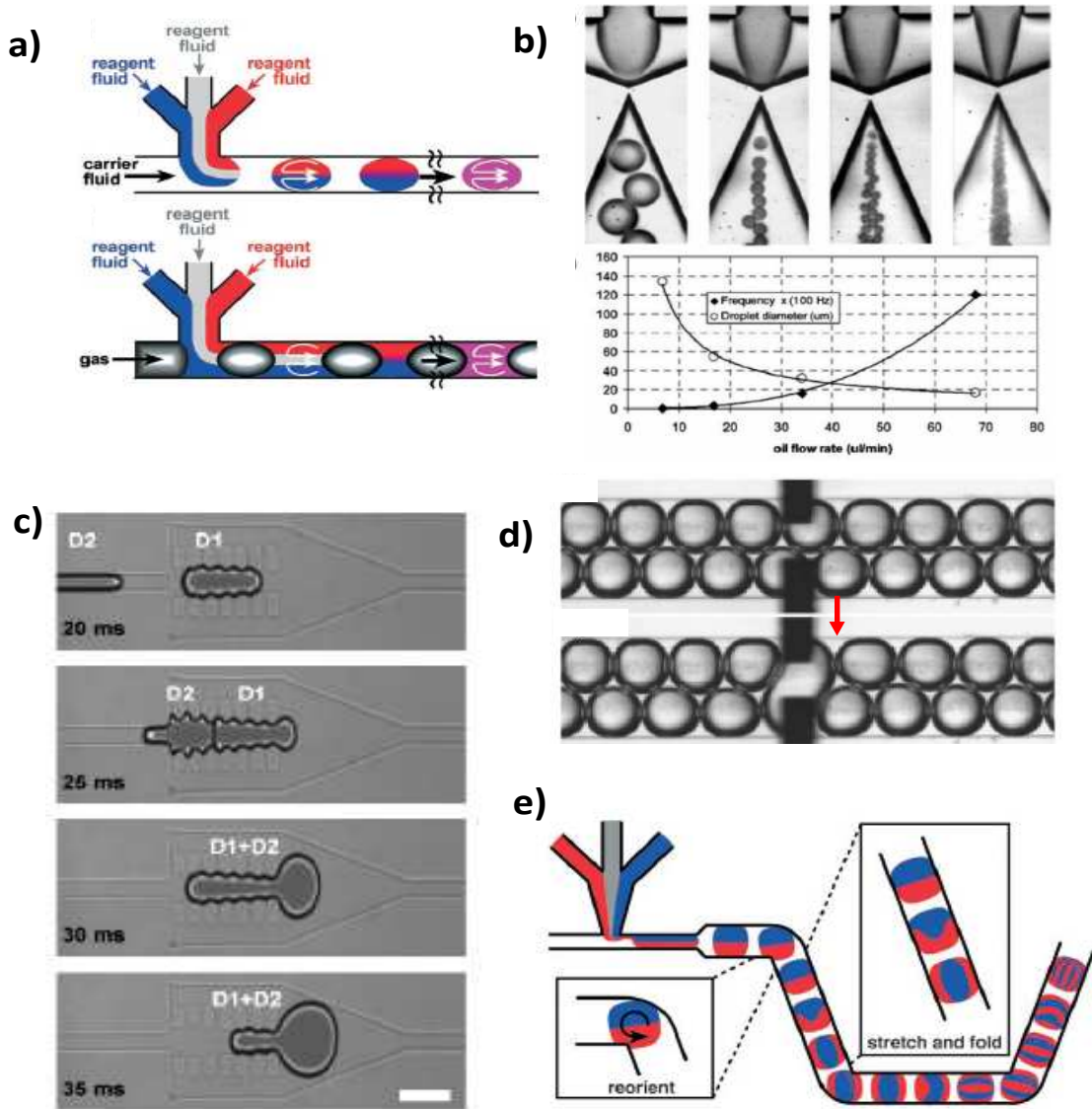


Figure 1.11: Droplet microfluidics. Top and bottom panels of (a) show schematics of T-junction based droplet generation using oil (top) and gas (bottom) continuous phases. Top panel of (b) show images of droplet generation via flow focussing, where a decrease in orifice diameter leads to the generation of smaller droplets. Lower pane of (b) shows a plot of the change in droplet size and frequency as a function of continuous phase flow rate. (c) The fusing of droplets, D1 and D2 over time using a pillar array, (d) shows droplet fusion using electrodes. (e) A schematic of the mixing which occurs in droplets when moving through a herringbone shaped channel. Images in (a), (b), (c), (d) and (e) have been reproduced from references [147], [148], [149], [150] and [144] respectively.

There are two basic formats for generation of droplets in microfluidic channels that depend on channel geometry: T-junctions [151] and flow-focusing [148]. Figure 1.11a and b illustrates both formats in operation. In the T-junction format the dispersed phase (usually aqueous phase in

biological experiments) and the carrier phase (oil phase) are injected from two branches of the “T”. Droplets of the disperse phase bud off into the carrier phase at the intersection due to shear force and interfacial tension at the fluid-fluid interface (Figure 1.11a). Typically, the phase that has lower interfacial tension with the channel wall is the carrier phase. Instead of a liquid carrier phase, gas may also be used to separate the droplets forming at the intersection as shown schematically in Figure 1.9a. Flow-focussing is achieved by employing a configuration where the carrier phase is injected from two side channels while the disperse phase is injected via central channel using a tapered orifice (Figure 1.9b). This format employs symmetric shearing of the disperse phase by the carrier phase. Although flow-focussing is more complex to implement, it provides the additional functionality of controlling droplet size. The plot in Figure 1.11b (lower panel) traces the change in droplet size and frequency of formation with increasing oil flow rate. Furthermore, especially small or viscous droplets may be generated using this format.

Aside from channel geometry other factors controlling droplet size are the viscosities of the immiscible phases, presence of surfactants and hydrophobicity or hydrophilicity of the device surface. The dimensionless capillary number (Ca) is used to determine the dynamics of droplet generation and can be defined as

$$Ca = \frac{\eta v}{\gamma}. \quad (1.21)$$

Where η and v are the viscosity and velocity of the continuous phase respectively and γ is the interfacial tension between the immiscible phases. A third, recently developed format for droplet generation uses a robotic head to draw carrier and disperse phase into the microchannel. This format of droplet formation is simple and can be used to generate droplets from multiple samples.

In order to function as microreactors, droplet manipulation post generation is crucial. Several operations have been developed enabling droplet fusion, mixing, dilution, splitting and size based sorting. Typically these operations are either performed in an active or passive format where the former is induced by an external stimulus and the latter by device geometry. Priest and co-workers [150], in 2006, described an active method for droplet fusion using electrodes placed parallel to the droplet channel. Droplet fusion occurred under application of both A.C. and D.C. voltages. In 2008, Niu *et al.* [149] demonstrated passive droplet fusion using a strategically designed pillar array

to induce selective and controllable droplet coalescence. Figure 1.11c and d show passive and active droplet fusion operations as described by Niu *et al.* and Priest *et al.* respectively. Furthermore both active and passive formats have been demonstrated by several groups for droplet fission, enabling controllable splitting of droplets for increasing experimental throughput and controlling reagent concentration. Mixing is an essential tool for sample preparation in most biological and chemical reactions. At the microscale, interfacial forces dominate and have to be overcome to promote mixing between samples. Under laminar flow conditions, parallel fluid streams only mix via diffusion and no turbulent mixing is observed. Passive mixing within droplets is induced using bends and turns (Figure 1.11e) in the channel geometry which encourage chaotic advection [144, 152] (the stretching and folding of droplet content). Active mixing in droplets has been primarily demonstrated on electrowetting-on-dielectric (EWOD) devices [153, 154]. Aside from the described operations other methods have been demonstrated for droplet manipulation including lasers[155], magnetohydrodynamic pumps[156] and electroosmotic flows [157]. Together these operations are providing the tools to perform and control sequential analysis within droplets. At present the most commercially successful microdroplet application is emulsion polymerase chain reaction (ePCR)[158]. Aside from an increase in throughput the use of droplets in PCR facilitates the isolation and amplification of single fragments from complex mixtures preventing competition between multiple amplicons. The segregation of template fragments also prevents the formation of artifactual fragments due to recombination between homologous sequences from different DNA templates.

1.6 Project outline

Recently a novel approach to integration has been described using droplet microfluidics to bridge continuous-flow devices [159, 160]. Here sample is fractionated into droplets post preparation, amplification or detection and transferred to the next step of the analysis. Integrated devices designed using these approaches adopt the advantages associated with both segmented and continuous flow microfluidics. Consequently, droplet interfaced devices can be designed such that each continuous flow device is an individual module which can be integrated on-demand using droplets. Herein two novel droplet-based interfaces are described and demonstrated. The first is a droplet to μ -CE interface where samples prepared in droplets were transferred to an electrophoretic separation channel without suspending the separation process. This enables multiple sample analysis on a single separation channel. Such a platform is much simpler in

operation than the conventional cross channel μ -CE and provides the desired capabilities of small sample handling, high throughput and quantitative analysis without cross contamination between droplets. Both CZE and CGE separation modes were successfully demonstrated, proving the potential of such a platform in a wide range of applications.

The second interface employs droplets to connect two macroscale instruments. Here the droplet interface provided an offline connection between a nano-liquid chromatography (nano-LC) instrument and a MALDI mass spectrometer. LC effluent was mixed with MALDI matrix and fractionated into droplets retaining the resolution obtained during the LC separation. Mass spectrometry results show that the interface provides results similar to those obtained using the traditional 'dried drop' technique. Furthermore, the performance of the interface device was compared to a commercially available automated spotter using the Trypsin digest of two proteins. In both cases the droplet interface device demonstrates superior performance.

Electrophoresis is an essential part of DNA analysis and is typically the last process in an analytical sequence. Herein several PEO based sieving matrices for both capillary and chip-based electrophoresis are investigated and discussed. The properties of each matrix were assessed to determine if they met the requirements of an ideal matrix. The effect of each matrix on the efficiency and resolution of the separation is also examined. Furthermore, electrokinetic sample injection employed in CE, on-chip and on the novel droplet-CE interface are compared and discussed. A novel study on the effect of SYBR Green I on the migration dynamics of dsDNA fragments was also carried out and compared to unlabelled fragments using UV detection. Additionally, a novel single-channel continuous-flow PCR device is also demonstrated with a view to enable integration to chip-based CE via the droplet-CE interface. The PCR device employed electroosmotic flows generated across the surface of immobilised diodes to propel the PCR reaction mix between temperature zones. Collectively the work described here goes some way in addressing fundamental problems associated with chip-based capillary electrophoresis and its integration to other biological and analytical procedures.

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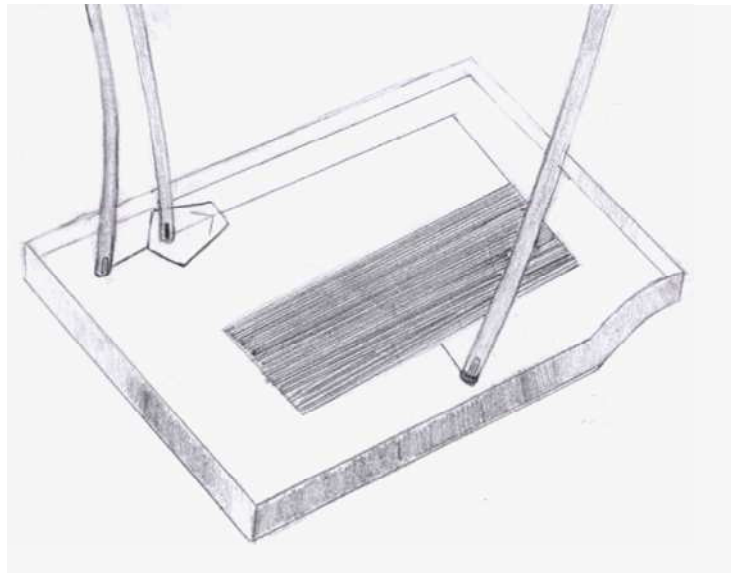
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Chapter II: Instrumentation and Fabrication



2.1 Microfabrication

One of the key tools in the fabrication of microfluidic devices is lithography. The term lithography has its roots in the Greek language, where *lithos* means ‘stone’ and *gráphein* means ‘to write’. The most widely used form of lithography is photolithography and pattern transfer (from the design to the device substrate) is typically initiated using this technique. The first example of pattern transfer by photolithography and chemical milling was demonstrated by a Parisian engraver, Lemaître in 1827. However, lithography as a technique is much older. It was invented by Aloys Senefelder in 1796, when he used chemically pre-treated Bavarian limestone to transfer a carved image on to paper with ink. The image and non-image areas on the stone became hydrophobic and hydrophilic respectively, thus attracting oil based ink to the hydrophobic areas. It was not until World War II that the first applications of printed circuit boards were reported [1]. By the 1960s methods allowing the mass production of transistors using photo-etching were devised, allowing the patterning of features with a resolution of 5 μm . Over recent decades pattern transfer and microfabrication techniques have been developed and refined to allow submicron accuracy in printing and feature production [2, 3].

Photolithography may be used multiple times during the fabrication of a microfluidic device. These devices are typically fabricated in quartz [4], glass [5, 6], silicon [7], ceramics [8], plastics [9], polymers [10], elastomers such as polydimethylsiloxane (PDMS) [11, 12] and metals. Unfortunately devices made of silicon or glass are expensive, time-consuming and tedious to fabricate. The use of elastomeric materials is advantageous since both the raw materials and the processing methods are cheap and facile. PDMS has been intensively studied and used within the microfluidics community for these reasons. Soft lithographic processing of PDMS enables high fidelity replica molding of features down 100 nm in size [13]. Moreover, PDMS is optically transparent down to 280 nm, and thus variety of detection schemes can be applied to monitoring biological and chemical reactions performed with devices. Importantly, its low surface energy and elasticity allow it to be released from the SU-8 master without feature distortion. In a study carried out by Chaudhury and Whitesides [14] in 1991, the surface free energy of oxidised PDMS was found to be 58.5 ergs/cm², three times higher than that of the unoxidised PDMS. Additionally, adhered layers of oxidised PDMS could not be separated without causing cohesive failure in the polymer samples. PDMS is also both thermally and electrically insulating. It has a thermal conductivity of 0.20 W/m K [12] compared to a value of 1.05 W/m K for glass and 16 W/m K for stainless steel.

Moreover, it has a breakdown voltage of 2×10^7 V/m [12]. The breakdown voltage of any insulating material is the minimum voltage at which a portion of the material becomes electrically conductive. PDMS is also impermeable to liquid water while being permeable to gases and non-polar organic solvents [15-17]. Furthermore, PDMS cures at low temperatures, is non toxic and can be deformed and sealed reversibly. The reversible sealing is provided by van der Waals contact between the PDMS and a range of materials and the seal is water-tight. However the seal cannot withstand pressures greater than ~ 5 p.s.i.[11]. Accordingly PDMS can also be sealed to itself and glass irreversibly through the formation of covalent bonds [18, 19].

2.1.1 PDMS Microdevice Fabrication

Fabrication of microdevices using PDMS involves a number of component processes. The required design must first be printed on to a transparency called a photomask. This transparency can be used directly to fabricate an SU-8 master, however it is typically converted to a chromium mask which is more durable. The patterned mask is used to create a master mold in SU-8 which can be then used to cast multiple devices made in PDMS. Once the chromium mask and master are fabricated they can be re-used multiple times.

2.1.1.1 Fabrication of Chromium Masks

Commercial chromium photomasks are conventionally fabricated using e-beam or direct laser writing [20]. Both processes produce high quality and high resolution photomasks. Prior to SU-8 soft lithography, a secondary UV mask can be patterned in chromium using the printed transparency. This is done to increase the lifetime of the mask, as chromium on glass is more durable than the transparency printed on acetate paper. In addition, the contact between mask and substrate is also better due to the weight of the glass, improving pattern transfer and handling. Gaps between the mask and SU-8 substrate during UV exposure will lead to a widening of the pattern on the final mold due to diffraction of light.

The fabrication process starts with the generation of a computer aided design (CAD) file which is printed on to an acetate film. The design is then transferred to the chrome mask using the process outlined in Figure 2.1. A commercially available glass wafer pre-coated with a positive resist layer

and a chromium layer (AZ1518, 5300 Å, Soda Lime, Nanofilm, USA) is used for device fabrication in this work. The acetate printout was used as the primary UV mask and the pattern transferred onto the UV sensitive positive photoresist layer. Following exposure to UV light, the micro-channel pattern was revealed by immersion of the substrate in a solution of photoresist developer, typically Microposit 351 Developer (Chectech Ltd, UK) diluted 1:5 in deionised water. The exposed chromium under the positive photoresist was then etched away using a chromium etch solution (Aldrich, UK). Finally, the top layer of photoresist is entirely removed using acetone leaving the transparent microchannel design on the chromium coated glass.

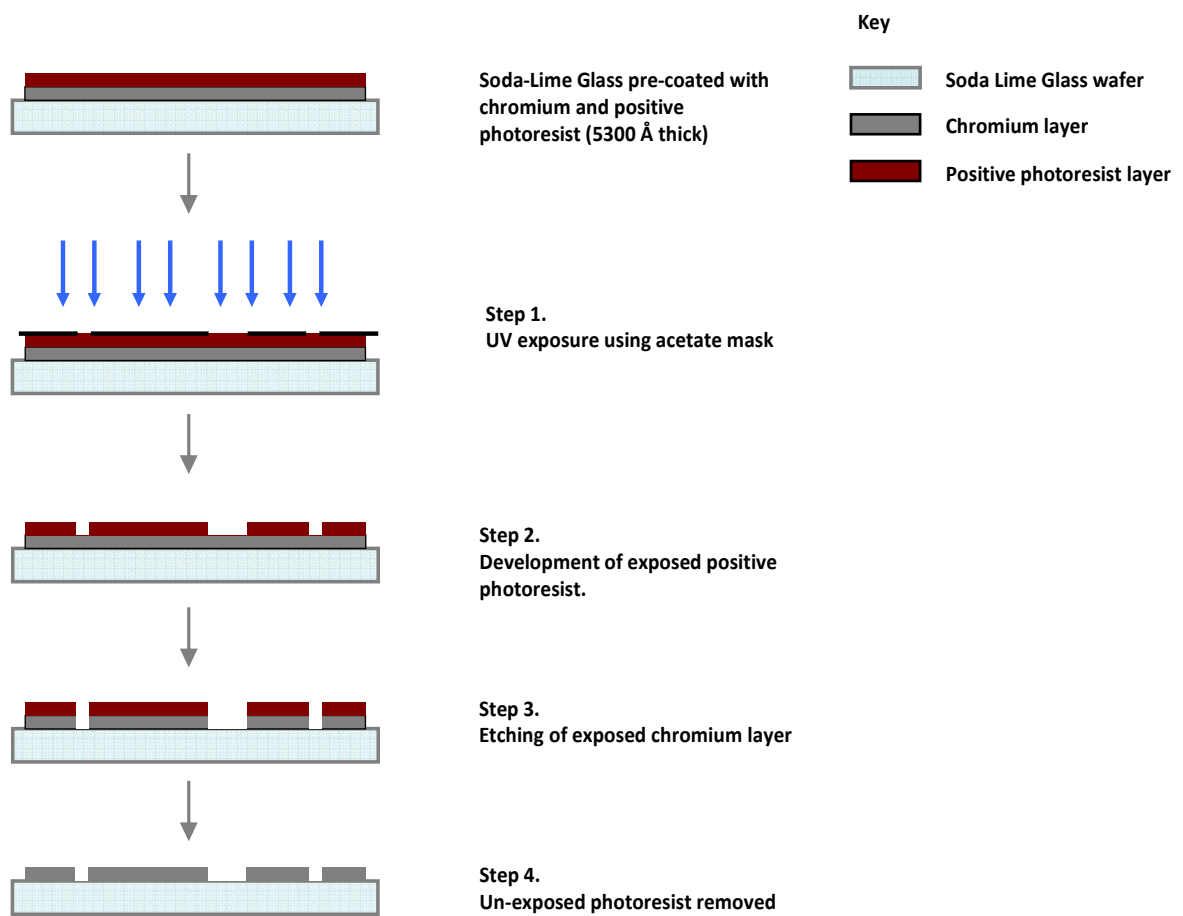


Figure 2.1: Schematic of the steps involved in the generation of a chromium mask. The pattern is transferred from the transparency on to commercially available chromium substrates and photoresist-coated glass. This is then developed and etched to reveal the desired pattern in chromium.

2.1.1.2 Generation of SU-8 Masters

An SU-8 master acts as a mould from which PDMS devices can be cast. SU-8 (Microchem, Chectech Ltd, UK) is an epoxy based photoresist developed by IBM in 1989 [21] for the

fabrication of high aspect-ratio MEMS devices. The SU-8 structure is detailed in Figure 2.2. Each monomer consists of approximately eight epoxy groups which allow for a high degree of cross-linking after photochemical activation. The photoresist consist of three parts, the first and second are a solution of Bisphenol A Novolak epoxy in gamma butyrolactone at various concentrations. The third is a photo-acid generator, triaryl sulfonium salt in a propylene carbonate solvent, this absorbs photons during exposure generating a strong acid which catalyses the cross linking reaction [22, 23].

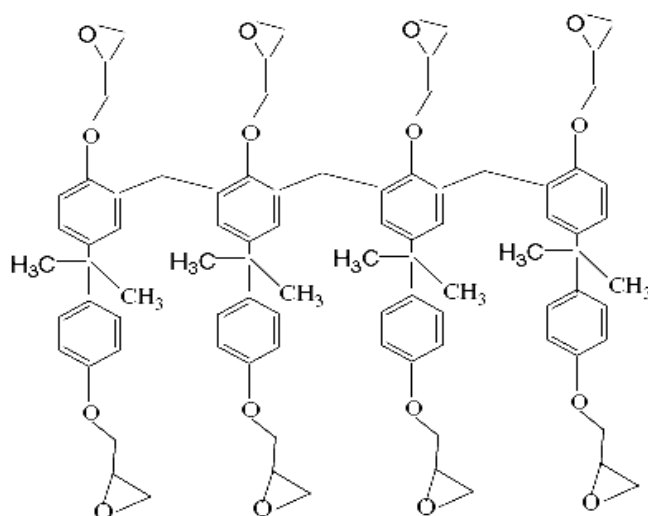


Figure 2.2: A Bisphenol A Novolak epoxy monomer contained in SU-8. Image reproduced from reference [22].

The basic steps involved in the production of an SU-8 master are listed in Figure 2.3; these must be performed in a clean room. The standard fabrication process includes a spin coat, soft bake, UV exposure, post exposure bake (PEB) and development steps. A clean room has temperature and humidity regulation and is permanently maintained at a higher pressure ensuring air flow out of the room. Clean room quality is defined by the number of particles of a size of less than $4\text{ }\mu\text{m}$ contained in a volume of cubic inch. Typically fabrication of microfluidic devices is carried out in clean rooms classified between 1000 and 10,000. While working in these rooms it is important to wear gloves, hair-nets and shoe covers to maintain the cleanliness of the environment.

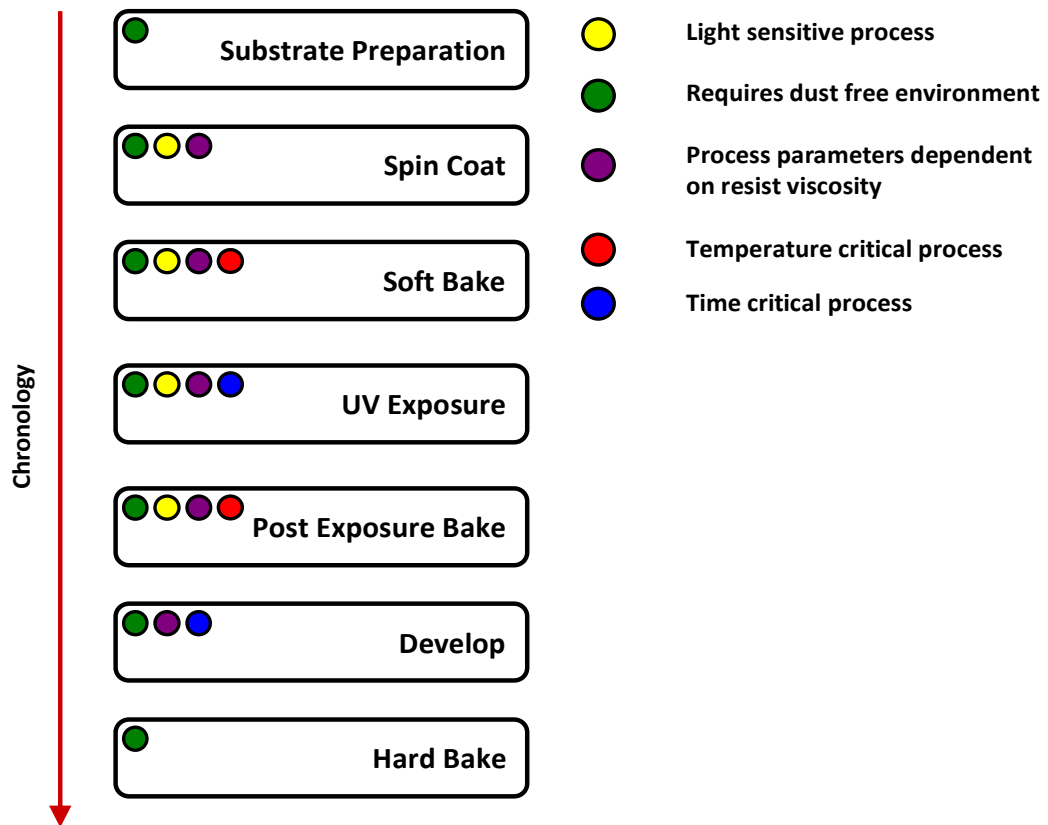


Figure 2.3: Schematic defining the processes involved in SU-8 master fabrication and their environmental sensitivity.

2.1.1.2.1 Spin Coating of SU-8

The SU-8 negative photoresist is deposited onto the surface of a 10 cm silica wafer (IDB Technologies Ltd, UK) in a thin layer. The thickness of this layer is controlled by the viscosity of the resist used and the spin speed applied during deposition. Typically a spin speed between 1,000 and 10,000 rpm is used to obtain resist layers between 10 μm and 200 μm thick. Spin coating is usually carried out as a two-step process where an initial stage involves spinning the wafer and photoresist at 500 rpm for 30–50 seconds. This allows the photoresist to spread across the surface of the wafer from the centre where it is initially deposited. In a second stage the wafer is accelerated to an optimum speed to obtain a uniform layer of photoresist of the desired thickness. The thickness of this layer will define the channel height. Consequently, to obtain channels with increasing height, lower spin speeds and higher viscosity photoresists are used. In this work PDMS channels of several thicknesses were prepared, the SU-8 type and viscosity, spin speeds employed

and thicknesses produced are listed in Table 2.1. The thickness, h of a photoresist film obtained in this way can be determined using the following relationship

$$h = \frac{kC}{\mu\omega}, \quad (2.1)$$

where k is a constant, C is the concentration of the polymer solution, μ is the viscosity of the resist and ω is the angular rotation velocity.

Product Name	Viscosity (in centistokes [cSt])	Thickness (μm)	Spin Speed (rpm)
SU-8 10	1050	15	2000
SU-8 50	12550	50	2000
		100	1000
SU-8 100	51500	150	2000

Table 2.1: Spin speeds and SU-8 type required to generate masters for these devices are listed.

2.1.1.2.2 Soft Bake of SU-8 Master

Product Name	Viscosity (cSt)	Thickness (μm)	Time at 65°C	Time at 95°C
SU-8 10	1050	15	2	5
SU-8 50	12550	50	6	20
		100	10	30
SU-8 100	51500	150	20	50

Table 2.2: Soft bake temperatures and times appropriate for each SU-8 type and thickness are listed.

After application of the photoresist the wafer is heated to evaporate the solvent in which the resist polymer is dissolved. During this step the solvent content drops from 30-40% to 5% increasing the density of the photoresist and preparing it for UV exposure. This soft bake is carried out in two stages. First the wafer is heated to 65°C and then the temperature is increased to 95°C. The time that the wafer is held at each of temperature depends on the viscosity of the photoresist and its thickness. Optimum times are catalogued in Table 2.2. The two stage process is carried out to prevent cracking or bubble formation in the resist. After heating, the wafer is allowed to cool to room temperature.

2.1.1.2.3 UV Exposure of SU-8 Master

Exposure is the pattern transfer step, where the chromium mask is placed above and in contact with the cooled photoresist coated wafer and exposed to UV light. In a negative tone resist like SU-8, the exposed areas become insoluble in the appropriate solvent, whilst the unexposed areas readily dissolve and can be removed. Exposed areas are made insoluble by a cross-linking reaction catalysed by a photo-acid present in the resist. The soluble SU-8 linear polymer is converted to an insoluble cross-linked network structure. This process is initiated during exposure but completed during the post-exposure bake.

In the current work exposure was carried out using a mercury lamp, whose typical emission spectrum contains lines at 436 nm (g-line), 405 nm (h-line) and 365 nm (i-line). The i-line represents 40% of the total emission between 460 and 320 nm. The SU-8 resist is optimised for use with the i-line [22, 24]. SU-8 is almost transparent and insensitive to wavelengths above 400 nm. Optimal exposure depends on the film thickness and the other process parameters. The absorption of SU-8 in the UV is very low compared to other resists of this kind, which allows the patterning of thicker coatings. If the optical absorption of a photoresist is high, even prolonged doses of UV light will not penetrate a thick layer of resist to generate clean, sharp profiles. While the SU-8 data sheets provided by the manufacturers give guidelines on soft bake, exposure and PEB times, it is ideal if the exposure, bake and UV dosage parameters, are calibrated on a case by case basis.

2.1.1.2.4 Post Exposure Bake and Development of SU-8 Master

Following exposure the wafer is heated in a two stage process, known as the post exposure bake. The wafer is first heated to 65°C and then to 95°C, the time at each temperature being determined by the thickness of the film (Table 2.3). Heating is initiated at a lower temperature as the properties of the resist film change due to the cross-linking reaction occurring at this stage. Shrinking of the film will occur due to loss of solvent and will increase resist density. If this occurs rapidly (as it would at high temperatures) the wafer would fracture destroying the master.

Product Name	Viscosity (cSt)	Thickness (μm)	Time at 65°C	Time at 95°C
SU-8 10	1050	15	1	2
SU-8 50	12550	50	1	5
		100	1	10
SU-8 100	51500	150	1	12

Table 2.3: Post exposure bake temperatures and times appropriate for each SU-8 type and thickness are listed.

Development transforms the latent resist image formed during exposure into a relief image which serves as a mould during downstream fabrication. After the PEB the wafer is immersed in a developer solution that dissolves the unexposed areas, leaving the exposed pattern in place. A developer solution for SU-8, consisting of 2-methoxy-1-methylethy acetate (Microposit EC Solvent, Chestech Ltd, UK) was used to remove the unexposed SU-8. For high aspect ratio moulds, the immersed wafer is strongly agitated and the developer solution refreshed after 10 minutes. This process speeds up the development process, but can increase the chances of mold detachment. That is, the SU-8 patterned on to the surface of the silica wafer has a higher probability of separating from the wafer. Post development the patterned wafer was cleaned with iso-propanol and then dried with nitrogen, before PDMS casting (Figure 2.4).

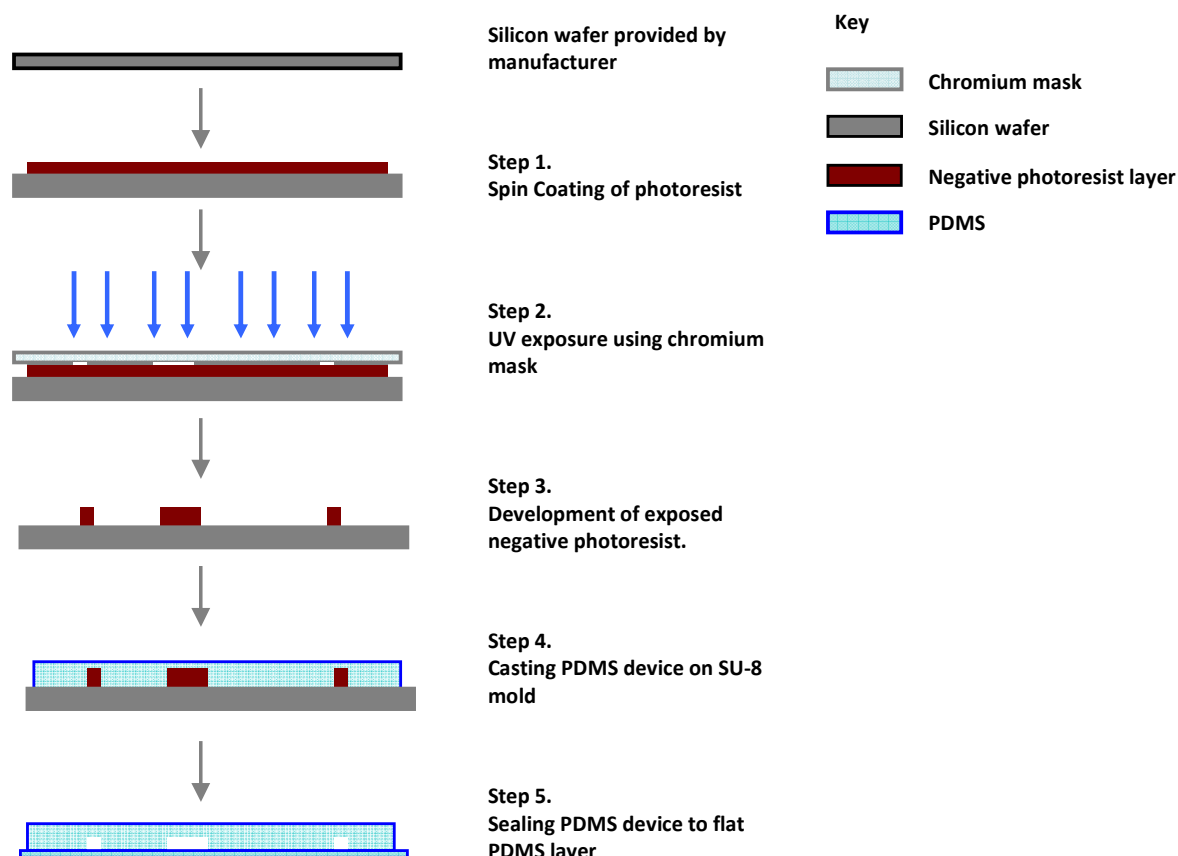


Figure 2.4: Schematic for SU-8 master and PDMS replica fabrication using standard photolithography.

2.1.1.3 PDMS Device Moulding

Once the SU-8 master has been fabricated, PDMS devices can be molded. PDMS is typically supplied as two components, a base (tetra(trimethylsiloxy)silane) and a curing agent (tetramethyltetravinylcyclotetrasiloxane), in a SYLGARD 184 Silicone Elastomer Kit (Dow Corning Ltd, UK). The base and curing agent are mixed together in a ratio of 10:1 and stirred vigorously to ensure even distribution of the curing agent. This mix is then poured over the SU-8 mold, degassed and cured at 65°C on a hot plate or in an oven for 2-4 hours. The resulting cross-linked polymer consists of repeating units of $-O-Si(CH_3)_2-$. Once cured the device is detached from the master using a scalpel. It is then sealed to another layer of PDMS or glass. The bottom sealing layer of PDMS is prepared by curing 12 gm of PDMS mix in a square Petri dish. Prior to curing the PDMS is levelled and degassed to ensure a flat, even and bubble free surface. The

bottom layers are then cut to the appropriate size prior to sealing. Furthermore, 1 mm access holes to channels and reservoirs are punched using a sterile dermal 1 mm biopsy punch (Kai Medical, Japan). These holes were used to introduce and recover fluids from the device. Polyethylene tubing (Harvard Apparatus Ltd, UK) of a slightly larger diameter (380 μm I.D and 1.09 mm OD) than the access holes was fitted to the device subsequent to sealing providing a water tight seal (Figure 2.5). To improve the sealing the area around the inserted tube was coated with Araldite epoxy (RS Components, UK) and cured.

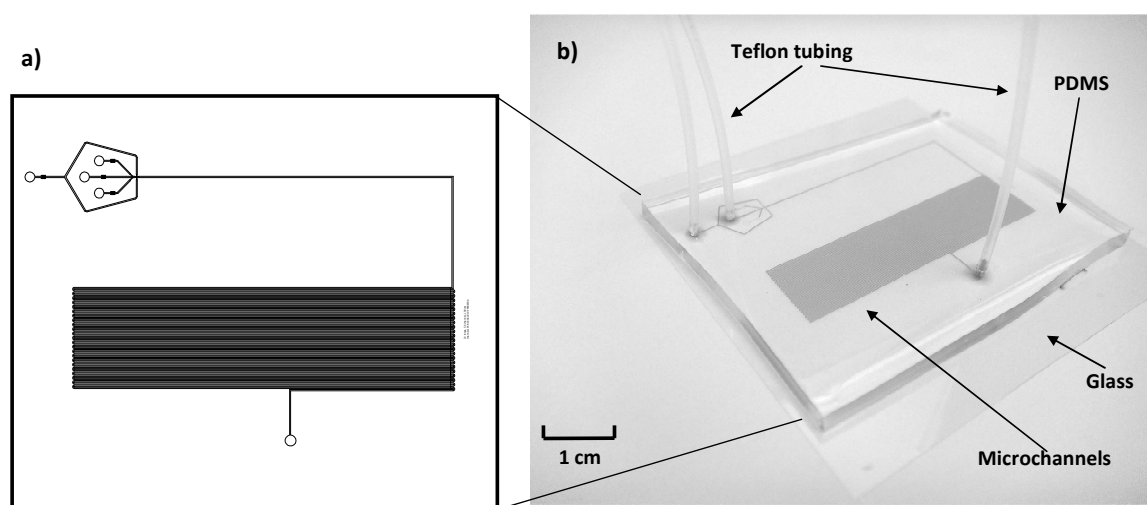


Figure 2.5: (a) A schematic of the CAD design for channels in a PDMS device and (b) the resulting micro-device post fabrication and assembly.

Two types of PDMS devices were employed herein. These included devices with PDMS sealing layers which afforded channels with all four walls constructed from the same material and devices sealed with borosilicate glass (VWR, UK). Exposing PDMS replicas to an oxygen plasma leads to the conversion of Si-CH₃ groups to Si-OH groups. The Si-OH groups in both layers interact to form a covalent bond via an -Si-O-Si- linkage with the loss of water [14, 25]. The covalent bonds allow for an irreversible seal withstanding pressures of between 30-50 p.s.i. [11] Plasma bonding may also be used to form an irreversible seal between PDMS and silicon, silicon oxide, silicon nitride, quartz or polystyrene layers [26]. PDMS substrates and sealing layers were cleaned by sonicating for 5 minutes in ethanol followed by a deionised water rinse. The replicas and sealing layers were then dried with nitrogen and exposed to an oxygen plasma (Harrick Plasma, USA) for 1 minute. The two surfaces were brought in to contact and a seal formed. The contact process should be carried out in under 1 minute as the surface of oxidised PDMS reconstructs in air [25].

2.2 Capillary Electrophoresis Instrumentation

2.2.1. PEREGRINE CE Instrument

Conventional capillary electrophoresis was performed on a PEREGRINE CE system (deltaDOT, UK). The instrument is an automated single capillary system incorporating a photodiode array detector. Samples and reagents are delivered using two, twenty four position, carousels on either side of the main cover. The capillary is mounted within a temperature controlled aluminium block housed under the main cover. Figure 2.6 is an image of the instrument showing the main cover, left-hand side carousel and status panel. The instrument is provided with two software packages, P3Controler and P3EVA. The P3Controler is used to run the instrument and collect data while P3EVA is used to analyse and compare the data. In operation the samples, rinse reagents and sieving matrices are loaded into glass vials (SMI-LabHut Ltd, Gloucestershire, UK) and inserted into vial holders in the carousels.

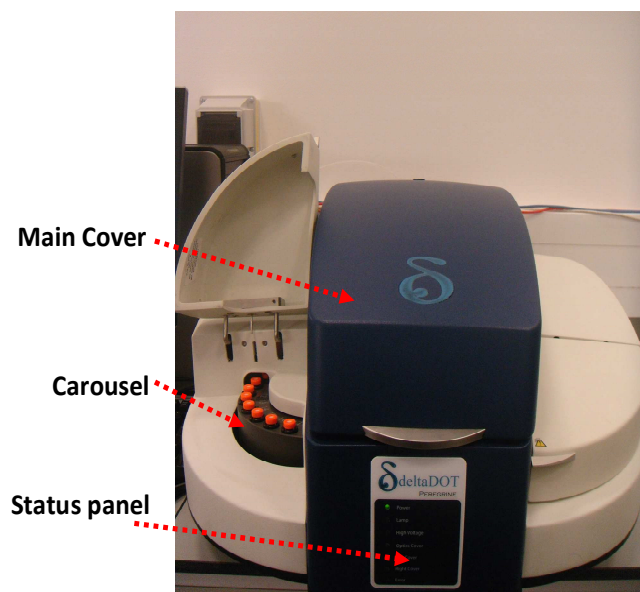


Figure 2.6: Outer view of the PEREGRINE illustrating the main cover, 24 position left hand side carousel and the status panel.

The optical rail along which all the main components of the CE and UV detection system are aligned is shown in Figure 2.7. UV light from a lamp is focused via a series of lenses on to the detection window in the capillary and then on to the detector. Analytes migrating past the window

absorb light leading to a reduction in light intensity reaching the detector. The electrical signal generated at the detector is converted into a trace called an electropherogram.

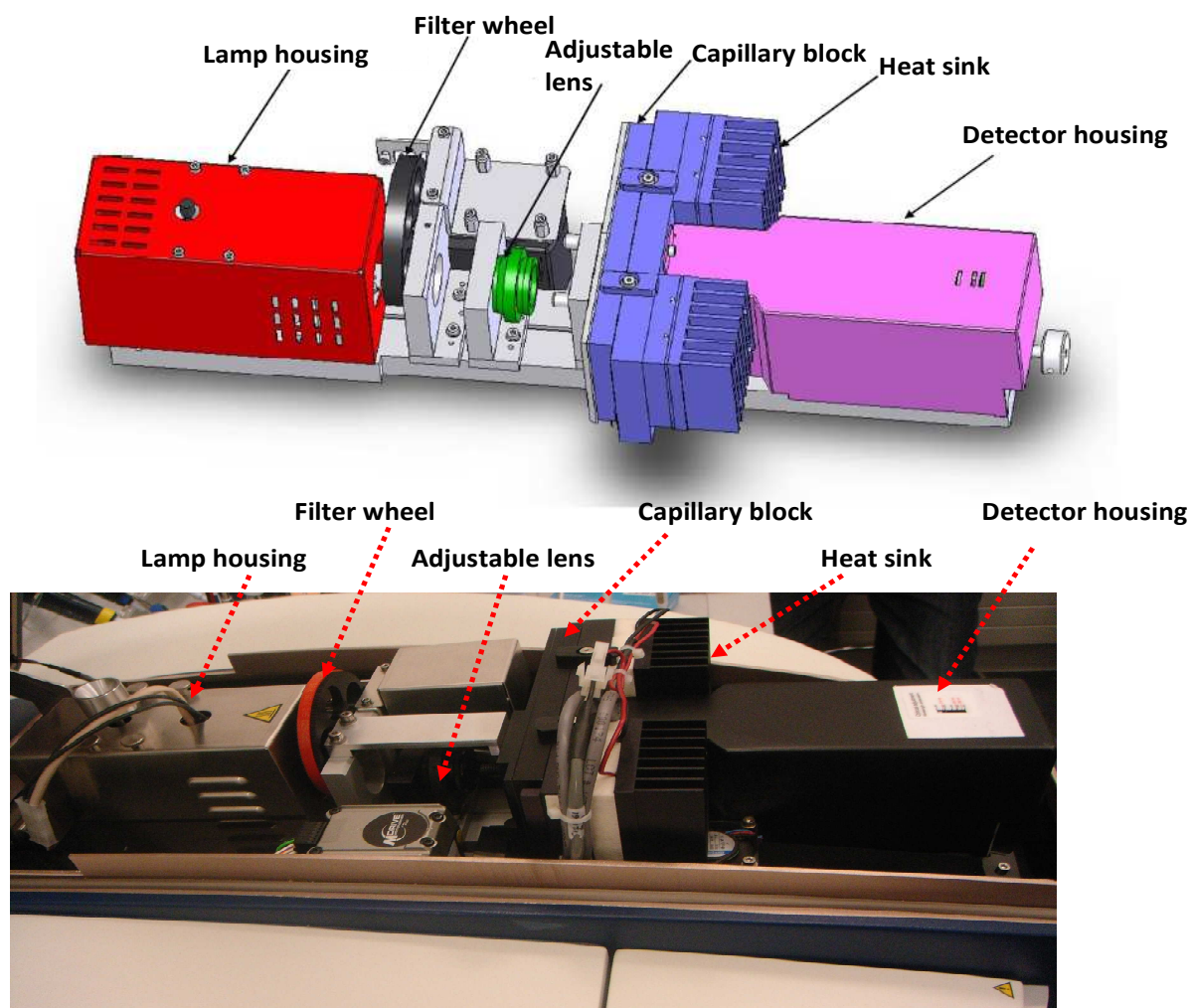


Figure 2.7: Schematic and photograph of the main components of the PEREGRINE capillary electrophoresis instrument. The schematic was reproduced from reference [27]. These components are located under the main cover of the machine and include the lamp, capillary block and detector aligned to allow UV detection of analytes migrating through the capillary.

2.2.2 Capillary Electrophoresis Detection System

In CE, DNA can be detected using UV absorption [28-30], Laser-Induced fluorescence (LIF) [31-33] and electrochemical [34] detection methods. Although fluorescence and electrochemical detection methods have demonstrable advantages in terms lower limits of detection and high

resolution [35], UV detection has remained in use due to its wide applicability, easy implementation and minimal sample preparation. A significant issue when using UV absorbance detection is the reduced sensitivity and poor concentration detection limits, typically in the range of 10^{-5} and 10^{-7} M [35] depending on the analyte. The Beer-Lambert law is employed to relate the absorption of light to the properties of the material it is travelling through. The absorption of light by an analyte solution of concentration (c) is described as

$$I = I_0 \times (10^{-\epsilon(\lambda)pc}), \quad (2.2)$$

where I is the intensity once the light has passed through the sample of thickness p , I_0 is the initial unobstructed intensity and $\epsilon(\lambda)$ is the wavelength-dependent absorption coefficient. The amount of light incident on the photo-detector is measured as voltage or intensity and is plotted as a function of time and the resulting trace is known as an electropherogram. In CE an electropherogram is composed of peaks that are raised above the background signal. The baseline represents the background noise level while a peak represents the signal obtained from the absorbing species as it travels past the detector. Signal-to-noise (S/N) ratio is used as a measure of signal strength relative to background noise. Typically S/N values above 3 are considered significant. Lowering detection limits in CE can be achieved using several approaches and assessed using S/N values. From the Beer-Lambert law it is evident that increasing the absorption path length will increase the amount of light absorbed thereby increasing the S/N value [36]. The use of Z-cells [37-39], bubble cells [40, 41] and multi-reflection cells has been demonstrated with an improvement in signal-to-noise by factors of 3 to 4. However, this increase in sensitivity is achieved at the expense of both efficiency and resolution. The widening of the channel to increase path length leads to band-broadening. An alternative approach to improving sensitivity is to increase the photon flux through the detection system. Bruin [37] and Moring [39] have demonstrated the use of a sapphire-ball lens to focus more light through the capillary at the detection window.

Sensitivity and limits of detection can also be improved by signal averaging across multiple measurements of an analyte. Multiple measurements can be made in several ways; the simplest is to run the CE separation multiple times. However, this approach is impractical as migration times in separate CE runs are not adequately reproducible. Furthermore, the time required to complete the necessary number of runs to sufficiently increase the signal-to-noise ratio would be prohibitive.

Signal averaging may alternatively be achieved during a single run, by means of multiple point detection along the capillary. The analyte signal from each detector may then be averaged to increase the signal-to-noise ratio. The signal-to-noise increase will be equal to the square root of the number of photodiodes if the detectors are random-noise-limited [42]. A linear photodiode array (PDA) may be used to generate multiple detection points. In 1991 Culbertson and Jorgenson [42] demonstrated signal averaging of data collected from a photodiode array consisting of 2024 pixels. Based on a detector set-up incorporating 1500 pixels (from pixel 250 to 1750) a x85 improvement in S/N was obtained for the signal averaged data compared to the results obtained from a single diode.

The PEREGRINE CE instrument employs a PDA with 512 pixel detectors configured linearly along the capillary. Each has a width of 25 μm and produces an array 1.28 cm long. Each detector produces an independent electropherogram, generating 512 electropherograms for signal averaging with the aim of lowering the concentration detection limit of UV absorbance detection in CE. Using such an array a S/N ratio enhancement of approximately 22 should be realized compared to that of a single diode.

2.2.3 CE Data Analysis

Signal processing applied to the data obtained from the PDA can generate several data outputs. Traces from three of these have been used in herein: single pixel electropherograms, Generalised Separation Transform (GST) electropherograms and Equiphase maps (Figure 2.8a, b and c respectively). These traces form part of the P3EVA analysis software provided with the PEREGRINE instrument. A single pixel electropherogram is the trace obtained from a single detector, Figure 2.8a shows the trace obtained from pixel 250 which is the default setting of the P3EVA analysis software. The GST algorithm is a method of signal averaging while it maximizes the signal-to-noise but retains analyte peak shape information.

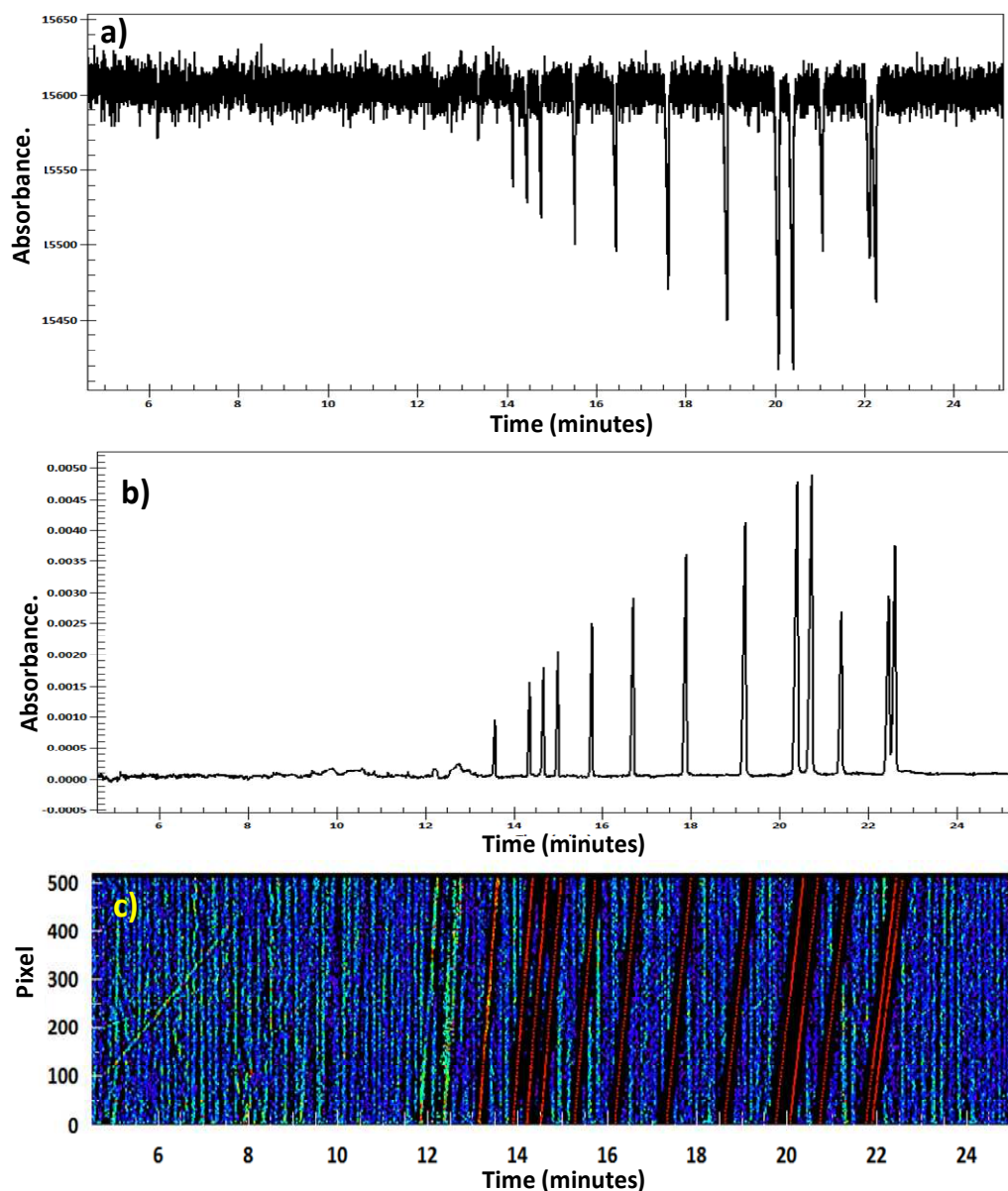


Figure 2.8: Capillary electrophoresis of Roche marker VIII dsDNA ladder using the PEREGRINE CE instrument. (a), (b) and (c) detail the single pixel (pixel 250), GST and equiphase map of the separation. Sample was separated using a 1.75% polyethylene oxide separation matrix.

In a multiple pixel array, the electropherograms generated at each pixel cannot simply be summed at a particular time point for all 512 single electropherograms. In each consecutive detector a distinct analyte will have a slightly longer migration time as each successive diode is further from the injection point. Furthermore, single electropherograms cannot simply be time-shifted relative to one another prior to averaging, as each analyte possesses a distinct velocity (based on their intrinsic characteristics). Thus, only one analyte will be correctly averaged for any specific velocity correction. To accurately signal average the single-diode electropherograms, a ‘vertexing’

constraint must be applied to the data. The last single-diode electropherogram (at pixel 512) is regarded as the base electropherogram. The position of the default ‘vertex’ of the analytes in distance and time is known, (t_0, z_0) . The vertex is the point of injection and is defined by the separation length or distance from the point of injection to the first pixel of the diode array and the time of the injection. This is typically not a point value as the injection occurs over a period of time generating a sample plug of some width. As the analytes have distinct constant velocities, the analyte velocity (v) can be established from the migration time (t_{512}) at which the analyte enters the last diode detector (n_{512}) at position (z_{512}) as

$$v = \frac{z_{512} - z_0}{t_{512} - t_0} . \quad (2.3)$$

The distance between each diode in the array is known, thus the distance between an arbitrary pixel (n) and the last diode detector is given as,

$$z_{512} - z_n = (512 - n)d , \quad (2.4)$$

where d is the diode width. Therefore migration time (t_n) can be defined as,

$$t_n = t_{512} + \frac{(n - 512)d}{v} \quad (2.5)$$

Here t_n is the time at which a particular point (e.g. an analyte peak) in electropherogram 512 will have passed pixel n . For each single pixel electropherogram, a normalization background light level (B_n) is calculated. This is taken as the average baseline voltage between t_{1+50} and t_{1+250} scans, which lies near the start of the electropherogram. To normalise, for all pixels, the individual electropherograms are transformed using,

$$V'(t_n) = 1 - \frac{V_n(t_n)}{B_n} , \quad (2.6)$$

where $V_n(t_n)$ is the voltage value measured on pixel (n) at the migration time (t_n). When the analyte migration time is calculated for each normalised single electropherogram, the analyte signal absorbance values can be signal averaged over all the single electropherograms using,

$$V_{SA}(t_n) = \frac{\sum_{n=1}^N (V'(t_n))}{N} \quad (2.7)$$

where $V_{SA}(t_n)$ is the signal averaged voltage value, and N is the total number of diodes in the array. This process is generalized to include not just the analyte peaks, but every data point on the base electropherogram. This allows all points on electropherograms to be signal averaged, subsequently leading to a single complete signal averaged GST histogram. As the noise component is as likely to be both positive and negative, only the real analyte velocities will be accumulated in the histogram, while the integrated noise in a given range of velocities grows much more slowly. Consequently, compared to the single pixel trace the GST shows an increase in signal to noise.

2.2.4 PEREGRINE: Signal Averaging for 512 Pixels

The improvement in signal to noise values between single pixel and signal averaging over multiple pixels was demonstrated using two peaks from a 25 bp dsDNA ladder (Figure 2.9). Peaks labelled 1 and 2 in Figure 2.9 were chosen as they show the highest and lowest signal in the electropherogram presented and because of the different polymer lengths (25 bp and 275 bp respectively). The S/N values for peaks 1 and 2 at pixel 250 were 12.5 and 5.6, whereas S/N values for the same peaks using GST processed data were 115 and 36.8. This indicates an order of magnitude increase in S/N between the detection approaches. However these values are still lower than the expected x22 enhancement. Variations in lamp intensity, electronic noise in the PDA, capillary vibration and any other sources of correlated noise which affect the whole PDA will reduce the signal to noise ratio.

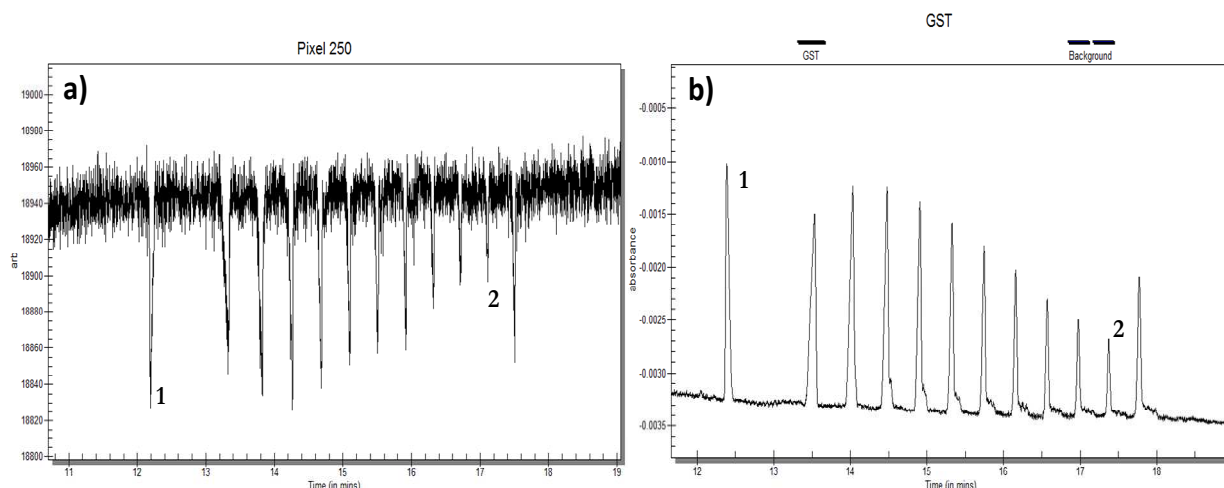


Figure 2.9: GST processed and single pixel electropherogram of the 25bp ladder (Promega Corporation, 36 μ g/ml in deionised water). (a) Single pixel electropherogram (pixel 250), the S/N on peaks 1 and 2 are 12.5 and 5.6 (b) GST processed electropherogram of the same separation where the S/N on peaks 1 and 2 are 115 and 36.8. The separation was performed using a 34cm long capillary of effective length 20cm, at 25°C and using an electric field strength of 203V/cm. The capillary was pre-coated with 10% PVP and the separation was performed in 4% PEO (300kDa) sieving matrix.

2.2.5 Equiphase Map

An equiphase map (Figure 2.8 c) is a three dimensional view of the separation, where the axes are distance, time and absorption. The equiphase algorithm searches for and calculates the size and position of all analyte peaks. The 3D image generated allows a macromolecule to be tracked as it migrates past the detector (Figure 2.10). The slope of the track generated is used to calculate the velocity of the analyte (as analyte velocity is equal to distance between pixel 1 and 512 divided by the time taken by the analyte to traverse the PDA). A schematic of an equiphase map is shown in Figure 2.9, with the associated single pixel electropherogram that may be derived from any pixel in the detector. An analyte traversing the detection window will appear in consecutive pixels of the PDA depending on its velocity. The velocity of an analyte is dependent on its intrinsic characteristics (e.g. molecular weight, pI and overall surface charge) and the applied field. Therefore, each analyte will generate a track across the detector with a specific slope; steeper slopes indicated analytes with higher velocities. Additionally, the tracks observed in the detection window may be extrapolated back to the injection zone (vertex) so that peaks not originating in the sample or moving in the opposite direction may be eliminated from the processed signal. By reversing the pixel order samples migrating from the outlet towards the detector can also be monitored. Thus, the presence of electroosmotic flow during a dsDNA separation can be

monitored. Moreover, the sample tracks can also be extrapolated forward to predict when any selected peak can be collected at the outlet end of the capillary after separation.

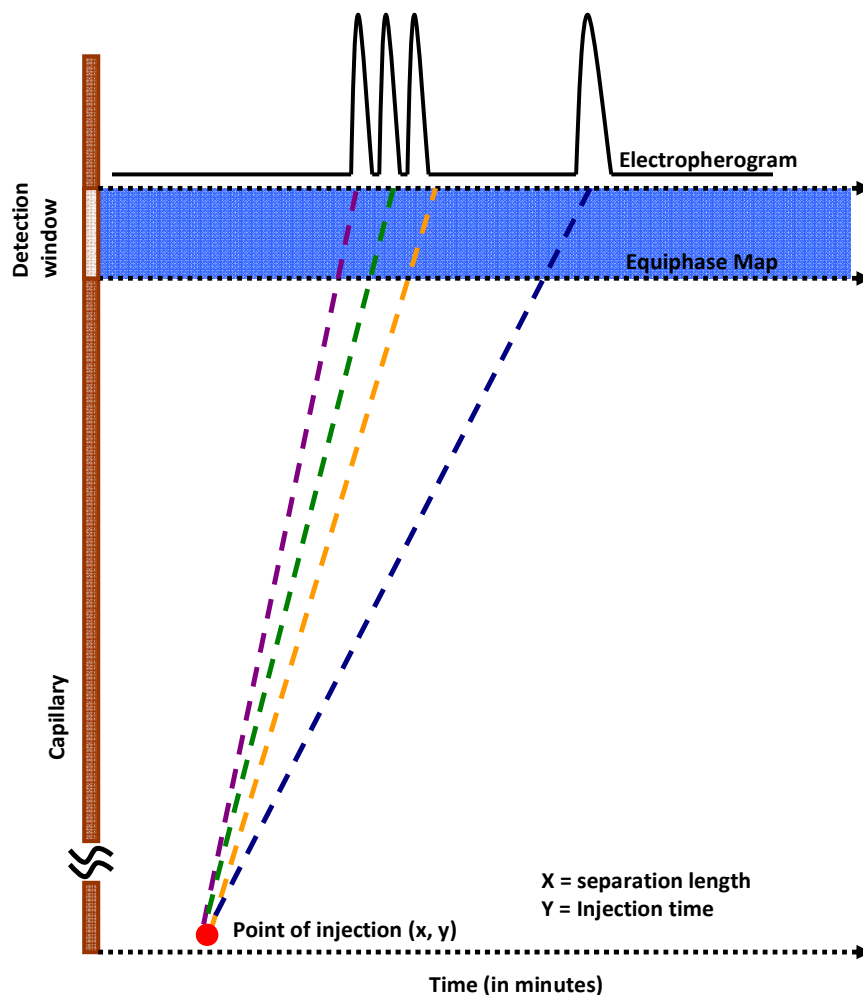


Figure 2.10: Schematic of equiphase map and single pixel electropherogram. Sample bands passing through the capillary at the detection window generate tracks that can be extrapolated back to the point of injection or vertex. Bands not part of the sample can be eliminated as their.

2.2.6 Separation Channel: Capillary

On the PERGRINE instrument the capillary is housed in a capillary holder. Figure 2.11a shows a schematic of all the components of the capillary holder while Figure 2.11b shows an image of the holder. The capillary holder is made from anodised aluminium with separation tracks from 10 cm to 70 cm carved into its surface. The tracks carved into the capillary block are plainly visible in Figure 2.11c and d. Separation lengths from 10 to 30 cm are symmetrical and therefore, for example, both inlet and outlet lengths can be set to 20 cm and samples may be injected from both

ends. This is often done to ascertain the presence of electroosmotic flow during a gel-based separation. The DNA sample is injected from the inlet end (typically the left hand side carousel) and an EOF marker, thiourea, from the outlet end. Consequently, the analytes are exposed to a similar electric field (E) and separation length (L_s), making mobility values determined based on E and L_s directly comparable. In addition, the equiphase map generated during such a separation permits the user to determine the direction of all other analyte bands and calculate the apparent and intrinsic mobility of each component.

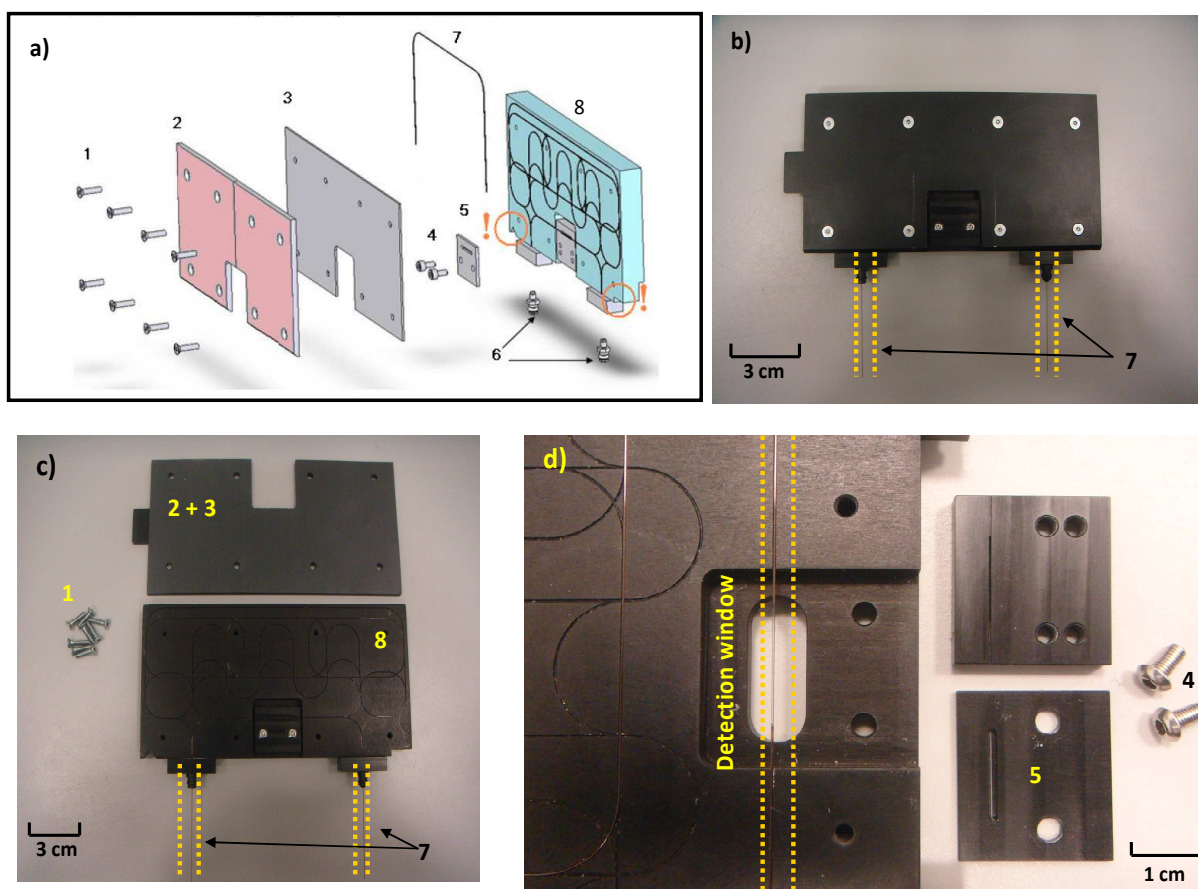


Figure 2.11: PEREGRINE capillary block where 1) Screws, 2) Top plate 3) Thermal pad, 4) Screws for detection window, 5) Cover plate for detection window with slit, 6) Ferule and valves, 7) Fused silica capillary and 8) Capillary tracks on base block are component parts. (a) A schematic of the capillary block with parts 1-8, reproduced from reference [27]. (b), (c) and (d) Images of the block whole, with top plate removed and highlighted detection window respectively. The capillary is highlighted using dotted lines. During capillary loading only parts 1, 2, 3, 5, 6 and 7 are dismantled; a new capillary is prepared and inserted into the capillary tracks following which the parts are re-assembled.

Capillaries are prepared by first measuring and cutting them to the appropriate length. For most dsDNA separations presented here a 40 cm separation length was employed, which had a total length of 54 cm and an internal diameter of 75 μm . After the 40 cm mark a 1.5 cm detection window was burned into the capillary using a flame. This removes the polyimide layer which is not UV transparent. The burning process should also be carried out in short steps where the capillary is exposed to the flame for only 30 to 40 seconds and then wiped down gently with ethanol soaked tissue (VWR International, UK). Once prepared the capillary was loaded on to the block, by inserting the capillary into the appropriate track. The capillary passes through an open section of the block which defines the detection window. The window burnt into the polyimide coated capillary is aligned with the detection window and secured (Figure 2.11d) before the capillary is fitted into the capillary block. Following which the capillary ends are trimmed such that they extend exactly 6 cm from the base of the capillary block. Due to the mechanism of sample loading on the PEREGRINE instrument, longer capillaries will fracture and shorter capillaries may suck in air during sample loading. Consequently the ends must be accurately measured and cut.

2.2.7 PEREGRINE: Standard Operating Protocol

The instrument was set up for the separation of dsDNA ladder and PCR amplicons. The capillary block was loaded and the filter wheel set to 254 nm. All the required flush reagents and samples were placed in their appropriate carousel positions. At the start of the day and prior to separation, the capillary was flushed with 1M HCl for 5 minutes at 60 p.s.i followed by a deionised water rinse for 3 minutes at 60 p.s.i. Between separations the capillary was rinsed in a similar way with the addition of a 0.1M HCl flush for 3 minutes at 30 p.s.i followed by a flush with 10 % polyvinyl pyrrolidone (PVP) for 5 minutes at 80 p.s.i. The separation matrix was then loaded into the capillary by applying a pressure of 100 p.s.i for 18 minutes. After loading the gel a conductivity check was always performed to ensure that the capillary was filled completely and that there were no air bubbles present, with a successful check generating a linear voltage to current plot. All DNA samples were injected electrokinetically, typically at 5kV for 18 to 30 seconds. Following injection, samples were separated by applying a potential of 10-25kV across the capillary. Specific values for injection and separation voltages are provided in the results section.

2.3 Chip-Based Capillary Electrophoresis: Standard Operating Protocol

2.3.1 Fluorescence Detection

Fluorescence detection was accomplished using two fluorescence microscopes; a Nikon Eclipse 400 and a Nikon Eclipse TS100 (Nikon Ltd. Surrey, UK). Briefly, light from a 100 W super high pressure mercury lamp was passed through a FITC filter cube before being focused on the chip detection region using x2, x4 or x10 objective lens. Fluorescence emission was collected with the same objective and detected using a CCD camera (C4742-96, Hamamatsu Photonic Systems, Bridgewater, NJ). Images were collected, stored, and analyzed with Image J software (NIH). Fluorescence intensities were extracted from the images to produce electropherograms. Bright field images were also captured using a USB Microscope (VMS-001, Veho Discovery, UK).

2.3.2 Electrophoretic Separation Device Set-up

Before use, microchips were conditioned by rinsing the separation channel with 1M NaOH or 1M HCl. NaOH was used for separations employing an electroosmotic flow, while an HCl rinse was applied prior to the loading of a sieving matrix. This treatment was followed by loading the separation channel with electrophoresis buffer. Prior to sample analysis, a conductivity check was performed by applying increasing voltages across the separation channel. The electric field was applied according to the direction of separation using a high voltage power supply (HVS448 3000V, Labsmith, CA, USA). Applied voltages were controlled using software provided with the power supply.

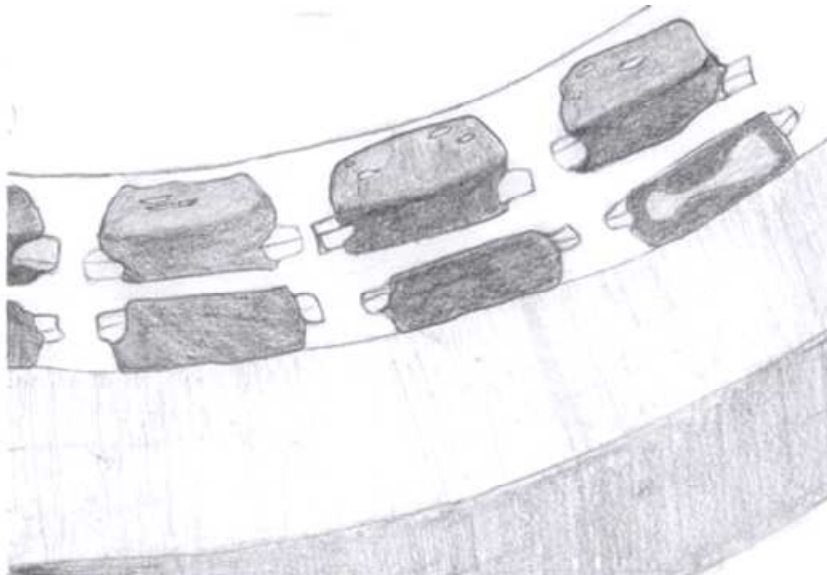
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Chapter III: Microchip Polymerase Chain Reaction



Synopsis

A circular, single channel PCR microdevice is described here, employing a novel in-channel pump to circulate the reaction components across the required temperature zones. The pump consists of fast-switching semi-conductor diodes fixed to the walls of the channel facing each other, with their cathode aligned in the same direction. Under an applied AC field, in the non-conducting direction of the diode a large potential drop is generated between the electrodes of the diode leading to the formation of an electrical double layer and an electroosmotic flow (EOF). An EOF generated in this manner is used to mobilise the PCR reaction mix around the channel. Compared to the single diode pair device described in literature, the multiple diode pair device employed in this work generated an EOF an order of magnitude stronger. Applying an A.C. field of 131.95 V/cm across a 16 pair diode device, a bead suspended in solution between the diodes travels at an average velocity of 0.43 mm/second and will complete one cycle in 1.47 minutes and 20 cycles in 29.43 minutes. The number of cycles depends on the length of time. To reduce cycle time the circumference of the circular channel can be reduced or the applied A.C. field increased. Polymerase chain reaction was conducted on a similar 16 diode device and compared to a bulk reaction performed on a commercial PCR instrument; both formats employed a 20 cycle amplification programme. Amplification proceeded on both commercial and diode formats, with the appropriately sized amplicon fragment (323 bp) observed for both procedures. The peak area obtained for the diode PCR amplicon was only 4.3 times lower than that obtained on the commercial instrument.

Initial experiments on the diode device led to the formation of a precipitate in the PCR solution on application of the AC voltage. To assess the cause of the precipitation, cation content of the PCR solution was measured using ICP-AES while anions were assessed using Ion Chromatography. Results indicated that components of the diode electrodes are transferred into the PCR solution via electrolysis. The presence of metal ions has been demonstrated to severely inhibit PCR. To reduce the effect of electrolysis on the diode electrodes the diodes were coated with a ~15 μm thick PDMS layer. The loss of components of the reaction to the surface of the PDMS via hydrophobic interaction was also investigated. Competitive agents like the protein bovine serum albumin (BSA), non-ionic surfactant Tween 20 and polymer polyvinyl pyrrolidone (PVP) were examined. Peak area results of the amplicon indicate that the presence of Tween 20 enhances the efficiency of the reaction, with a 16 % increase in peak area compared to that

Chapter III

observed for the standard, while the addition of 2% PVP increases the efficiency of the reaction by 36%. However, PVP has an inhibitory effect on EOF; therefore Tween 20 was used as a competitive agent for PCR performed on the diode device. Other experiments described here show that an A.C. field does not have a detrimental effect on the constituents of a PCR mixture and describe the effect of BSA and SYBR Green I on the amplification reaction.

3.1 Introduction

In nature, all organisms copy their DNA by the process of replication. In humans and most mammals this process occurs at body temperature with the aid of enzymes and other co-factors. The polymerase chain reaction (PCR) mimics this process, via temperature cycling and the use of a thermostable enzyme. In PCR amplification is exponential with the DNA produced in the n^{th} cycle being used as a template in the $n+1^{\text{th}}$ cycle [1]. Since a large amount of DNA is necessary for most genetic analyses, studies of isolated pieces of DNA are often impractical without PCR amplification. PCR has been essential in the progression of many areas of science, including mapping the human genome [2], molecular archaeology [3], forensic science [4,5], molecular ecology, disease diagnosis [6,7] and drug discovery [8]. In addition, the basic process of PCR has been modified to allow different types of DNA to be analysed. For example, inter-simple sequence repeat PCR (ISSR-PCR) [9] is a method for DNA fingerprinting that amplifies regions between some simple sequence repeats to produce a unique fingerprint of amplified fragment lengths. It is extensively used in forensics to help identify individuals. Quantitative PCR (qPCR) [10, 11] is used to quantify the original amount of DNA in a sample. Here, intercalating fluorescent dyes like SYBR Green I are used to monitor the amplification in real time [12, 13]. Accordingly, PCR is an important tool in the biological laboratory. The polymerase chain reaction was developed in the 1980's [14-16] by Kary Mullis but was originally described by Saiki *et al.* in 1986 [17]. Mullis was awarded the Nobel Prize for Chemistry in 1993 [18, 19] for this invention.

Conventional PCR systems are in use in most biological laboratories today. These systems are well characterised, form part of many routine biological procedures and are generally available in two formats. The first is a combined heating and sample stage (a block) which is ramped between two or three appropriate temperatures. The second format involves the use of three or four heating stages pre-set to defined temperatures, with a robotic arm transporting sample to each temperature zone in the appropriate sequence. In each, the total reaction time is typically between 1 and 2 hours. Micro Total Analysis Systems (μ -TAS) have facilitated the next step in the progression of many scientific methods including PCR. Miniaturization has key advantages, such as low sample and reagent consumption, faster reaction times due to higher mixing rates and increased portability. However, miniaturization of PCR is particularly significant as it is an important molecular biological tool. Compared to conventional systems chip-based PCR systems have smaller thermal masses which allows significantly higher temperature ramping rates. Heat transfer

between the temperature controller and the PCR reaction mix is faster, reducing the total amplification time from hours to minutes. In addition, the PCR mixture is exposed to more uniform temperatures due to fast mixing and rapid heat transfer, thus improving the efficiency of the reaction. Chip-based PCR also allows parallel amplification with minimal sample cross-talk and facile integration into a more complex μ TAS (reducing the manual handling of the sample between techniques).

PCR microdevices have been fabricated using a wide variety of materials. Initially, glass and silicon [20-28] were the most commonly employed due to well characterized fabrication techniques. Additionally, silicon and glass have excellent thermal conductivity enabling fast ramping times and efficient heat dissipation. Furthermore, metal film heaters are easily patterned on to the surfaces of these materials enabling integration of PCR heating and temperature sensing components. However, even in the face of all these advantages PCR microfluidics has moved away from using silicon or glass as a substrate material. The high thermal conductivity of silicon has a negative aspect, in that thermal insulation is required to prevent excessive loss of energy to the surroundings. Addition of thermal insulation to a PCR device complicates both its design and fabrication. Silicon has also been proven to inhibit PCR, reducing its efficiency or completely disabling amplification [29]. In addition, the silicon substrate is not transparent, hindering on-line optical detection. Glass, however, does not suffer from any of the above mentioned disadvantages. On the contrary, it has a well understood surface chemistry; it is optically transparent and does not inhibit PCR if passivated. Nonetheless, devices made in silicon or glass cannot be marketed as single use devices due to their high cost of fabrication. This shortcoming has hindered the use of these materials in commercially relevant applications. In recent years, polymer-based PCR microchips have been investigated as they can be fabricated at low cost and in high numbers, they are disposable and most are optically transparent [30]. Polymers like polydimethylsiloxane (PDMS) [31-34], polycarbonate (PC), polymethylmethacrylate (PMMA) [35], poly(cyclic olefin), SU-8 [36, 37] and polyethylene terephthalate (PET) have been used to construct PCR devices. However, based on current knowledge no single material can meet all the stringent requirements for PCR microdevices, including biocompatibility, optical transparency over a wide range of wavelengths, low cost and ease of fabrication and disposability.

To date several layouts have been suggested and tested by various research groups. There are two basic formats which include stationary chamber PCR devices with single and multiple chambers.

This is the simplest microchip PCR format, and was first demonstrated by Northrup and co-workers [38] in 1993 and Wilding *et al.* in 1994 [39]. Both groups employed silicon devices etched with micro-wells. The heating element and the entire device were cycled through the three temperatures required for amplification. Compared to conventional PCR, Northrup *et al.* completed 20 cycles four fold faster, due the reduced thermal mass of the microdevice. Since its introduction, many groups have presented chip-based PCR using the well plate format. However, there have been only a few devices which differed significantly from the original model presented by Northrup *et al.* Stationary chamber devices have been fabricated from materials like glass [40], poly(dimethyl)siloxane [33,41], silicon, SU-8 [36], poly(methyl)methacrylate [42] and polycarbonate. Nevertheless, single chamber PCR devices are not suitable for high-throughput applications and sequential use leads to cross-contamination between PCR samples. Disposable multi-chamber devices overcome these limitations; however special care should be taken over their design. Inhomogeneous temperature distributions across chamber arrays lead to differences in amplification rates which will affect reliability, reproducibility, specificity and efficiency of the reaction. In addition, on-chip integration of upstream and downstream processes would require the potentially challenging undertaking of handling and processing of multiple low volume samples.

The second micro-PCR format is the continuous flow device [27]. Here the PCR reaction mix is moved through a channel traversing heating blocks held at the three appropriate temperatures. The initial working version of this device was described by Kopp *et al.* in 1998 [27]. A flow-through layout has several advantages. It allows extremely rapid heat transfer as the heaters and substrate are not cycled between temperatures. Cross contamination between samples is minimal compared with multi-chamber layouts and it is more feasible to integrate this format into a μ TAS device. A significant limitation in conventional flow-through devices is that the number of cycles is dictated by the fixed channel layout. Furthermore, direct contact of the PCR mixture with the walls of the device and the increased surface-to-volume ratio compared to the well based format leads to loss of sample components to the surface of the device. Additives to the PCR mixture and permanent and dynamic coatings can aid in alleviating the adsorption. Furthermore, higher volumes of reaction mix are required. Some of these issues may be addressed using a variation of the continuous flow format that employs water-in-oil droplets. In droplet-based PCR reactions the parent reaction mix is fractionated into droplets separated by an oil phase and then amplified. The aqueous droplets are enveloped by the oil phase preventing loss of PCR components to the

microdevice walls. In addition, multiple samples can be processed and integrated with other processes without cross-contamination. Furthermore, the droplet format reduces the thermal mass reducing the total amplification time. A recently developed macroscale application of droplet PCR is emulsion PCR [43, 44]. Here the aqueous phase is prepared with primer loaded beads, Taq polymerase, PCR buffer and dNTPs, and template DNA. This is mixed with an oil phase, and agitated to create an emulsion. Monodisperse, paramagnetic, Streptavidin-coated micro-beads are pre-loaded with a dual Biotin forward primer. Ideally each droplet will have one bead, and a single molecule of template DNA. PCR is then performed to amplify the template DNA within each emulsion droplet. Consequently, depending of the immobilised primer, multiple sequences within the template strand can be amplified.

Accurate control of flow rates in a flow-through microfluidic PCR device is critical to the success of the reaction. In flow-through PCR devices, the flow rate, cross-sectional area and channel length control the residence time of the sample at each temperature zone [45]. In addition the cycle time may be defined as the residence time of sample in each temperature zone. Consequently, maintaining a stable flow rate ensures reproducible residence and cycle times, and in turn amplification yield. The minimum cycle time is controlled by the processivity of the polymerase, as both denaturation and annealing occur rapidly. PCR flow-through devices require a mechanism to drive sample through the channels. This is usually performed using syringe or peristaltic pumps external to the device. Several groups have demonstrated the use of syringe pumps to control the flow of PCR mixture through microchannels. Peristaltic pumps were used in 2002 by Chou *et al.* [46] and Sadler *et al.* [47] to drive PCR solution through a microdevice. These external pumps not only increase the footprint of the device but also have considerable dead volumes and are a great hindrance to integration of other techniques on-chip. They also increase the total reaction volume and the total energy input. In addition, flow-through PCR devices driven by pumps typically have long serpentine channels guiding the PCR solution through the various temperature zones. The long microchannels generate a significant backflow, requiring high pressures to drive the solution towards the exit reservoir. Indeed two layer polymer-based PCR devices show delamination under high pressure, leading to device failure. Some on-chip pumps have been trialled successfully, like rotary pumps, however, these are difficult to fabricate and require specialised equipment to control.

Aside from the use of pumps, PCR mixture may be driven through microfluidic devices using thermally induced convection and electrically induced electroosmotic flows (EOF). Thermal convection driven micro-PCR was first demonstrated by Krishnan *et al.* in 2002 [48]. A Rayleigh-Bernard cell filled with PCR solution was placed between two heaters maintained at 97°C (bottom) and 61°C (top). The temperature gradient developed between the heaters led to convection of the solution, or cycling between temperature zones. Hence the conditions for PCR were provided. Although PCR was achieved, a significant cost in terms of energy was incurred as the two halves of the chamber had to be maintained at high temperature. In 2006 Hu *et al.* [49] described a single channel microdevice to amplify DNA employing electrically induced EOF to oscillate the solution between the reservoirs situated at either end of the channel. The applied current also induced Joule heating which provided the temperature gradient necessary for PCR. Cycles of high and low current were employed to heat the PCR solution in the sequence of temperature required for amplification. The effect of the DC field on the reaction or its components was not described. However it may be assumed that as PCR was achieved, the field did not significantly affect either components or reaction kinetics. The EOF formed due to application of an electric field appears to be merely a by-product of system design rather than a requirement. As the liquid within the entire length of the channel was heated under the applied field, an EOF to transport the reaction mixture between reservoirs was unnecessary.

Chang *et al.* in 2007 and 2008 [50, 51] described the use of semiconductor diodes as pumps in microfluidic systems. By applying a global external A.C. field, a D.C. voltage is induced across the electrodes of the diode as a result of rectification of the A.C. field. The potential drop across the diodes leads to the generation of an electroosmotic flow which pumps the liquid adjacent to the surface. Such an electrically driven flow allows complete control over the direction of fluid flow in microfluidic system. In addition, there are no dead volumes as typically observed when using off-chip peristaltic pumps. Here a single channel continuous flow device is demonstrated, where electroosmotic flows generated across the surface of immobilised diodes are used to propel the PCR reaction mix between temperature zones. This format removes the need to have complex spiral channels which exhibit high back pressure and the reaction mixture can be cycled as many times as required. Chang *et al.* demonstrated fluid motion generated by a pair of diodes immersed in water. Here devices employed over 16 pairs of diodes placed parallel to drive the PCR mix across the appropriate temperature zones successfully amplifying template DNA.

3.2 Amplification Reaction

PCR is a cyclic process where each cycle consists of three steps, denaturation, annealing and extension, which occur at different temperatures (Figure 3.1) [52]. Theoretically, after each cycle (n) the amount of DNA in the sample doubles, leading to $2^n - 1$ copies. Therefore, 20 cycles will yield over a million copies from a single copy of the original target DNA. Typically around 25-40 cycles are required to obtain quantities at which detection and quantification can be conducted. Increasing cycle number will eventually lead to an “amplification plateau” where additional cycles do not increase amplicon concentration. This results from the exhaustion of deoxynucleotide Triphosphates (dNTPs) in the reaction mix and a drop in enzyme activity of the polymerase.

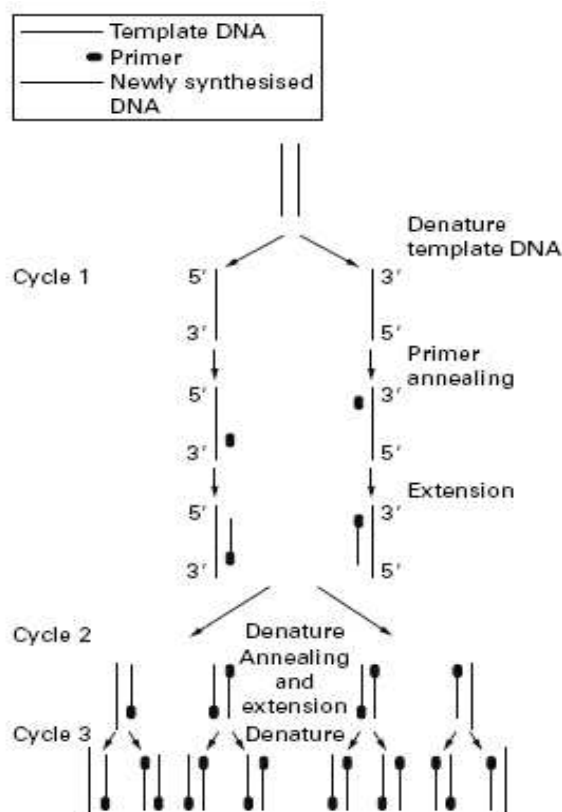


Figure 3.1: Schematic of the basic steps of the polymerase chain reaction. In a single cycle the sample is exposed to three different temperatures. Initially, double stranded template DNA is separated into single strands at high temperature (95°C). Then at 55°C upstream and downstream primers anneal to the single stranded template DNA. The upstream and downstream primers are composed of sequences which are complementary to the region flanking the amplification sequence. Finally, extension occurs at 72°C when the polymerase binds to the primer –template complex and extends it by adding on deoxynucleotide triphosphates complementary to the template sequence. The process repeats itself, exponentially increasing the DNA concentration with every cycle. Typically, 20 to 30 cycles are performed. Image reproduced from reference [1].

The effectiveness of PCR can be determined by its specificity, efficiency and fidelity. The fidelity and specificity of PCR depend on the polymerase used to catalyse the amplification reaction. PCR fidelity may be defined as the enzyme's capability to faithfully replicate a DNA strand without adding, substituting or deleting a base. Specificity defines the enzyme's ability to produce a single amplification product (amplicon) which is an exact copy of the target sequence. The efficiency of PCR is defined by its yield. A more efficient amplification will result more product from fewer cycles. The specificity and efficiency of PCR can be determined by electrophoretic analysis of the amplicon [53]. There are three basic methods for measuring the fidelity of PCR; forward mutation assay, reverse mutation assay and denaturant-gradient gel electrophoresis [53].

3.2.1 Standard Temperature Protocol

Macroscale PCR is generally performed on an automated instrument, Figure 3.1 illustrates the three temperature defined steps incorporated in one cycle. The reaction starts with a denaturation step, this ensures that the template strand is completely separated into single strands. During denaturation double stranded template DNA melts into two single strands. In addition, primer strands and associated extension product also detach from template DNA. Denaturation occurs at approximately 95°C, due to the disruption of hydrogen bonds between the nitrogenous bases. This is the first step of the reaction and all enzymatic action ceases during this step. After this the temperature is lowered to 50-60°C and at this stage the primers anneal to the 3' end of the single stranded DNA. Both strands of double stranded DNA can be copied simultaneously using upstream and downstream primers. The DNA polymerase then binds to the primer-template complex. The temperature of the elongation step depends on the DNA polymerase used. Optimum activity is usually observed around 72°C. In the presence of Mg^{++} and Mn^{++} ions, dNTPs complementary to the template strand are added to the primer strand. The reaction is generally terminated by maintaining the temperature at 72°C for several minutes enabling all the strands to be extended to their full length. The reaction is then cooled to 4 °C and stored at this temperature until further analysis. At this temperature the DNA is in double stranded conformation. In operation a typical temperature programme would read:

1. 95°C for 5 minutes (Initial denaturation).
2. The reaction mixture is then cycled between 20-30 times through three temperatures:

- 2a. 95°C for 20-30 seconds (Denaturation)
 - 2b. 55°C for 30-60 seconds (Annealing)
 - 2c. 72°C for 30-60 seconds (Extension)
3. 72°C for 2 to 3 minutes (Final extension)

3.2.2 Reaction Components

DNA Polymerase is the enzyme used to catalyse the polymerization of deoxyribonucleotides into a DNA strand. The polymerisation occurs in the 5' to 3' direction only and requires a pre-existing primer attached to the template from where the reaction can be initiated. In evolutionary terms, DNA Polymerase enzyme is highly conserved, with almost every organism displaying at least one form. The first polymerase reaction performed by Mullis and co-workers employed the Klenow fragment of DNA Polymerase I derived from *E. coli* [16]. However this enzyme is not heat stable and as the PCR reaction exhibits higher fidelity at high temperatures, a thermostable version of this enzyme was sourced from organisms (*Thermus aquaticus*) living in hot springs. The enzyme used in PCR is more commonly known as Taq Polymerase. It shows optimal activity at 72 °C within a pH range of 7.0-7.5, adding about 100 nucleotides per second with high fidelity in this environment. Taq Polymerase is a hydrophobic protein, hence the addition of non-ionic surfactants like Tween 20, Triton-X and Nonidet P-40 or other proteins help stabilise it in solution. Other reagents such as Urea, DMSO, DMF, formamide and SDS are well documented inhibitors of Taq Polymerase.

The template is the length of double stranded DNA to be amplified; it can be sourced from fresh tissue, hair, nails and bone marrow cells. PCR can tolerate poor quality template sample and has been applied to DNA sample extracted from archaeological finds. However, these samples typically contain short template fragments and require specially designed primers. Primers are short sections of single stranded DNA which are complementary to specific sections of the template. Primers are usually designed to be complementary to the sequences flanking the amplification region [54]. Taq polymerase only catalyses reactions in the 5' to 3' direction, consequently, for double stranded DNA two primers are required for sense and anti-sense strands of the dsDNA. These are typically known as upstream and downstream primers. Primers should not contain sequences that are complementary to one another or themselves as this will lead to

primer-dimer formation. Primers are typically between 15 and 30 bases in length, with a 50–60 percent guanine-cytosine base composition. Primers should not exhibit any secondary structure; therefore no palindromic sequences are used. The annealing temperature of the PCR reaction depends on the primer melting temperature (T_m) which in turn depends on its length and composition. The melting temperature is the temperature at which half the double stranded DNA dissociates to its single stranded form. The annealing temperature (50 - 80°C) is generally set between 1°C and 5°C above the primer T_m .

Deoxynucleotide triphosphates (dNTP) which are the building blocks of the new DNA strand are added to the PCR mix at concentrations between 50 μ M and 200 μ M. These molecules are thermostable and have a half life of more than 40 cycles. The dNTPs also sequester magnesium ions which are critical to the PCR reaction as they act as enzymatic co-factors. Magnesium ion concentration influences the shape, overall charge and binding efficiency of Taq Polymerase. For this reason the magnesium ion concentration is maintained at about 2.0 mM above the dNTP concentration. Generally, a lower concentration of magnesium cations will reduce PCR yield, while in excess they trigger the production of non-specific amplification products. The PCR buffer is critical in maintaining the pH of the reaction mix. Typically, a 10mM solution of Tris pH 8.5 to 9.0 at 25 °C is used. As the pH of Tris decreases by approximately 0.3 units for every 10°C rise in temperature, at 72 °C the reaction mix will pH 7.2 if prepared as pH 8.8 at 25°C. In addition, the salt content of the reaction mix is important; at high concentrations the salt ions may inhibit Taq polymerase function. Furthermore, the presence of phosphates and EDTA in the reaction mix will either precipitate or chelate magnesium ion reducing their concentration which in turn affects polymerase activity.

3.3 Methodology

3.3.1 Diode Device

The PCR diode device was a single circular channel 38 mm in circumference, 1.5 mm wide and 1.0 mm high with two reservoirs 2 mm in diameter to accommodate electrodes (Figure 3.2a). The device was fabricated using a purpose made aluminium mould (Figure 3.2c). PDMS base and curing agent were mixed together in a ratio of 10:1, poured over the metal mould, degassed and then cured at 65°C in an oven for 2-4 hours (Chapter 2 discusses standard PDMS device

fabrication). The PDMS replica was then cut away from the mould following which diodes were mounted on to the side walls using araldite epoxy (RS Components, UK). Here 1mm surface mount fast switching diodes (Digikey, UK) were used. As illustrated in Figure 3.2b the diodes were arranged parallel to one another with their cathodes facing the same direction.

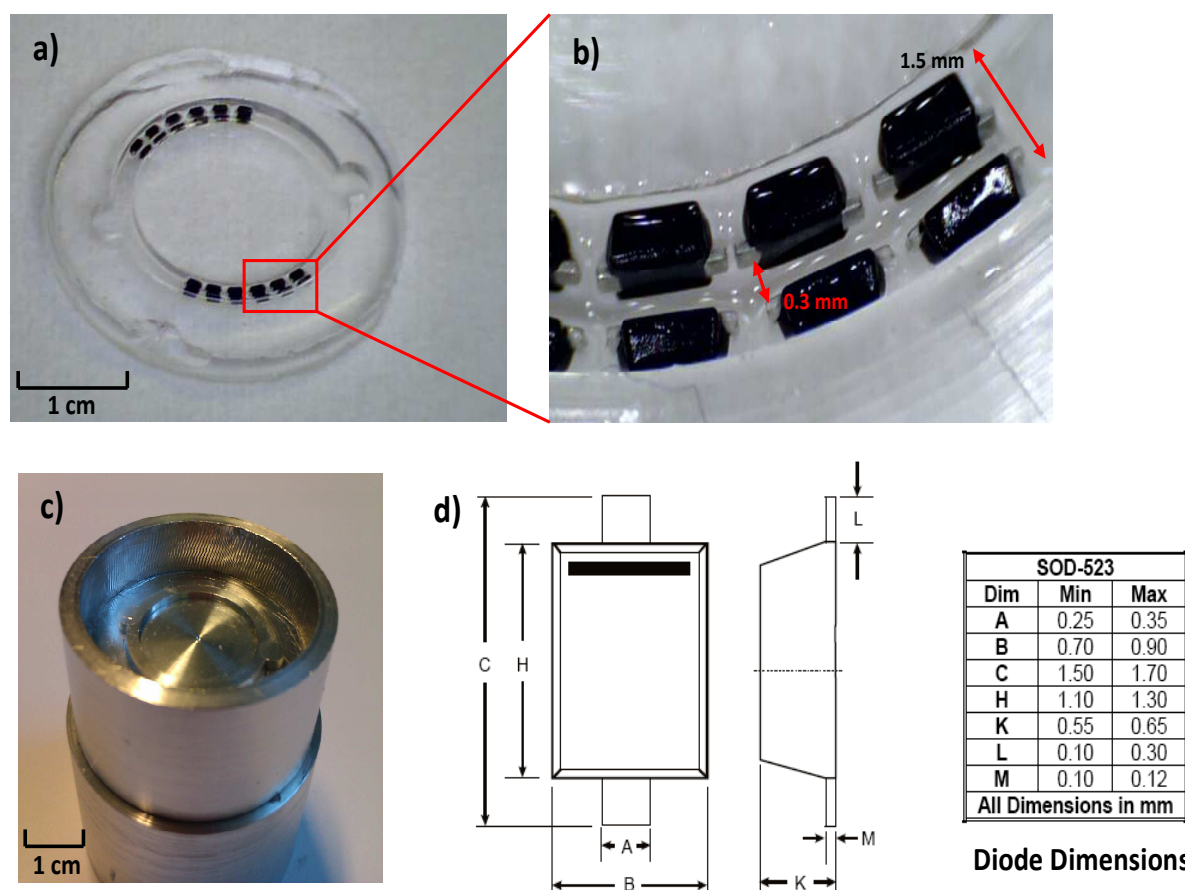


Figure 3.2: Images of the PCR diode device. (a) and (b) are photographs of the diode device cast in PDMS with a total of 12 diode pairs. The diode device had a circular channel ~ 38 mm in circumference, 1.5 mm wide and 1mm deep with two reservoirs 2 mm in diameter to accommodate electrodes. The diodes were glued to the inner surface of the channel ensuring the cathodes aligned in a single direction. The PDMS template was cast in a specially designed metal mold detailed in (c). (d) A schematic of the 1 mm diodes employed in this work along with the diode dimensions. Panel D has been reproduced from the manufacturers' specification sheet.

Typically, twenty to forty diodes were mounted for each operational device. The dimensions of the 1 millimetre diodes were provided by the manufacturers and are detailed in Figure 3.2d. The total length of the diode, including electrodes was about 1.6 mm, while the plastic main body of the diode was between 1.1 to 1.3 mm long. The width of the diodes was about 0.6 mm. Therefore when placed in the PDMS channel parallel to one another they form an inner channel

approximately 300 μm wide (Figure 3.2b). In operation, the circular channel of the PCR diode device is sandwiched between two borosilicate glass slides (VWR, UK). The glass slides were coated with a 300 μm thick layer of PDMS while the diodes and the channel were coated with a 15 μm thick layer of PDMS. The bottom layer is sealed to the PCR device using plasma bonding while the top layer is held in place using thin strips of double sided tape. Access holes were drilled into the top layer to permit insertion of A.C. electrodes.

3.3.2 A.C. Electric Field Generation

AC electric fields were generated using a TG2000 function generator (TTi, RS Components, UK) and a LPA400 laboratory power amplifier (N4L, UK). Figure 3.3a shows the experimental set up without the heating stage. The function generator output was connected to the amplifier input. Platinum electrodes were used to apply the external A.C. field. They were inserted into the electrode reservoirs on the diode device and connected to the amplifier output. The function generator was set to deliver a square waveform at 1 to 10 kHz and amplitude of 4 to 12 V peak to peak (V_{pp}). Figure 3.3b shows the variation of applied voltage as a function of the amplified voltage. The function generator settings used to achieve PCR on the diode device were a square waveform at 1 kHz frequency and amplitude of 10 V peak to peak with 50x amplification.

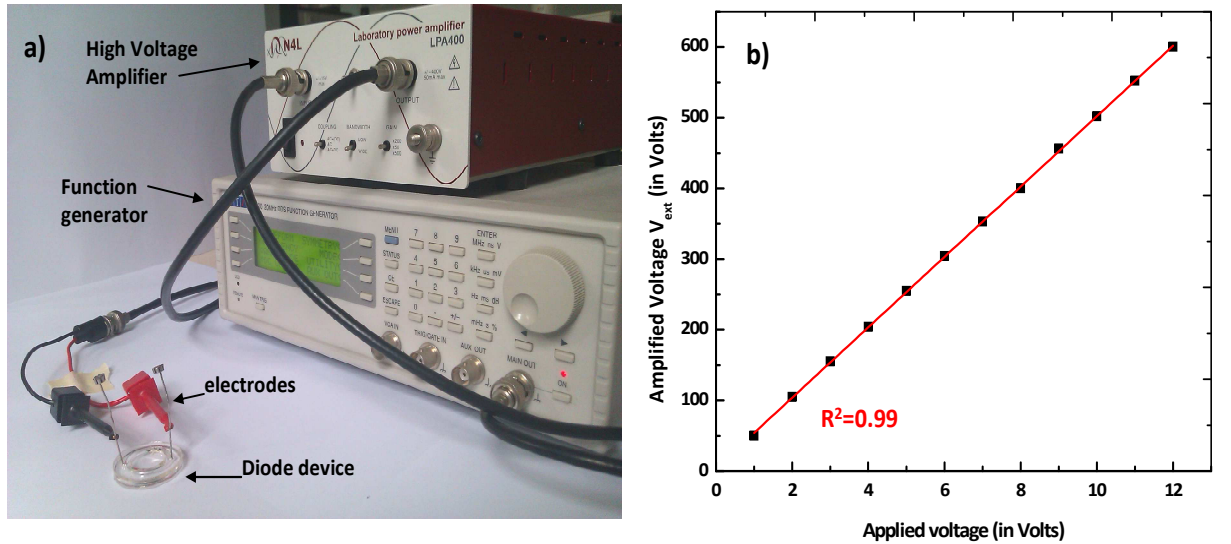


Figure 3.3: Experimental set up for diode device. (a) Illustration of the experimental set up, where the waveform generated by the function generator was amplified and applied to the diode device via platinum electrodes. The electrodes were inserted into the device at the reservoirs on the device. (b) A plot of applied voltage versus amplified voltage.

3.3.3 Heating Stage and Temperature Measurement

The PCR process is temperature sensitive and inefficient heating of the reaction mixture can lead to failure at any point in the amplification[30]. The choice of heating method depends on the substrate material and the chip design. Heating methods employed in PCR microfluidics can be divided into two groups; contact and non-contact heating [30]. The former includes metallic [55, 56] and Peltier-effect based ceramic heating blocks [56] and thin film heating elements like platinum [57, 58], polysilicon [59], aluminium [60] and indium tin oxide (ITO) [31, 32]. Non-contact based heating techniques include hot-air cycling [61, 62], infra-red light [63] and laser mediated heating. These allow heating of specific regions and thus problems associated with thermal isolation of other devices on the μ TAS are circumvented.

Herein two Peltier effect thermo-electric ceramic heat pumps (RS Components, UK) were used. The larger 3.0 x 3.0 cm Peltier was set to 95°C, while the smaller 1.5 x 1.5 cm Peltier was set to 75°C (Figure 3.4a). Temperature is monitored and maintained using a proportional–integral–derivative (PID) controller employing thermocouples. Each Peltier was powered by 4M power supplies (RS Components, UK) which were connected to the heat pumps via the PID controller. Feedback thermocouples were placed on the edges of the peltiers (Figure 3.4a). The peltiers were mounted on an aluminium stage specifically designed to hold them. A fan, placed below the stage, was used to cool the stage and allow the Peltier heat pumps to maintain their temperature. In addition, the fan cooled the stage between the heaters created the temperature gradient required for PCR. The PCR microdevice was mounted on the heating stage with each electrode reservoir positioned on top of a heat pump. The diode pump was used to cycle the PCR solution between each temperature zone, permitting amplification of the DNA target.

Temperature within the diode device was monitored using K-type thermocouples (RS Components, UK). The experimental conditions necessitated measurement of temperature in the presence of an external A.C. field at a minimum frequency of 1 kHz. This interfered with the temperature measurement generating an extremely noisy signal. Accordingly, the section of the thermocouple exposed to the field was coated to shield it from electrically induced noise. Thermocouples were tested coated with transformer varnish (RS Components, UK), PDMS and Kapton tape (Farnell Ltd., UK). All three coatings reduced electrical noise, however in terms of ease of use PDMS proved the best. The PDMS coated thermocouples were easy to insert between

parallel diodes to monitor the temperature of the solution. The PDMS coating was applied by dipping the thermocouple probe in uncured PDMS and heating at 90 °C for 5 minutes (Figure 3.4 b). A small difference (2-4°C) in measured temperature was noted between the coated and uncoated thermocouple when tested in the absence of an A.C. field.

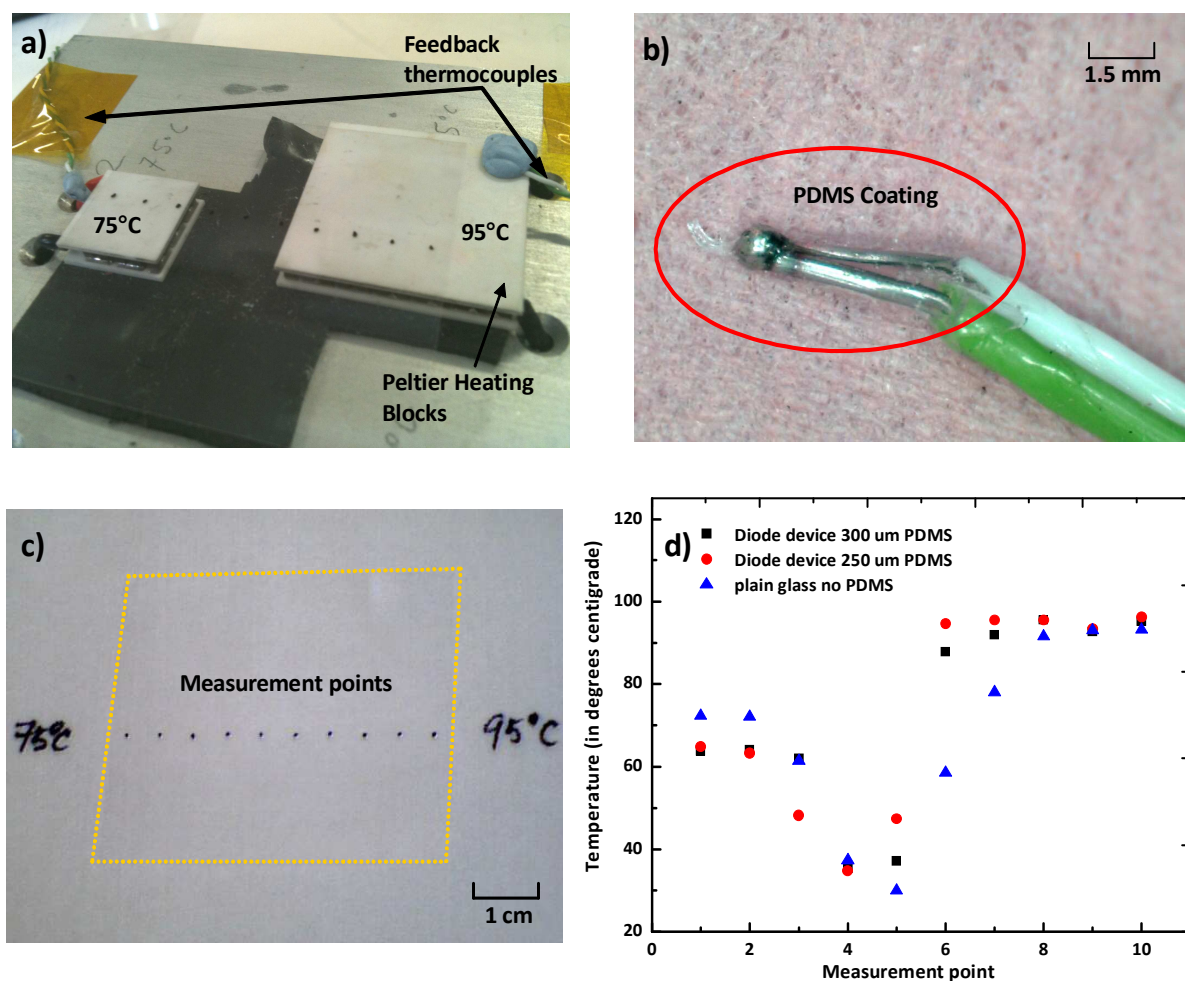


Figure 3.4: Heating stage and temperature monitoring. (a) A specifically designed aluminium stage which is mounted on a fan to allow efficient heat dissipation and prevent leakage of heat from one temperature zone to another. The stage holds two Peltier effect heat pumps set to 75°C and 95°C, with their heating surface facing away from the stage. Temperature of each heat pump was monitored and maintained using thermocouples and a PID controller. Thermal putty is placed below the heat pumps for superior thermal contact. The PCR diode device was mounted on the Peltiers such that each electrode reservoir sat on one Peltier. Thermocouples were also used to monitor the temperature of the solution within the diode device. (b) A thermocouple junction coated with PDMS, which prevented interference from the A.C. field applied to the diode device. The temperature gradient developed between the two Peltiers was determined using a glass slide with 10 equidistant measurement points as depicted in (c). In addition the temperature values within the PCR diode device when placed between the heat pumps were also determined. The temperature was measured at 10 equidistant points along one arm of the device. (d) A plot of the temperature values obtained on the glass slide and PCR diode device.

The temperature gradient across the heat pumps was measured using the PDMS coated thermocouples. A borosilicate glass slide (VWR, UK) was marked with 10 equidistant points (~ 0.5 cm) and placed between the Peltier heat pumps (Figure 3.4 a and c). The temperature at each point was measured five times and the average value at each point was plotted on the graph detailed in Figure 3.4d. The temperature gradient across the PCR diode device was also determined. Measurements were taken across a single arm of the circular channel, again at 10 equidistant points approximately 2 mm apart. The bottom of the PDMS diode device was sealed using borosilicate glass coated with a PDMS layer 300 μm or 250 μm thick. The PDMS device was loaded with 60 μL deionised water and placed on the heating stage for 5 minutes before measurements were recorded. The results obtained are plotted in Figure 3.4d. A maximum 20% difference in temperature was observed between the coated and uncoated measurements. The bottom layer with a 300 μm thick coating was used for all further work.

3.3.4 PCR Reaction and Analysis

The PCR reaction was carried out using a GoTaq[®] PCR Core System II reagent kit (Promega, Southampton, UK). The kit was supplied with a proprietary PCR buffer, deoxynucleotide mix (dATP, dGTP, dCTP and dTTP), magnesium chloride solution, upstream and downstream primers specific to the control template provided and DNA polymerase. The template fragment length was 323bp. Reaction mixture was prepared in deionised water as specified by the manufacturer and amplified using a RoboCycler (Stratagene, Agilent Technologies, USA) using a standard three step temperature programme. Table 3.1 lists the reagents, their final concentration and the volumes used to generate PCR mixtures of total volume 50 μL , 100 μL and 200 μL . Generally, a 200 μL master mix was prepared and fractionated into 50 μL sample volumes and then amplified. Following amplification samples were desalted using desalting columns (Pierce, UK) and then analysed using capillary gel electrophoresis on a PEREGRINE instrument (deltaDOT, UK). All CGE analysis was performed using a 1.75% polyethylene oxide (PEO) sieving matrix prepared in x1 TBE solution. Separation was performed on a bare fused silica capillary 54 cm long, having a separation length of 42 cm and internal diameter of 75 μm . The capillary was rinsed with 10% polyvinyl pyrrolidone (PVP) prior to loading the sieving matrix. PVP coats the internal surface of the glass capillary neutralising the EOF and enabling reproducible separations. Samples were injected electrokinetically at a voltage of 5 kV for 20 seconds and separated under a voltage of 11 kV.

PCR components	Final Concentration	Total volume 50 μ l (in μ l)	Total volume 100 μ l (in μ l)	Total volume 200 μ l (in μ l)
Flexi PCR Buffer	x1	10	20	40
PCR Nucleotide mix (dNTP mix)	0.2 mM each	1	2	4
Upstream Primer	0.99 μ M	3.3	6.6	13.2
Downstream Primer	0.99 μ M	3.3	6.6	13.2
Magnesium chloride	1.5 mM	3	6	12
Taq Polymerase	1.25 u/50 μ l	0.25	0.5	1
Control plasmid	0.02 ng/ μ l	1	2	4
Deionised water		28.15	56.3	112.6

Table 3.1: Components of the GoTaq® PCR Core System II reagent kit and their final concentration in standard PCR mixture used in this work. The volume required for each component to prepared reactions of total volume 50 μ l, 100 μ l and 200 μ l.

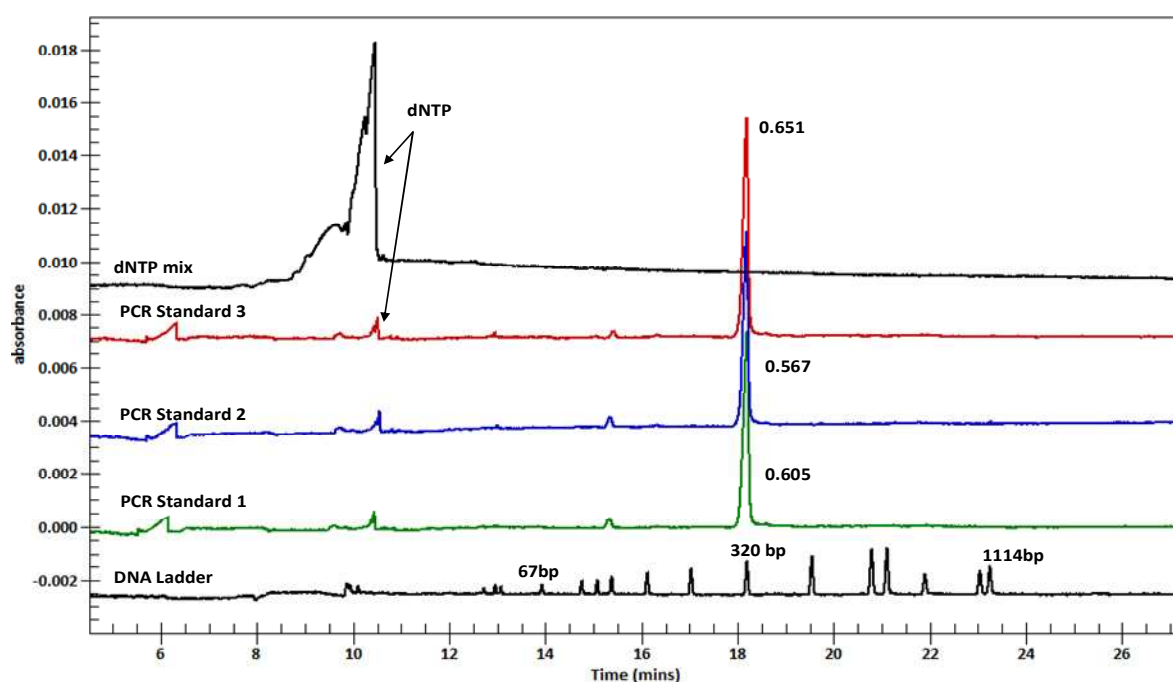


Figure 3.5: Analysis of PCR amplicons on Peregrine HPCE instrument. The image is an overlay of GST processed electropherograms obtained from the analysis of PCR amplicons using capillary gel electrophoresis. Analysis was performed to estimate the reproducibility of the standard PCR mixture used in this work. The traces compare the signal obtained from three separate amplifications of sample sourced from the same PCR master mix. The results are also compared to the Roche Marker VIII dsDNA ladder (25 μ g/ml in deionised water) and the dNTP mix. The standard PCR reaction mix was prepared as detailed in section 3.3.4, the reaction produces an amplicon fragment 323bp in length. All separations were performed using a 54 cm long capillary of effective length 40 cm, at 25°C and using a field strength of 203.7 V/cm. Separations were performed in the 1.75% PEO (5 MDa) sieving matrix.

Results obtained for the separation of double stranded DNA ladder (Roche Marker VIII, Hoffman La Roche, Basel, Switzerland) and the 323 bp PCR amplicon prepared under standard conditions and detailed in Figure 3.5. The PCR master mixture was divided into three 50 μ l samples, amplified and then analysed. The electropherograms show the peak areas obtained for standard 1, 2 and 3 to be 0.605, 0.567 and 0.651 respectively, resulting in a relative standard deviation (RSD) value of 6.7%. Additionally, the dNTP mix was analysed to establish elution times using the standard CE separation protocol. Several peaks were observed with an average migration time of 9.8 minutes. In comparison the signal obtained for the dNTP peaks in the PCR samples was much lower. This is expected as they are consumed in the reaction to produce the complementary strand of DNA. The dNTP signal was used to assess if PCR had proceeded on the diode device.

3.4 Results and Discussion

3.4.1 Fluid Flow Control

Chang and co-workers [50, 51] described the effect of an immobilised pair of diodes immersed in a low salt aqueous solution under an applied A.C. field in publications in 2007 and 2008. Figure 3.6a shows a schematic of their device including an equivalent circuit diagram used to establish the voltage drop across the diodes. The diodes rectify the applied voltage. In the conducting direction most of the current flows through the diode which offers a lower resistance compared to the surrounding liquid. The potential drop across the diode in this direction depends on the forward voltage drop, typically 0.7 V, generating a 0.63 V/mm field across the face of the diode. However, in the non-conducting direction, the resistance across the diode is very large and the current flows through the solution. The potential drop established between the diode electrodes is now defined by the current flowing through the solution and the resistance of the solution. The potential drop is larger in the non-conducting direction of the diode. The presence of ions in the solution leads to the formation of an electrical double layer (EDL) at the surface of the diode. The EDL is mobilised under the applied potential generating an electroosmotic flow. An EOF is established in both the conducting and non-conducting direction of the diode. As a larger potential drop is obtained in the non-conducting direction, it generates a stronger EOF pushing the liquid in that direction (Figure 3.6 c). Under standard conditions with buffer pH above 8, EOF is driven in the direction of the diode cathode.

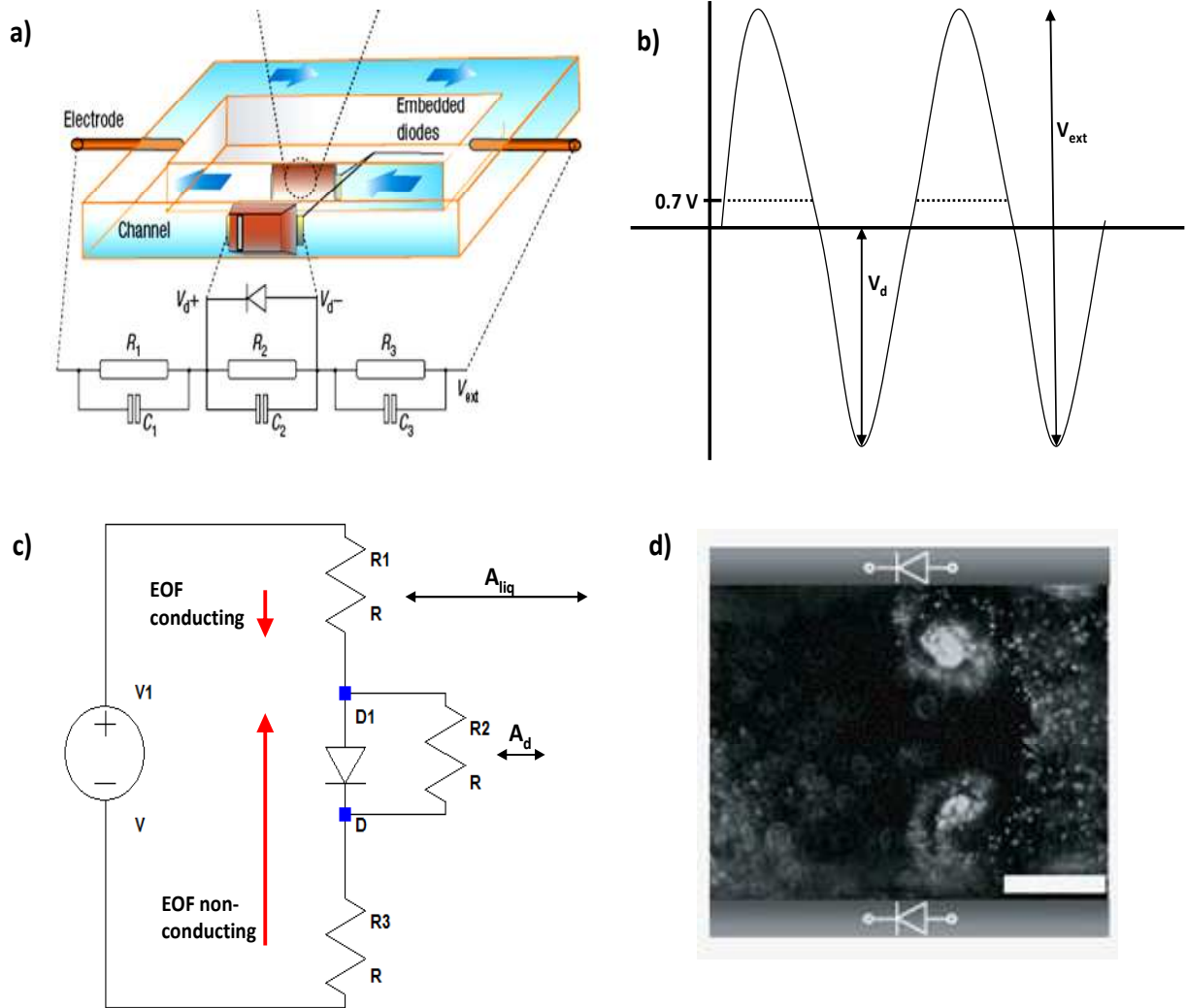


Figure 3.6: Generation of electroosmotic flow when applying an A.C. field across a diode immersed in an electrolyte solution. (a) A schematic of the diode device layout and corresponding circuit diagram used by Chang et al [51]. The resistance of the liquid adjacent to the diode pair are referred to as R_1 and R_3 , while the resistance of the liquid between the diodes is referred to as R_2 . (b) An illustration of the voltages applied across the diode device. The applied A.C. field (V_{ext}) is rectified at the diodes, where in the conducting direction the voltage obtained is the diode forward voltage drop, approximately $0.7V$. In the non-conducting direction the voltage drop across the diode is V_d . (c) A circuit diagram corresponding to a single diode pair device, with reduced area of cross-section. The resistance of the liquid adjacent to the diode pair are referred to as R_1 and R_3 , while the resistance of the liquid between the diodes is R_2 . The area of cross-section of the channel is A_{liq} while the area of cross section between the diodes $D1$ (and $D2$ not shown) is A_d . (d) Pumping action generated within microchannel by applying an A.C. field across diodes oriented in a similar direction. Images in (a) and (d) were reproduced from reference [50, 51].

The authors explain the origin of the D.C. field using an equivalent circuit diagram of the experimental system (Figure 3.6a). The liquid surrounding the diodes and between the diodes are defined in terms of resistance. The channel has some background resistance R_1 and R_3 while the region between the diode pairs it has resistance R_2 . In the conducting direction of the diode, it

offers little resistance to the flow of current. Consequently, the current flows through the diode instead of the surrounding liquid and the voltage drop across the diode is defined by its forward voltage drop. In this way the A.C. field is effectively rectified into a D.C. voltage across the diode. This value was measured for the 1 mm diode used in this work using a voltmeter and an average of 0.574 V was obtained over 10 randomly chosen diodes. However, in the non-conducting direction the diode offers nearly infinite resistance to the flow of current.

The current I flowing through the liquid in which the diode is immersed and may be defined as:

$$I = \frac{V}{R_1 + R_2 + R_3} . \quad (3.1)$$

Where V is the applied voltage (Figure 3.6 b), R_1 , R_2 and R_3 are the total resistance of the liquid due to ionic conductance. Specifically R_2 is the resistance of the liquid between the diode pair and R_1 and R_3 are the resistance of the liquid adjacent to and either side of the diode pair. Therefore, the D.C. voltage across the diode V_d in the non-conducting direction (Figure 3.5 b) maybe defined as

$$V_d = IR \quad \text{or} \quad V_d = \frac{R_2}{R_1 + R_2 + R_3} V . \quad (3.2)$$

Assuming that the material is uniform and homogenous the resistance is proportional to length (L) and inversely proportional to cross-sectional area (A) and can be defined as:

$$R = \frac{\rho L}{A} , \quad (3.3)$$

where ρ is specific resistivity. On their devices, Chang *et al.* immobilised a diode pair at a distance of 300 μm or 600 μm from each other. The diodes were attached such that the cross-sectional area of the channel remained the same. Therefore, the resistance was assumed to be linearly proportional to the length of the channel. Replacing resistance with the total channel length (L_T) and diode length (L_d), the D.C. voltage induced between the diode electrodes can be defined as:

$$V_d = L_d \frac{1}{2} \frac{V_{ext}}{L_T} = \frac{L_d}{2} E_{ext}, \quad (3.4)$$

where V_{ext} is the external A.C. voltage and E_{ext} is the A.C. field determined from V_{ext} and L_T . The factor of $1/2$ accounts for the A.C. voltage being rectified by the diode. Figure 3.5d shows the pumping action generated by the EOF when the diodes are immobilised with their cathodes facing the same direction. The width of the channel (600 μm) employed did not permit the EOF generated at the diode surfaces to overlay causing a backflow to develop in the central section of the channel. In the current work, the diode device had a minimum distance of 300 μm between parallel diodes. A basic circuit diagram of this device using a single diode pair is shown in Figure 3.6c. Without the diodes, the channel has some background resistance R_1 and R_3 , while the region between the diode pairs has resistance R_2 . The resistance is inversely proportional to the cross-sectional area of the channel and its variation will affect the resulting V_d . If the resistivity of the solution surrounding the diode remains constant, the resistance across the device ($R_1 + R_3$) without diodes and the resistance across the channel parallel to the diodes (R_2) can be defined as:

$$R_2 = \rho \frac{L_d}{A_d}, \quad (3.5)$$

$$R_1 + R_3 = \rho \frac{L_T - L_d}{A_{liq}}, \quad (3.6)$$

where A_d is the cross-sectional area of the channel formed between parallel diodes and A_{liq} is the cross-sectional area of the channel in the absence of diodes. Replacing R_2 and $R_1 + R_3$ in **equation 3.2**, the potential drop across a diode pair in a channel of reduced width is given by:

$$V_d = \frac{1}{2} V_{ext} \left(\frac{\frac{L_d}{A_d}}{\frac{L_T - L_d}{A_{liq}} + \frac{L_d}{A_d}} \right), \quad (3.7)$$

Re-arranging **equation 3.7** to define V_d in terms of electric field E_{ext} yields

$$V_d = \frac{1}{2} \left(\frac{A_{liq}}{A_d} \right) L_d E_{ext} \left(\frac{1}{1 + \frac{L_d}{L_T} \left(\frac{A_{liq}}{A_d} - 1 \right)} \right), \quad (3.8)$$

where A_{liq} and A_d are the cross-sectional areas in the open sections of the channel and between the parallel diodes respectively and L_T is the total length of the channel (38 mm). A_{liq} and A_d for the diode device are 1.5 mm and 0.3 mm respectively. Therefore, the V_d observed using this device format is 4.48 times greater than that observed in a channel of constant cross-section (equation 3.9)

$$V_d = \frac{4.48}{2} L_d E_{ext}. \quad (3.9)$$

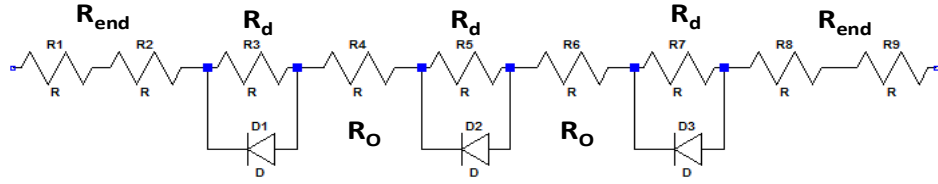


Figure 3.7: Simple circuit diagram of multiple diode device. Sections of the device contributing to resistance have been identified.

The diode device employed here had a single circular channel with multiple diode pairs arranged parallel to each other on either arm of the device. A simplified circuit diagram of this device is illustrated in Figure 3.7 while an image of the device is shown in Figure 3.2a. The resistance at the terminal ends of the device (R_{end}), between adjacent diode pairs (R_o) and between parallel diodes (R_d or R_2) is given as:

$$R_{end} = \frac{\rho_{end} L_{end}}{A_{liq}}, \quad (3.10)$$

$$R_o = \frac{\rho_o L_o}{A_{liq}}, \quad (3.11)$$

$$R_d = \frac{\rho_d L_d}{A_{liq} - A_p}. \quad (3.12)$$

Where ρ_{end} , ρ_o and ρ_d are the resistivity of the electrolyte at the terminal ends of the device, between adjacent diode pairs and between diode pairs respectively. L_o is the distance between adjacent diodes, L_d is the diode length and L_{end} is the length of the terminal ends of the device. A_p is the total cross-sectional of the diode pair. Therefore, the total resistance (R_{tot}) across the diode device is,

$$R_{tot} = 2R_{end} + \left(\sum_{i=1}^n (R_d + R_o) \right) - R_o. \quad (3.13)$$

The resistance at the ends of the device is doubled to account for two terminal ends housing the AC electrodes and n is the number of diode pairs in series. Replacing R_1 , R_2 and R_3 in **equation 3.2** with R_{tot} in terms of length and cross-sectional area gives,

$$V_d = \frac{\frac{1}{2} \frac{L_d}{A_{liq} - A_p} V_{ext}}{\frac{2L_{end}}{A_{liq}} + n \left(\frac{L_d}{A_{liq} - A_p} - \frac{L_o}{A_{liq}} \right) - \frac{L_o}{A_{liq}}}. \quad (3.14)$$

The numerator and denominator in **equation 3.14** were multiplied with A_{liq} . A new term α was then defined as the ratio of whole cross-sectional area and the restricted area (**equation 3.15**).

$$\alpha = \frac{A_{liq}}{A_{liq} - A_p}. \quad (3.15)$$

Replacing α in **equation 3.14** gives,

$$V_d = \frac{\frac{1}{2} \alpha L_d V_{ext}}{2L_{end} + nL_d \alpha + nL_o - L_o}. \quad (3.16)$$

To simplify, a new term ε was defined; ε represents the fraction of the total length taken up by the diodes and is given by,

$$\varepsilon = \frac{nL_d}{2L_{end} + nL_d + (n-1)L_o}. \quad (3.17)$$

Thus,

$$V_d = \frac{\frac{1}{2} \alpha L_d V_{ext}}{\frac{nL_d}{\varepsilon} + n \alpha L_d - nL_d}. \quad (3.18)$$

Equation 3.18 can be further simplified by multiplying numerator and denominator by nL_d and ε , to yield

$$V_d = \frac{\frac{1}{2} \frac{\alpha \varepsilon}{n} V_{ext}}{1 + (\alpha - 1) \varepsilon}. \quad (3.19)$$

In the case of devices with a high diode packing fraction and narrow internal channels formed by diode pairs the value of ε is close unity and the value of a is much greater than unity. Consequently, **equation 3.19** can be simplified to give a V_d proportional to the applied peak-to-peak voltage and inversely proportional to the number of diodes, i.e.

$$V_d = \frac{1}{2} \frac{V_{ext}}{n} \quad (3.20)$$

Equation 3.19 was used to determine the potential drop across a diode pair on a PCR diode device containing 16 diode pairs for V_{ext} between 50 V and 600 V (Figure 3.8 c). The potential drop across the diode was also determined using **equation 3.20** for a device with 16 diode pairs (Figure 3.7 c). A value of 5 was obtained for the term a , assuming a total cross-sectional area (A_{liq}) of 1.2 mm^2 and total diode cross-sectional area (A_p) of 0.96 mm^2 . The value of ε was determined to be 0.36, assuming that diode the length is 1.1 mm, the terminal length (L_{end}) is 12 mm and the length between adjacent diodes is 0.4 mm.

3.4.2 Effect of Frequency and Voltage on Flow Velocity

Fluorescently labelled 1 μm latex beads (Invitrogen, UK) were used to monitor the strength of the EOF. Initial work with these beads indicated that they varied both in size and surface charge. Therefore, their motion is related to their inherent electrophoretic mobility (due to their charge) and the EOF generated across the diode device.

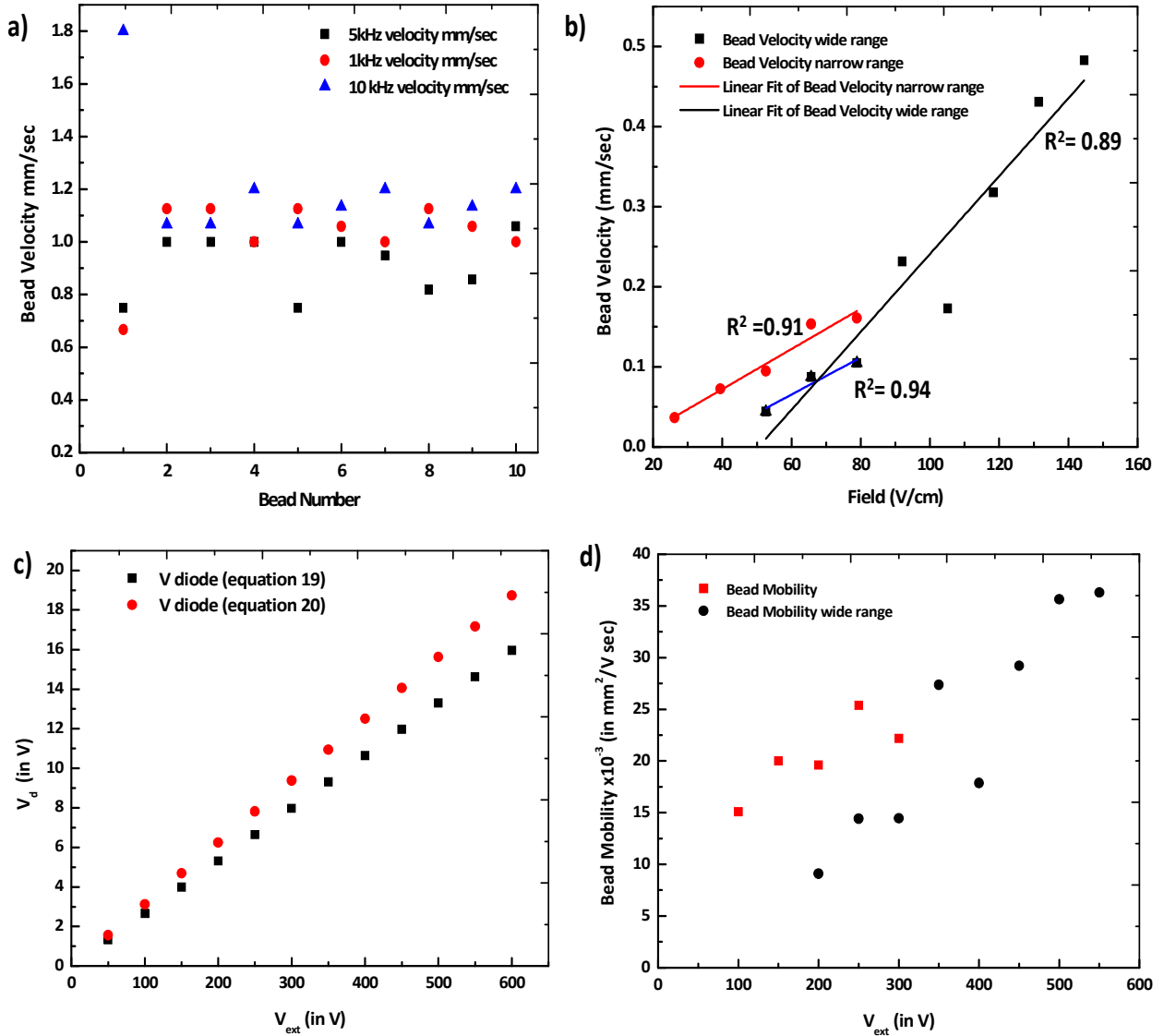


Figure 3.8: PCR diode device calibration. (a) and (b) Illustrate the effect of increasing A.C. frequency and amplitude on bead velocity. (c) A plot of applied voltage versus voltage drop across the diode as determined using equations 3.19 and 3.20. (d) The electroosmotic mobility of the beads determined from their bead velocity and the voltage drop across the diode.

The velocities of the beads were measured for increasing values of V_{ext} . The results obtained are illustrated in Figure 3.8b, with each data point being an average value obtained from the velocities of 10 beads. The wide range reading monitored the bead velocity induced in the A.C. voltage range of 200 V to 550 V peak-to-peak. The low range reading monitored the velocity in the range of 100 V to 300 V peak-to-peak A.C. voltages. The A.C. voltage applied was a square wave of 1 kHz frequency. Both results indicate that an increase in velocity is observed with an increase in applied field. Additionally, the beads were not observed to move at an applied A.C. voltage of under 50 V. The flow velocities at each V_{ext} were used to estimate the time required for beads to complete one revolution. A single revolution would represent one PCR cycle. The time required to complete a single cycle, 20 cycles and 30 cycles are listed in Table 3.2. At an A.C. field strength of 131.95 V/cm, a bead travels at an average velocity of 0.43 mm/second and will complete one cycle in 1.47 minutes and 20 cycles in 29.43 minutes. Furthermore, extrapolation of the trendline to the x-axis for each set of bead velocity data should identify the external field strength at which bead velocity has a zero value. This voltage represents the “turn on” voltage of the diodes (V_t). The estimated V_t from the narrow range trendline was 11.3 V/cm or 42.9 V peak-to-peak A.C. voltage while the wide range trendline gave a value of 28 V/cm or 106.52 V A.C. voltage. The disparity in values obtained is due to greater ion density at higher voltages. At low voltage and low power aqueous solutions will behave Ohmically. Power is directly proportional to the current and voltage ($P = IV$). However, at high powers (larger IV), the current will rise more quickly than the applied voltage, because the ion density is higher due to greater dissociation.

External A.C. Field strength (V/cm)	1 cycle (in minutes)	20 cycles (in minutes)	30 cycles (in minutes)
53.62	14.43	288.56	432.83
67.03	7.28	145.65	218.47
79.91	6.05	121.01	181.51
92.78	2.74	54.75	82.13
105.14	3.67	73.37	110.06
119.86	1.99	39.89	59.84
131.95	1.47	29.43	44.15
145.09	1.31	26.27	39.41

Table 3.2: The time required for a single cycle, 20 cycles and 30 cycles in minutes based on the applied external A.C. field strength (V_{ext}). The values were determined based on the velocity of beads at increasing field strengths.

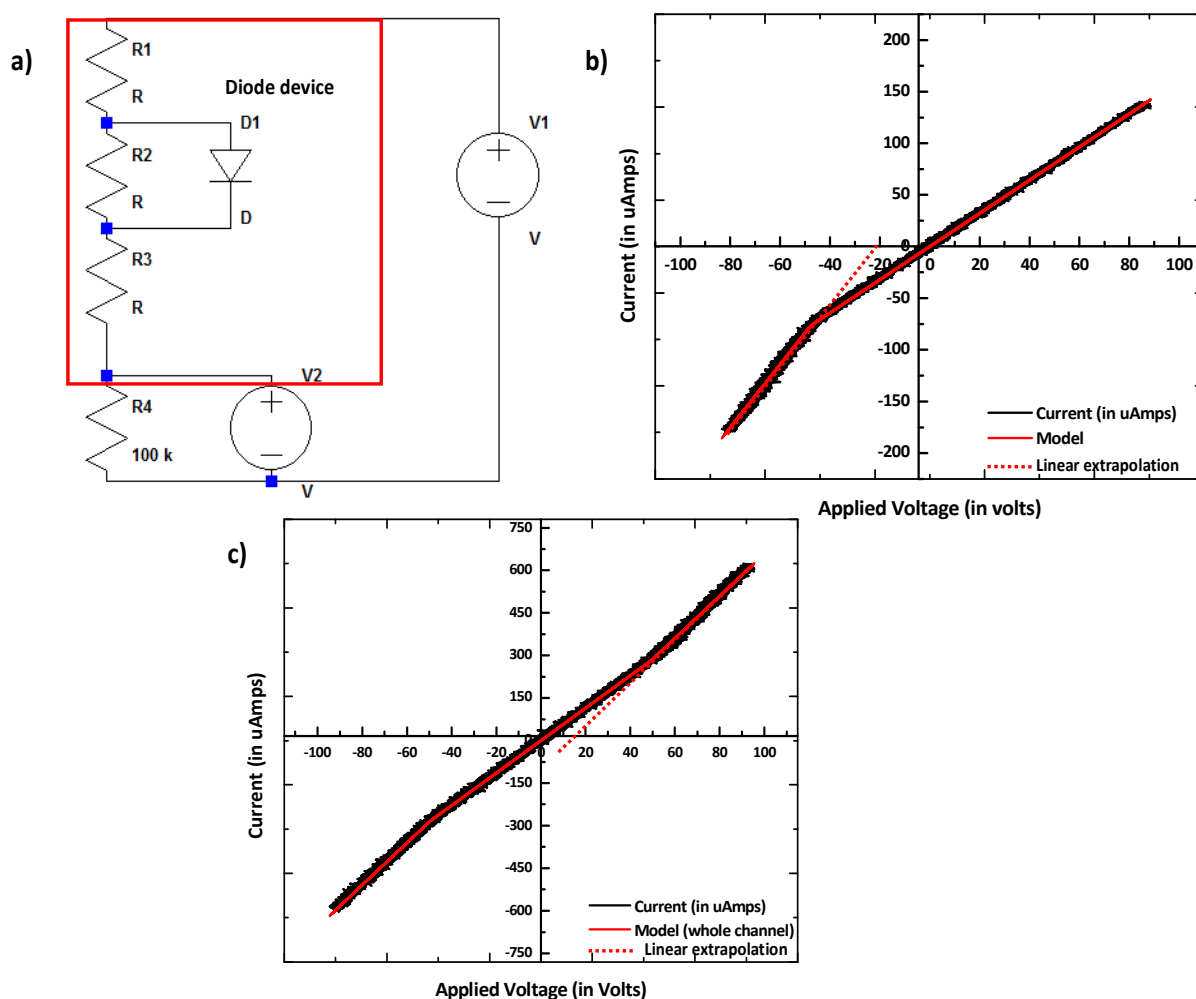


Figure 3.9: Determining diode threshold voltage. (a) A circuit diagram equivalent to an experimental set up where the voltage was measured across a 100 kOhm resistor (V_2) and across the 100 kOhm resistor in series with the PCR diode device (V_1) using an oscilloscope. The results obtained were plotted voltage versus current plots illustrated in (b) and (c). Measurements were recorded across a single arm of the circular device (half channel) shown in (b) and across the whole channel shown in (c). A model was fitted to the data used to determine the threshold voltage for diode turn on.

The velocity of the beads and the voltage drop across the diode (V_d) as determined using **equation 3.19** were used to ascertain the apparent mobility of the beads (Figure 3.8d). Mobility is defined as the ratio of velocity of the molecule and the applied electric field strength (Chapter 1). The field strength across the diode was determined by dividing the values obtained for V_d by the length of the diode (1.1 mm). Apparent mobility values were plotted against V_{ext} and show a clear trend of increasing mobility with an increase in applied voltage. Large variations in mobility values obtained at similar applied voltages are due to differences in surface chemistry of the walls of the diode and

the PDMS channel the device was immersed in 0.5 M sodium chloride and sonicated for 15 minutes. Following which it was air dried under nitrogen and placed on the heating stage. Additionally, the effect of frequency of the external A.C. field on bead velocity was assessed in the range of 1 kHz to 10 kHz. Other waveform parameters were maintained as constant. A square waveform of 500 V peak-to-peak amplitude, 0.0 V D.C. offset and 50 % symmetry was employed for the analysis. The results indicate that there is no correlation between the A.C. frequency and the velocity of the beads.

To evaluate the minimum applied voltage required for diode turn on or the diode threshold voltage V_{th} , a set of measurements was performed using an oscilloscope. A 100 k Ω resistor was placed in series with the PCR diode device and voltage measurements were acquired across the whole device (V_1) or across half the device (one arm of the circular channel also V_1) and across the 100 k Ω resistor (V_2). Figure 3.9a illustrates an equivalent circuit diagram of the set up, while panel (b) of the same figure plots the results from the half device, obtained in terms measured applied voltage (V_1) and current (I). Current (in μ A) was determined from V_2 as the value of resistance is known (100 k Ω). The result shows that at low voltages (below the diode threshold) the plot is Ohmic. The total resistance across the device is the sum of R_1 , R_2 , R_3 and R_4 . However as the applied voltage increases, the diodes turn on, its resistance reduces to zero and it takes more current, increasing the slope of the graph. Consequently, the overall trace obtained when applying an A.C. field across a channel lined with diodes has a ‘hockey’ stick appearance.

A model was fitted to the data using three components, where $R_1 + R_3$ defines the resistance of the channel (except near to the diodes), R_2 is the resistance of the channel sections that overlap and is electrically in parallel with the diodes. Assuming that above some threshold voltage the diode does not conduct, the current through the channel may be described as:

$$I = \frac{V}{(R_1 + R_3) + R_2}. \quad (3.21)$$

When the voltage is sufficiently negative, there is a threshold voltage V_t across all the diodes together that is sufficient to turn them all on. Once turned on, this voltage stays fixed across R_2 . Therefore, R_2 no longer controls the amount of current in the entire channel. Instead, the current

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is limited by the voltage drop across the remaining $R_1 + R_3$ of the channel, which is now $V - V_t$, because there is V_t drop across the diodes and resistance R_2 giving

$$I = \frac{V - V_t}{R_1 + R_3} \quad (3.22)$$

To estimate the value of V_t consider that a minimum current flowing through R_2 is required to develop an adequate voltage drop across R_2 in order to switch the diodes on. The voltage across R_2 at the point where the diodes begin to conduct is:

$$V_t = I_t R_2, \quad (3.23)$$

where " I_t " indicates the threshold values. The voltage that is available across R_2 is given by $R_2 * I_t$. Because the resistance $R_1 + R_3$ is in series, there should be $(R_1 + R_3) * I_t$ drop across $R_1 + R_3$. The total voltage is:

$$V = V(\text{across } R_1 + R_2) + V(\text{across } R_3) \quad (3.24)$$

$$V = I_t (R_1 + R_3) + I_t R_2. \quad (3.25)$$

Consequently,

$$I_t = \frac{V}{(R_1 + R_3) + R_2}. \quad (3.26)$$

Hence the threshold condition occurs when

$$V_t = I_t R_2 = \frac{V R_2}{(R_1 + R_3) + R_2}. \quad (3.27)$$

The three quantities $R_1 + R_3$, R_2 , and V_t are not independent, therefore defining a new variable c which is equivalent to R_1/R_2 . In summary,

$$I = \frac{V}{(R_1 + R_3) + R_2} \text{ for } \frac{V R_2}{(R_1 + R_3) + R_2} > V_t, \quad (3.28)$$

$$I = \frac{V - V_t}{R_1 + R_3} \text{ for } \frac{VR_2}{(R_1 + R_3) + R_2} \leq V_t. \quad (3.29)$$

The plot and a fit to this model (half channel) are detailed in Figure 3.9b. The values obtained for $R_1 + R_3$ and ϵ were 327 kOhms and 1.11 respectively. Therefore R_2 was 295 kOhms and V_t was -22.6 V. For the measurements obtained for the whole channel device, the right hand side diodes conduct when the voltage is above V_t while the left hand side diodes conduct when the voltage is below $-V_t$. The total current is then the sum of the two currents. A plot and a fit to this model are detailed in Figure 3.9c. Based on the fit the values obtained for $R_1 + R_3$ was 219 kOhms and ϵ was 1.68. Therefore R_2 was 130 kOhms and V_t was ± 18.5 V.

3.4.3 Overall Fluid Flow through the Device

Compared to the single diode pair device described by Chang *et al.* [50, 51] the multiple diode pair device employed in this work generates a stronger EOF. Figure 3.10a and b are stills from Video 3.1 showing bead motion under an applied field. The number of cycles depends on the length of time the A.C. field is applied. To reduce cycle time the circumference of the circular channel can be reduced. In operation, the diode device had a minimum of eight pairs of diodes in each arm of the circular channel with all cathodes aligned in the same direction. As described earlier, the EOF generated in the non-conducting direction is stronger than that observed in the conducting direction. Consequently, on the circular device, in the positive cycle of the A.C. field (Figure 3.10c); the left hand side (LHS) diodes are conducting while the right hand side (RHS) diodes are non-conducting.

The EOF generated on each arm is directed towards each other, however the RHS EOF is stronger. Therefore the net EOF drives the solution clockwise towards the LHS. In the negative cycle, the left hand side (LHS) diodes are non-conducting while the right hand side (RHS) diodes are conducting (Figure 3.10c). As before, the EOF from each arm opposes the other. However, now the LHS EOF is stronger driving the solution clockwise towards the RHS. During the positive or negative cycle, although the flows move towards each other the incompressible fluid in the channel does not overflow. This is due to the electrode reservoirs which behave as capacitors, permitting a swell of liquid to appear on each half cycle and then get picked up and moved around

on the other half cycle. In effect, the reservoirs smooth out the motion. The overall effect is to drive the solution within the channel clockwise round the device. Figure 3.10d illustrates this using an equivalent circuit diagram. The net EOF generated in the non-conducting direction of the diode pumps the liquid around the device. Additionally, it was observed that between adjacent diodes vortices were formed interfering with the EOF. This effect was minimised when the diode device was spin coated with PDMS.

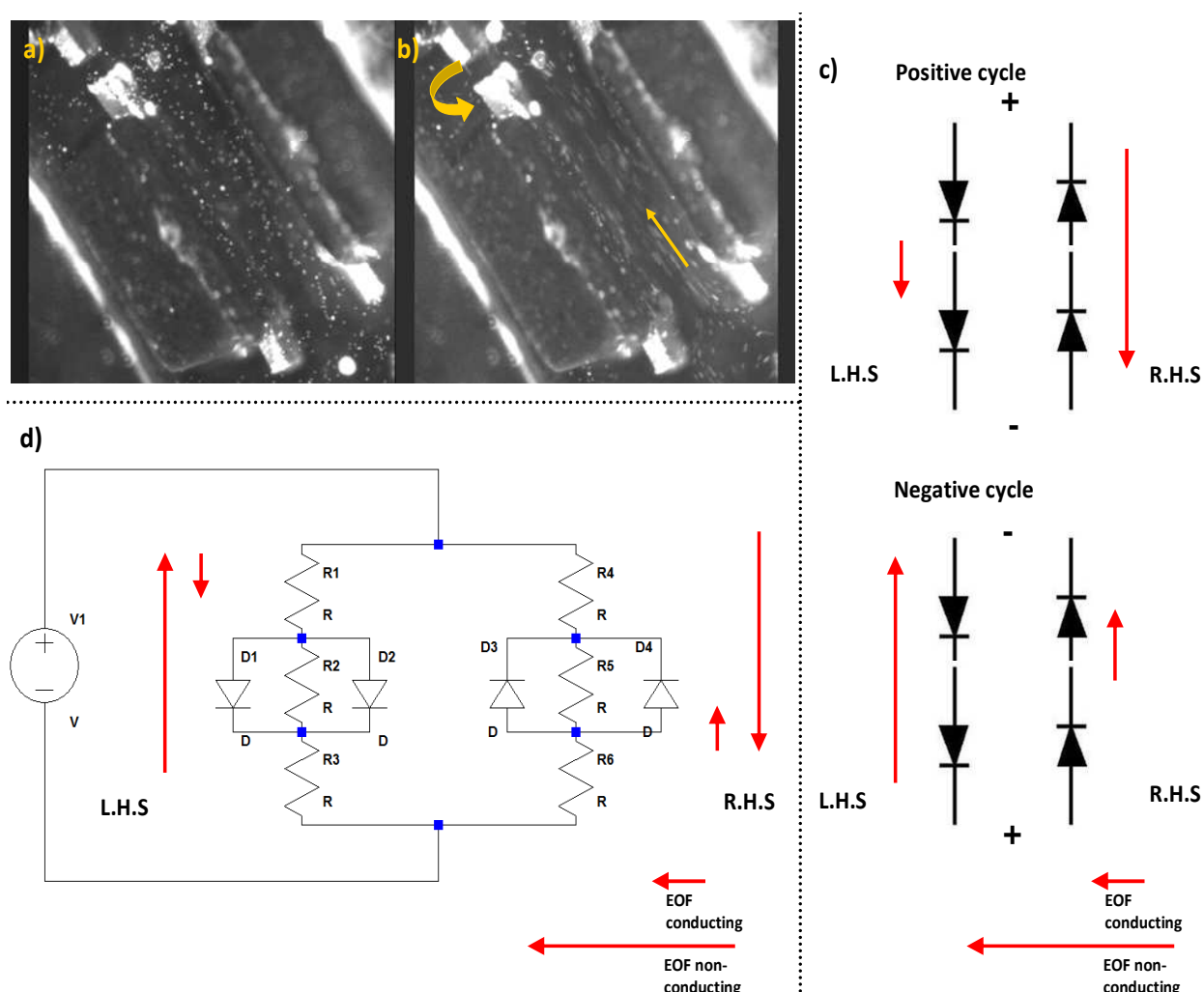


Figure 3.10: Flow of liquid within the PCR diode device. (a) and (b) are stills from Video 3.1, taken when the A.C. field was off and on respectively. The device was prepared with diodes attached to the channel wall with the same orientation permitting pumping of liquid around the device. Fluorescent beads were used to monitor the motion of the liquid. (c) Illustration of the EOF generated on the left hand side (L.H.S) and right hand side (R.H.S) of the diode device during the positive and negative cycle of the A.C. voltage. EOF in the non-conducting direction is always greater due to the higher voltage drop across the diodes. The diodes on the L.H.S and R.H.S alternate between conducting and non-conducting when the A.C. voltage changes from positive to negative. (d) An equivalent circuit diagram of the whole experimental system demonstrating the overall effect of the EOF. Following the larger EOF arrows the fluid is driven round the device in a clockwise direction.

3.4.4 Effect of PCR Mix on Diode Pump

The PCR diode device was filled with 60 μ l of standard PCR mixture and a layer of mineral oil was applied above the surface. The power supply was set to deliver 500 V peak-to-peak across the device. On application of the A.C. voltage the PCR solution developed a precipitate, which coloured the solution green (Figure 3.11a). To assess the cause of the precipitation, samples were analysed using a transmission electron microscope (TEM) with energy dispersive X-ray (EDX) detection, ion chromatography and inductively coupled plasma – atomic emission spectroscopy (ICP-AES). The analysis performed on the TEM-EDX proved unsuccessful as the instrument did not have sufficient resolution to identify all the ions present. Two sets of samples were analysed, samples 2 to 5 were controls and were not treated with an A.C. field. Sample 2 consisted of diluted PCR buffer while samples 3, 4 and 5 were prepared as standard PCR mixtures with variable concentrations of magnesium ions. The PCR buffer solution was diluted to one-fifth its standard concentration in deionised water prior to analysis due to the high concentration of precipitate formed during the initial tests. Samples 7 to 9 were the A.C field treated counterparts to samples 2, 3 and 5. The samples were exposed to A.C. voltage and consequently a diode rectified D.C. voltage for 5 minutes. Two magnesium salts were tested to determine if the ‘sleepers’ ion (chlorine or nitrate) contributed to the precipitation. Sample 3 contained 1.5 mM of magnesium chloride, while sample 5 contains 1.5 mM magnesium nitrate. Sample 3 reflects the standard concentration and salt typically used in PCR. In addition, PCR reactions with both salts were independently tested (Figure 3.11c) and analysed using CE. The reaction proceeds as normal, with peak areas for amplicon produced in the presence of magnesium chloride and magnesium nitrate being 0.901 and 0.661 respectively. A decrease in efficiency of the reaction was observed in the presence of nitrate.

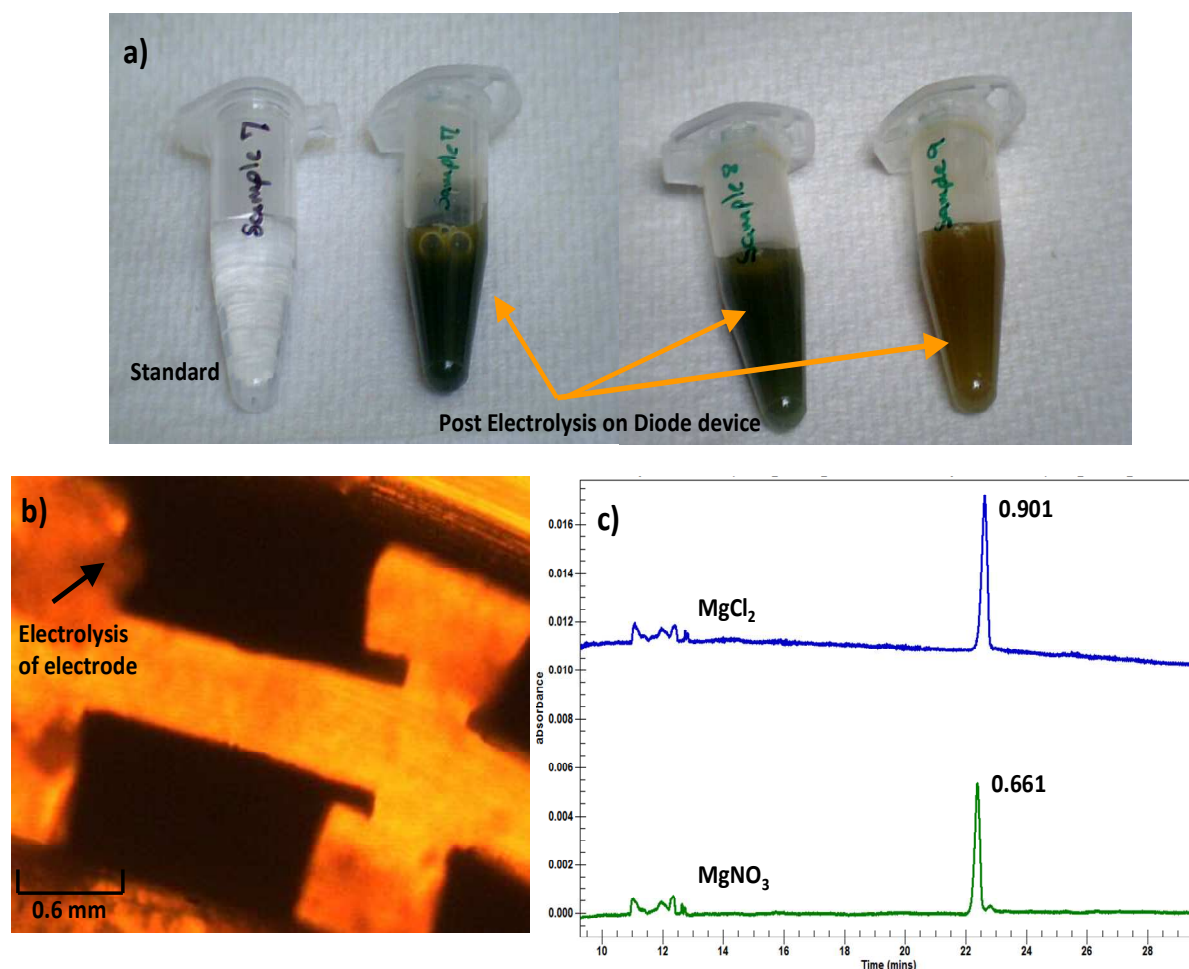


Figure 3.11: The effect of diode device on PCR solution. When loaded on to the diode device and exposed to an A.C. field the PCR solution turned green and a precipitate appeared. (a) A photograph of vials containing PCR solution exposed to A.C. field on the diode device compared to a standard untreated solution. The PCR solution depicted here is the PCR buffer diluted to one fifth its in use concentration with or without magnesium chloride. Sample number corresponds to those listed in Tables 3.2 and 3.3. (b) An image of the diodes on the diode device after the PCR precipitate and solution was removed. The same electrode on all diodes in the device had disappeared. (c) An overlay of GST processed electropherograms obtained for the analysis of a standard PCR sample compared to PCR solution prepared with magnesium nitrate.

Metal cations cobalt (Co), chromium (Cr), copper (Cu), iron (Fe), potassium (K), magnesium (Mg), manganese (Mn) and nickel (Ni) were measured by ICP-AES (Varian Inc, UK) employing a CCD detector. Multi-element calibration standards were made in 2% HNO_3 from single element stock solutions. Anions in the PCR solution were analysed using Ion Chromatography on an ICS-3000 (Dionex Corporation, USA). They were quantified by conductivity measurements in suppressed mode after separation on an AS-19 analytical column using KOH solution (10-50 mM gradient) produced by automatic eluent generation. Anions tested for included fluoride, chloride, nitrite, nitrate, bromide, sulphate and phosphate. The results obtained using ion chromatography and

ICP-AES for all samples are listed in Tables 3.3 and 3.4 respectively. In addition, an inspection of the PCR diode device after application of the AC field to the PCR mixture showed that most of the diodes in the device had lost their anodes (Figure 3.11b). According to the manufacturer's, specifications the electrodes were made from "Alloy 42". A basic composition of this alloy is detailed in Table 3.5. On comparison to the ICP-AES results, the presence of cobalt, iron, manganese, nickel and copper in the solutions analysed suggested that on application of an A.C. field components in the diode electrodes are transferred into the PCR solution. The most likely route is via electrolysis.

Sample	Ion Chromatography (ppm)						
	Fluoride	Chloride	Nitrite	Bromide	Nitrate	Sulphate	Phosphate
Sample 2 - PCR Buffer (1/5) Control sample	<	1666	<	<	35.7	1352	0.51*
Sample 3 - PCR Buffer (1/5) with 1.5mM MgCl ₂	<	1964	<	<	<	1445	<
Sample 4 - PCR Buffer (1/5) with 0.75mM MgCl ₂	<	1983	<	<	traces**	1517	<
Sample 5 - PCR Buffer (1/5) with 1.5mM MgNO ₃	<	1864	<	<	271	1497	<
Sample 7 - PCR Buffer (1/5) post electrolysis	<	1660	<	<	30	1023	<
Sample 8 - PCR Buffer (1/5) with 1.5mM MgCl ₂ post electrolysis	<	2281	<	<	<	1536	<
Sample 9 - PCR Buffer (1/5) with 1.5mM MgNO ₃ post electrolysis	<	1975	<	<	302	1554	<
LOD	5	5	10	10	12	10	10

Table 3.3: Analysis of the anion content of PCR solutions using Ion Chromatography.

Sample	ICP- AES (ppm)							
	Co	Cr	Fe	K	Mg	Mn	Sn	Cu
Sample 2 - PCR Buffer (1/5) Control sample	<	<	<	322	0.39	<	<	<
Sample 3 - PCR Buffer (1/5) with 1.5mM MgCl ₂	<	<	<	324	37.1	<	<	<
Sample 4 - PCR Buffer (1/5) with 0.75mM MgCl ₂	<	<	<	333	19.4	<	<	<
Sample 5 - PCR Buffer (1/5) with 1.5mM MgNO ₃	<	<	<	334	34.9	<	<	<
Sample 7 - PCR Buffer (1/5) post electrolysis	1.59	<	303	356	<	2.41	8.6	1.75
Sample 8 - PCR Buffer (1/5) with 1.5mM MgCl ₂ post electrolysis	1.42	<	287	357	32.6	2.45	<	2.2
Sample 9 - PCR Buffer (1/5) with 1.5mM MgNO ₃ post electrolysis	2.02	<	386	345	33	3.01	<	3.29
LOD	1	0.5	0.5	0.5	0.1	0.05	1	1

Table 3.4: Analysis of the cation content of PCR solutions using ICP-AES.

Electrolysis is a process in which a liquid solution containing salt ions forms part of an electronic circuit [65]. During electrolysis, under an applied voltage, charges move between the electrodes via salt ions in solution. The anode or positive electrode, typically has a deficit of electrons and will accept electrons from the ions in solution around it, hence oxidation reactions occur at the anode. While the cathode or negative electrode has an excess of electrons and will donate them, catalysing reductive reactions. The ions in solution in turn either accept or donate electrons at the appropriate electrode. During this process the ions in solution can be made to participate in chemical reactions. The products of these reactions depend on the electrolyte composition, electrode composition, applied voltage and current and the temperature of the electrolytic cell. In specific applications, such as electro-refining, the material of the electrodes is critical to the reaction within the electrolytic cell. Here the anode is consumed as its metal constituents are oxidised and go in to solution as cations. These cations migrate to the cathode where they accept electrons producing metal ions that deposit on the surface of the cathode. The presence of high concentrations of metal ions in the PCR mixture which are typically found in “Alloy 42”, indicate their transfer through some process. One explanation for the precipitate observed in the PCR solution is that the metal of the diode anode oxidises in solution. From the images taken it was not

possible to determine if any of the metal was deposited on the cathode as part of the electrolytic process, though a large quantity of it remained in solution in the form of a precipitate.

Alloy 42 Basic Chemical Composition (Diode electrodes)										
Weight %	Ni	C	Mn	P	S	Si	Cr	Al	Co	Fe
Alloy 42	42	0.005	0.8	0.025	0.025	0.030	0.250	0.15	1	
		max	max	max	max	max	max	max	max	Remainder

Table 3.5: Chemical composition of Alloy 42 as detailed by the manufacturer. The electrodes of the 1mm diode are fabricated from alloy 42. The main components include nickel, iron, cobalt, manganese and chromium.

Typically electrolysis does not occur when using A.C. voltage. The high frequency oscillation of electrode state, between anode and cathode, balances out reactions leading to a net equivalent of no reaction. However, on the diode device the diodes rectify the voltage generating a D.C. voltage across their electrodes. A minimal current flows in the conducting direction of the diode while a larger current flows in its non-conducting direction. The current in the non-conducting direction is dependent on the resistance offered by the electrolyte solution and the voltage drop across the diode. Therefore, although the potential gradient across the diode reverses with the positive and negative cycle of the applied A.C. field, the current flowing in the non-conducting direction is greater. According to Faraday's first law of electrolysis the quantity of substance liberated at an electrode is directly proportional to the quantity of current flowing through the electrolytic cell. Consequently, the anode in the non-conducting direction oxidises at a faster rate. This is corroborated by the fact that only one diode electrode vanishes.

3.4.5 PCR Mix Optimisation

At higher concentrations of electrolyte, bubble formation at the diode electrodes increased. This bubble formation is part of the electrolytic process. Above a threshold voltage of approximately 1.2 V (at 25 °C) electrolysis of water will lead to the formation of oxygen and hydrogen gas at the anode and cathode respectively [65]. Chang *et al.* [50, 51] performed bead velocity measurements in 1 μ M aqueous solutions of sodium chloride. The working solution of the standard PCR mixture used in this work contained 200 μ M of each deoxynucleotide triphosphate, 1 μ M of upstream and downstream primer, 1.5 mM magnesium chloride and x1 solution of the PCR buffer. The x1 PCR

buffer contained 50 mM potassium chloride, 10 mM Tris HCl pH 9.0 and 0.1% Triton X. At these concentrations, a strong EOF was observed, however, rapid bubble formation and nucleation at the diode electrodes led to channel blockages, which arrested the EOF.

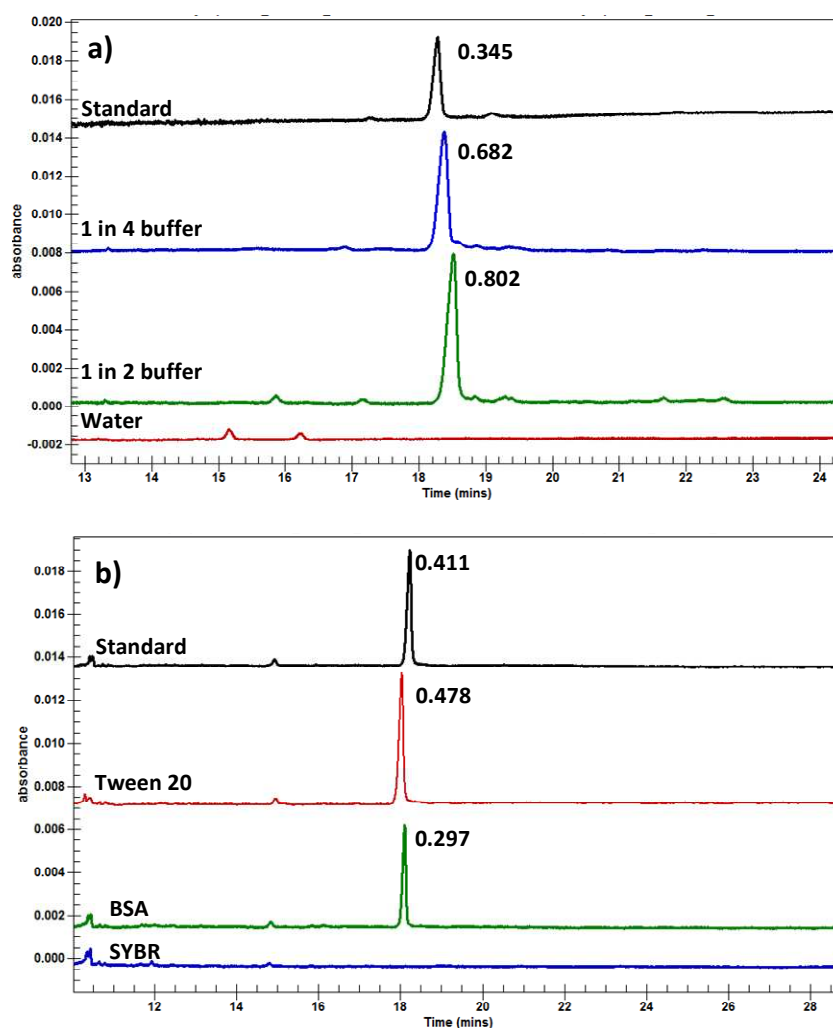


Figure 3.12: Analysis of the effect of salt and additives on the efficiency of PCR. (a) and (b) are overlays of GST processed electropherograms obtained from the analysis of PCR amplicons using capillary gel electrophoresis. All separations were performed using a 54 cm long capillary of effective length 40 cm, at 25°C and using a field strength of 203.7 V/cm. Separations were performed in the 1.75% PEO (5 MDa) sieving matrix. The standard PCR reaction mix was prepared as detailed in section 3.3.4, the reaction produces an amplicon fragment 323bp in length. (a) The effect of reducing the salt content of the PCR buffer. The PCR buffer was diluted to one half and one fourth its standard in-use concentration in deionised water or the PCR buffer was entirely replaced with deionised water. (b) The effect of additives such as Tween 20, Bovine serum albumin and fluorescent dye SYBR Green I (labelled SYBR).

To enable PCR to proceed on the diode device for a minimum of 20 cycles (~30 minutes) the concentration of the PCR buffer was reduced. Figure 3.12a illustrates the effect of reducing salt

concentration in PCR buffer for bulk reactions. PCR samples were prepared with half, one-fourth and standard PCR buffer concentrations. In addition, the buffer was entirely replaced with deionised water and tested. All other components of the PCR reaction were prepared as a stock and distributed to their appropriate vials prior to the additions of the modified PCR buffer. The results reveal that in the absence of PCR buffer the reaction fails to proceed and no amplicon was generated. However, reducing the level of salt in the reaction increases the amount of amplicon produced. Peak area values show that compared to the standard preparation whose amplicon peak area was 0.345, the reaction mix with half the salt showed a peaks area of 0.802, approximately 2.5 times that of the standard. While the reaction mix with one-fourth the level of salt showed a peak area of 0.682, double that observed with the standard preparation. Overall, reducing the concentration of salt in the PCR mixture appears to improve the efficiency of the reaction. The lower salt level also reduces electrolyte conductivity decreasing the amount of bubble formation.

Polydimethylsiloxane is a hydrophobic elastomer and forms the main body of the PCR diode device. To reduce the effect of electrolysis on the diode electrodes the diodes were also coated with PDMS. Work by other groups has demonstrated the adverse effect of PDMS on the PCR reaction using microfluidic devices. The loss of components of the reaction to the surface of the PDMS via hydrophobic interaction is one of the most widely accepted explanations for the lower amplicon levels observed. This problem is often exacerbated in a microfluidic environment due to the increased surface-to-volume ratio. Various groups have examined competitive agents like the protein bovine serum albumin (BSA) [24, 63], non-ionic surfactant Tween 20 [66] and polymer polyvinyl pyrrolidone [27, 67]. These agents compete with the polymerase and other PCR components for the surface of the PDMS, reducing the level of loss to the walls of the reaction chamber. Here the effect of 100 µg/ml BSA, 0.1% Tween 20 and 2% PVP on PCR using the standard amplification program (30 cycles) was examined. The PCR reaction was prepared as standard with the addition of BSA, Tween 20 and PVP maintaining the overall volume and concentration of all other reaction components. In addition, the effect of PDMS on PCR was also examined in the presence and absence of 2% PVP. Figure 3.12b and Figure 3.13 detail an overlay of the electropherograms obtained in each case. Peak area results of the amplicon indicate that the presence of Tween 20 enhances the efficiency of the reaction, with a 16 % increase in peak area compared to that observed for the standard. However a drop in reaction efficiency was observed in the presence of BSA. Most proteins including BSA adhere to hydrophobic surfaces due to their hydrophobic core. In addition, BSA is not thermostable and will denature at the high temperatures

applied during PCR, exposing its hydrophobic core. Therefore, at higher temperatures BSA should adhere more strongly to hydrophobic surfaces. However, if the protein is in excess it will sequester hydrophobic Taq polymerase, preventing it from participating in the DNA extension process. Consequently, at 1 mg/ml concentration BSA appears to reduce the efficiency of the reaction by 27 % compared to the standard. Accordingly device specific concentration calibration should be performed to ensure that excess BSA does not interfere with amplification process.

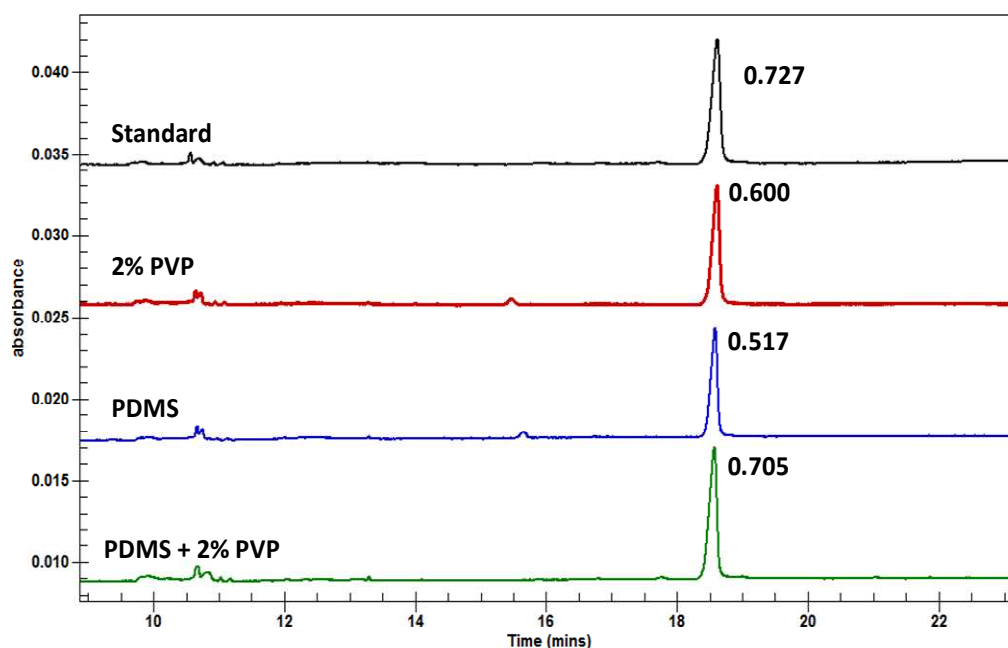


Figure 3.13: Analysis of the effect polyvinyl pyrrolidone (PVP) and PDMS on the efficiency of PCR. The image is an overlay of GST processed electropherograms obtained from the analysis of PCR amplicons using capillary gel electrophoresis. PVP was added at a final concentration of 2% to the standard PCR mixture which was amplified in a standard micro-centrifuge vial and one coated in PDMS. The results were compared to a sample not containing PVP. The standard PCR reaction mix was prepared as detailed in section 3.3.4, the reaction produces an amplicon fragment 323bp in length. All separations were performed using a 54 cm long capillary of effective length 40 cm, at 25°C and using a field strength of 203.7 V/cm. Separations were performed in the 1.75% PEO (5 MDa) sieving matrix.

The effect of the fluorescent reporter molecule SYBR Green I on PCR was also tested (Figure 3.12). This dye forms part of the quantitative PCR assay, allowing real time monitoring of the reaction. However, the addition of SYBR Green I at a final concentration of 1 µg/ml (x1 SYBR Green I, Chapter 4) to a standard PCR mixture, led to a CE electropherogram with no amplicon peak. It is important to note that the dNTP peaks associated with the progress of the reaction are small and comparable to those observed in the presence of other additives. A possible explanation

for this effect is that the dye modifies the overall charge of the DNA altering its behaviour during PCR and CE injection.

The internal surface of the PCR micro-centrifuge tubes were coated with PDMS and tested. The coating was applied by filling the tube with PDMS, heating the tube for 30 seconds at 90 °C following which the uncured PDMS was removed using a pipette. The tubes were then inverted and placed in an oven to cure overnight at 90°C. Four reactions were performed, the first two were controls where the PCR reaction was prepared and run under standard conditions and with 2% PVP in uncoated micro-centrifuge tubes. The second set was a similar experiment but was performed in coated tubes. Figure 3.13 illustrates the electropherograms obtained in each case. A standard reaction performed in a PDMS coated tube shows a 28% decrease in peak area compared to an uncoated tube. The decrease in efficiency can be attributed to loss of sample components to the surface and reduction in efficiency of heat transfer to the reaction mixture due to increase thickness of the tube and the low thermal conductivity of PDMS (0.15 W/ m K) [41]. The addition of 2% PVP to the PCR reaction performed in the PDMS coated microcentrifuge tube increases the efficiency of the reaction by 36%. In addition, the variation in peak area between standard and the reaction with 2% PVP performed in a PDMS coated vial was 3%. PVP is routinely used in capillary electrophoresis (CE) to retard the EOF, as will be demonstrated in the following chapters. PVP adheres to the surface of the silicon, glass or PDMS preventing the formation an EDL giving the surface a neutral character. Its addition, the PCR reaction loaded on the diode device would significantly reduce the EOF formed across the surface of the diode. This would have the effect of increasing cycle time and preventing amplification from proceeding.

3.4.6 Diode Pump with PDMS Coating

The performance of PCR is extremely sensitive to its environmental conditions. The presence of metal ions has been demonstrated to severely inhibit PCR. Taylor *et al* [68] showed that chromium was detrimental to PCR amplification. Chromium concentrations between 0.1M and 1 nM lead to little or no amplification. To contain the effect of electrolysis the diodes were spin-coated with PDMS. The PDMS applied was prepared as standard (Chapter 2) where the base and polymer were mixed in a 10:1 ratio and then diluted in toluene in a 1:1 or 2:1 ratio. Initially, the diode device channel was filled with PDMS (diluted 1:1 in toluene), spin-coated at 6000 rpm for one

minute and then allowed to stand for 10 minutes prior to baking overnight at 90 °C. This procedure resulted in a PDMS layer approximately 2 µm thick covering the diodes. The thickness of the coating was estimated by reproducing the coating on a glass slide and profiling the surface using a DekTak stylus profiler (Veeco, New York, USA). A decrease in bubble formation at the diode electrodes was observed for the coated device (Video 3.2). The function generator setting applied during the experiment was a square waveform of 1 kHz frequency, 10 V peak-to-peak amplitude and no D.C. offset. The amplifier was set to it x50 setting generating a voltage of ~ 500 V peak-to-peak across the device. A greater decrease in the rate of bubble formation was observed for high salt buffers (e.g. x0.1 TBE) with the addition of the double coat. The double coat consisted of PDMS (diluted 2:1 in toluene) and spin-coated twice. After the first round of spin coating the device was heated at 150 °C for five minutes. Following which it was cooled to room temperature and then spin-coated with PDMS for the second time. The coated device was then allowed to stand for 10 minutes following which it was baked overnight at 90 °C. The double coat gave a layer ~15 µm thick, reducing the rate of bubble formation and area of cross-section between parallel diodes.

With the addition of the PDMS layer the surface chemistry (leading to the EOF) has now been changed. Electroosmotic flows on PDMS are not well understood and are difficult to both maintain and control. However, it was observed that post treatment with an oxygen plasma or sodium hydroxide a strong EOF was generated. In addition, post coating the PCR mixture is exposed to a consistent surface chemistry.

3.4.7 Effect of AC Electric Fields on PCR Reaction

PCR mixtures were exposed to increasing A.C. field strengths prior to amplification on a conventional PCR thermal cycler. These samples were prepared under standard conditions with the exception of the PCR buffer, which was diluted to one half its original concentration. A 60 µL sample was loaded into a device without diodes and a 100 µL of mineral oil was applied to seal the liquid in the channel and prevent evaporation due to heating at high voltages. The A.C. voltage applied was a square waveform of 1 kHz frequency and amplitudes of 250 V, 350 V and 500 V peak-to-peak voltage was applied. Figure 3.14 illustrates an overlay of the GST processed electropherogram obtained at each amplitude compared to an untreated sample and a 50 bp dsDNA step ladder.

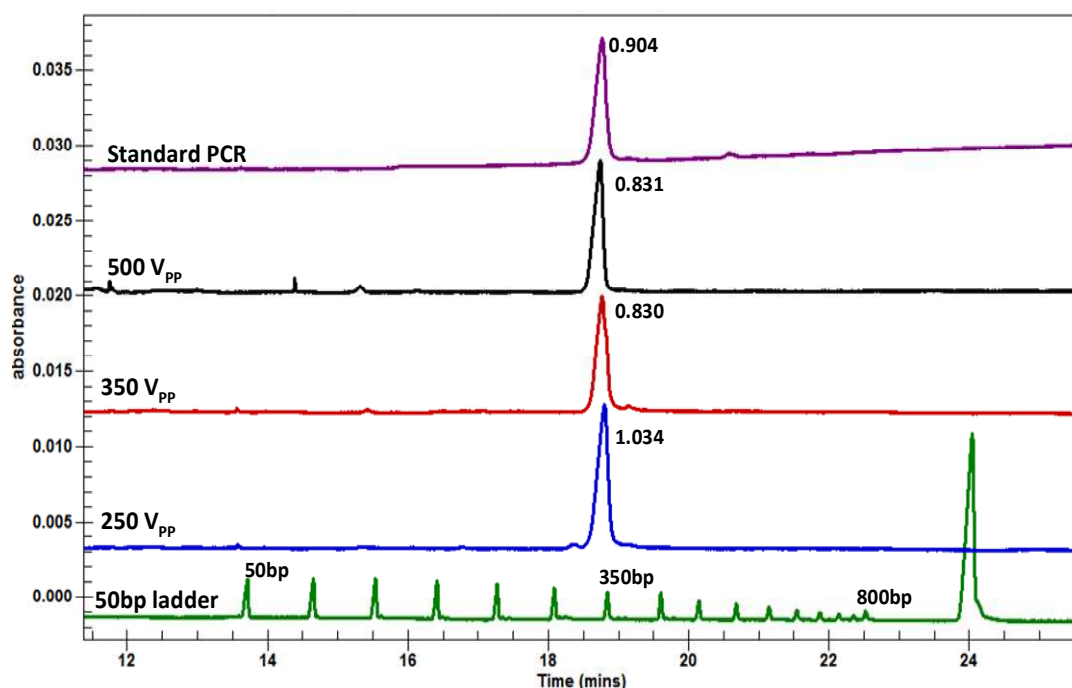


Figure 3.14: Analysis of the effect of A.C. field on the efficiency of PCR. The image is an overlay of GST processed electropherograms obtained from the analysis of PCR amplicons using capillary gel electrophoresis. All samples were prepared under the standard conditions as detailed in section 3.3.4, however the PCR buffer was diluted to one half its in-use concentration. The reaction produces an amplicon fragment 323bp in length. PCR mixture in 60 μ L batches was exposed to A.C. voltage prior to amplification. Samples were exposed to A.C. voltage of peak to peak (V_{pp}) value 250 V, 350 V and 500 V. All separations were performed using a 54 cm long capillary of effective length 40 cm, at 25°C and using a field strength of 203.7 V/cm. Separations were performed in the 1.75% PEO (5 MDa) sieving matrix.

In 2006, Hu *et al.* [49] described a microfluidic PCR device which employed Joule heating to achieve two-temperature PCR thermal cycling. Joule heating was induced by the flow of D.C. through the PCR mixture. Higher current heated the solution to 95 °C and low current along with rapid heat transfer helped realise the lower temperatures required for PCR. The effect of D.C. voltage on the components of PCR was not demonstrated. However, the authors proved that the amplification reaction proceeds using real time PCR with a fluorescent reporter molecule. Consequently, aside from heating the solution the applied current did not appear to have an adverse affect on the reaction. Results obtained in the current work corroborate this assertion and prove that an A.C. field (in the voltage range tested) does not have a detrimental effect on the constituents of a PCR mixture. The 323 bp amplicon fragment was observed in all three samples treated with an A.C. voltage. In addition, the peak area for all three samples was comparable to that obtained for the untreated sample.

3.5 PCR on Diode Device

The polymerase chain reaction was conducted using a diode device with a total of 16 diode pairs. The diodes and the channel were coated with a 15 μm thick layer of PDMS. Following which the device was sonicated in a 0.5 M solution of sodium chloride for fifteen minutes and the dried under nitrogen. The device was then rinsed twice with 100 μL of deionised water and dried under nitrogen. 60 μL of PCR mixture was loaded on to the device and degassed to ensure that no bubbles remain trapped between the diodes. Mineral oil was then applied above the PCR solution and diodes to minimise evaporation. The PCR mixture used was prepared as standard, however the PCR buffer was diluted to one half its original concentration prior to preparation and Tween 20 was added at a final concentration of 0.1% . A 200 μL sample was prepared from which 60 μL was used on the diode device and 60 μL was used to perform a control reaction using a commercial thermal cycler. The amplification program was set to 20 cycles to match the diode device protocol. Post PCR the control sample was processed as detailed earlier.

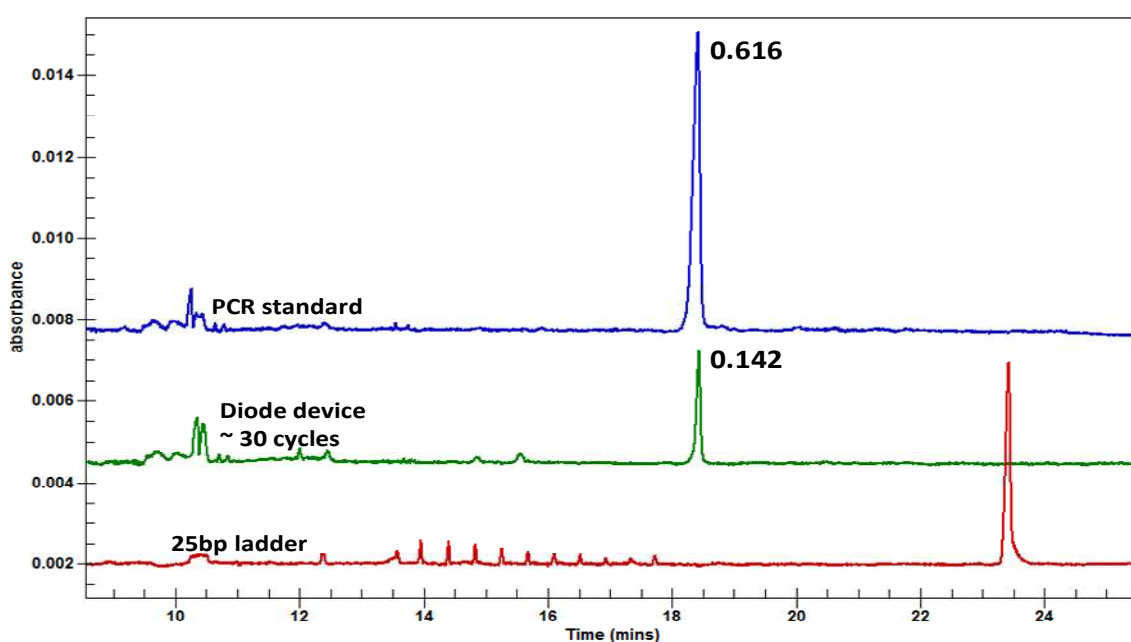


Figure 3.15: DNA amplification on PCR diode device. Overlay of GST processed electropherograms obtained from the analysis of PCR amplicons produced on the PCR diode device (green trace) and macroscale PCR thermo-cycler (blue trace) using capillary gel electrophoresis. Both samples were prepared under the standard conditions as detailed in section 3.3.4, however the PCR buffer was diluted to one half its in-use concentration. The reaction produces an amplicon fragment 323bp in length. The PCR results were compared to a 25bp dsDNA step ladder (red trace). All separations were performed using a 54 cm long capillary of effective length 40 cm, at 25°C and using a field strength of 203.7 V/cm. Separations were performed in the 1.75% PEO (5 MDa) sieving matrix.

The heating stage was turned on, allowed to get to temperature and stabilise before the diode device was placed on top. The PCR solution within the device was allowed to get to temperature before the A.C. field was applied. This took approximately 4 minutes and varied depending on the ambient temperature. The A.C. field was applied for 30 minutes or approximately 20 cycles, following which the diode device left on the heating stage for a further 3 minutes. The entire liquid volume was removed from the device using a pipette. The device was then rinsed with 50 μ L deionised water, this was also collected and added to PCR mixture. The oil was removed using a commercially available oleophilic and hydrophobic membrane (Chapter 5). The aqueous fraction was desalted using a desalting column (Pierce, UK) and then concentrated using a vacuum concentrator. The sample is then re-suspended in 50 μ L of deionised water and analysed using capillary gel electrophoresis on the PEREGRINE HPCE instrument. The results obtained are detailed in Figure 3.15, where the GST electropherograms for the control amplification and diode amplification are compared and plotted along with a 25 bp dsDNA step ladder. An amplicon fragment is observed for both control and diode amplification procedures. However, the peak area obtained for the diode PCR is 4.3 times lower than the control. Lower amplification efficiency could be due to contamination by metal inhibitors leaking through the PDMS coat, loss of reaction components to the channel surface and inefficient recovery of product from the device. Increasing the PDMS coating and Tween 20 concentration may help improve the amplification efficiency. In addition, the positioning of feedback thermocouples within the device should improve the heating of the PCR solution. Furthermore reduction of the channel circumference will decrease the cycle time permitting reactions with higher cycle number.

3.6 Conclusion

A single circular channel microfluidic device with embedded diodes was employed herein to move liquid round the channel. Fluid motion was induced by the electroosmotic flow generated across the face of each diode in the presence of a rectified D.C. field. Previously other authors have proposed a device using a single pair of diodes to pump a low salt solution through a microfluidic device. The strength of the flow generated on the multi-diode device demonstrated here was an order of magnitude higher than that cited in the literature. Additionally, the arrangement of the diodes in the circular channel established a peristaltic pumping action moving the fluid from adjacent arms sequentially. The multi-diode driven pump demonstrated here was used to perform PCR. CE analysis performed on the reaction mix after amplification on the multi-diode device

indicated that reaction had proceeded efficiently. Indeed the expected 323 bp amplicon fragment was observed. The yield obtained was only 4.3 times lower than that obtained on a conventional PCR instrument.

Biological reactions are extremely sensitive to the ambient environment and the polymerase chain reaction is a frequently used exemplar of this truism. On the diode device the high salt levels of traditional PCR mixtures lead to electrolysis. Bubble formation during electrolysis blocked the channels hindering fluid motion. Furthermore, the electrolysis also led to oxidising of the diode electrodes contaminating the PCR mixture with reaction-inhibiting metal ions. Additionally, temperature measurement within the device using thermocouples was hindered due to interference from the applied A.C. field. Most of these problems were addressed enabling DNA amplification to proceed on the multi-diode device. The multi-diode device was coated with PDMS reducing the effect of electrolysis and preventing most of the metal inhibitors from contaminating the PCR solution. Temperature measurements were achieved by shielding the thermocouples inserted in the device using PDMS. In addition, the PCR mixture was optimised for amplification performed on-chip by reducing the salt content and adding 0.1% Tween 20 to the reaction mixture. These changes reduced electrolysis and prevented reaction components from adhering to the microchannel.

3.7 References

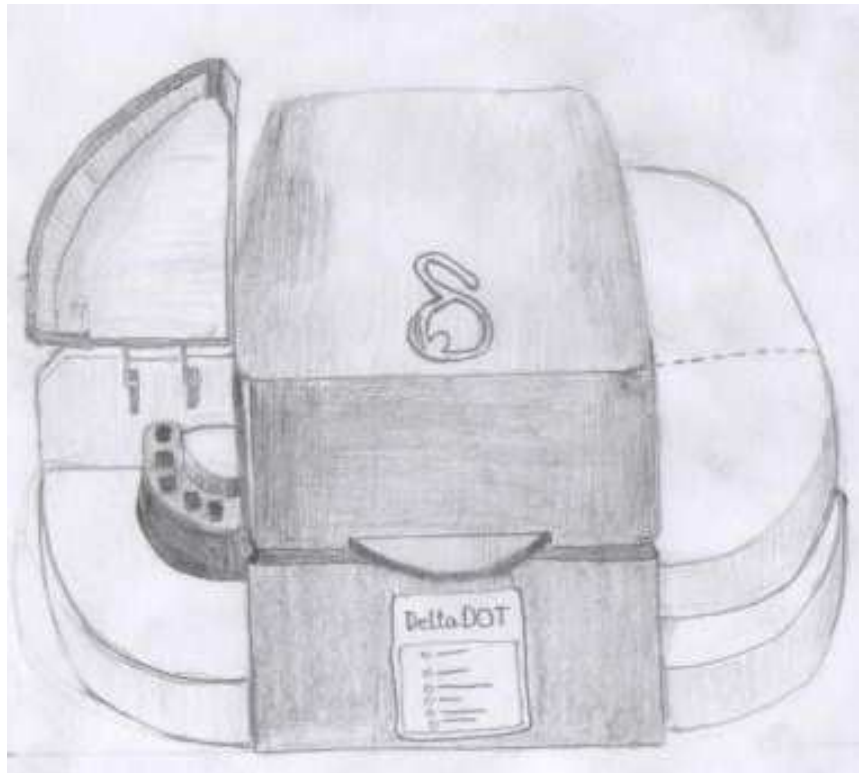
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Chapter IV: DNA Electrophoresis: On capillary and microchip



Synopsis

Successful transfer of electrophoretic separation protocols to microdevice requires optimisation of several separation parameters. This chapter investigates the parameters critical to component resolution during a separation, these include, initial injection plug size, separation length, sieving matrix and applied voltage. Separation lengths are typically shorter on micro-fabricated separation channels; therefore obtaining results comparable to commercial CE instruments is dependent on optimising the other controlling factors. Several PEO-based sieving matrices are investigated and discussed in this study. Through this process a novel, low viscosity, low polymer length matrix was developed enabling the separation of a wide variety of dsDNA fragment length ladders with baseline resolution. Complete resolution of all fragments of a 50bp ladder was also achieved in this buffer using a capillary of 10cm effective length. Aside from the low viscosity matrix a high molecular weight based PEO sieving matrix is also demonstrated. The separation protocols were successfully transferred on to chip-based devices employing sieving matrices with longer polymer lengths. Increasing polymer length and viscosity using glycerol improved the resolution between fragments and was especially effective across shorter separation lengths.

Electrophoretic separations performed on chip are theoretically more efficient than those performed on capillary. This is primarily attributed to the devices injection geometry which produces a small, reproducible sample plug. Smaller sample plugs reduce peak widths and increase both efficiency and resolution. However, this approach soon reaches its limitation with detector sensitivity; trace sample analysis and fabrication constraints. The 2.5% PEO (5 MDa) in x0.1 TBE sieving matrix, developed on a CE instrument was successfully used to separate 25bp and 50bp dsDNA size ladders on-chip. However, baseline resolution was not achieved and as demonstrated here once all the parameters controlling performance on a chip-based device have been optimised further improvement in resolution can only be achieved by increasing the channel length. A novel hybrid cross injector chip with capillary separation channel is also demonstrated, where sample plugs generated using a cross-piece assembly are then separated in the attached capillary. The separation length is defined by the length of capillary attached to the device and can be altered depending on the application. Furthermore, the surface chemistry of fused silica is better understood than PDMS and more suited to electrophoresis. This allows existing CE protocols to be transferred to the hybrid device with minimal optimisation. The attached capillary can be pre-loaded with buffer and then inserted permitting the use of high viscosity buffer typically used for

high resolution separations in CE. Moreover, all detection formats currently in use on CE instrumentation can be applied to this set up, including detection at wavelengths below 260 nm. The work described in this chapter is developed further in chapter 5 where the cross-piece injector is replaced with an interface allowing whole droplet injection into a capillary.

This chapter also includes a novel study on the effect of SYBR Green I on the migration dynamics of dsDNA fragments. A comparison between the separation of labelled and unlabelled fragments is achieved using UV detection. To pursue DNA separations in devices fabricated from PDMS it is necessary to fluorescently label samples. The effect of increasing concentration of intercalating dyes, principally SYBR Green I, on the electrophoretic mobility of dsDNA is investigated. The mobility value for each fragment was plotted against its dye-to-base pair ratio (dbpr), which is defined as the moles of dye per mole of DNA base pair. The presence of excess dye is shown to affect mobility, peak width, resolution and quantification of dsDNA fragments. Furthermore the effect of dye on fragment sizing was also determined using a 323bp PCR amplicon. At 100 $\mu\text{g/ml}$ ($\times 100$) SYBR Green I concentration a fragment size of 550 bp was obtained, indicating a polymer length over 200bp longer. Consequently, microchip and traditional electrophoretic separations employing labels can only generate accurate data when all the samples and controls are prepared at standard dye to base pair ratio and all sample concentrations are known.

4.1 Introduction

Electrophoresis has been used for many decades to separate and characterise biopolymers [1-4]. Its application to the analysis of double stranded DNA (dsDNA) has led to major developments in the fields of molecular biology, medicine, forensic science, agriculture etc [5-12]. The original slab gel format, although still in use, has been largely replaced over the years by capillary electrophoresis (CE). The use of capillaries to contain the separation matrix has led to shorter separation times, minimal sample and matrix usage and facile automation and integration of downstream analysis [13, 14]. With the advent of microfluidics, chip-based electrophoresis was an early application introduced by Harrison *et al.* in 1992 [15]. Since then the number of research laboratories working on chip-based CE has dramatically increased leading to a large variety of separation protocols that can be carried out on-chip [16,17]. Miniaturized CE systems are characterised by significantly lower sample and reagent consumption, shorter analysis times, higher efficiencies, decreased manual handling and improved portability than conventional CE systems.

The charge-to-mass ratio of DNA is essentially constant, owing to the structure of repeating nucleotide subunits [18]. Accordingly, when assayed using capillary zone electrophoresis (CZE), fragments of different size will have almost identical migration times and will not be resolvable. To accomplish size selection or sequencing of nucleic acid fragments using capillary electrophoresis, gel-based matrices are usually employed to provide for size selection. Initially, methods were directly adapted from traditional slab gel formats with cross-linked gels being prepared in a similar manner. Unfortunately, several disadvantages are associated with cross-linked gel filled capillaries. These include the time and complexity associated with preparation, the propensity for gel shrinkage during polymerisation, reduced operational lifetimes and the inability to replace the matrix in a facile manner [19, 20]. A solution to these issues involves the use of uncrosslinked polymer solutions [21, 22]. The properties of such polymer solutions, along with the associated migration dynamics of DNA have been previously reviewed in several papers [23-26]. There are a number of water soluble polymers which may be used in the preparation of entangled polymer solutions which allow the separation of DNA. Some of these form part of commercially available capillary separation buffers and can be used for sequencing, restriction fragment mapping and the separation of short tandem repeats (STRs).

In capillary electrophoresis, solutions of uncrosslinked polymers have also been found to have separation potential over a wide range of concentrations [25]. These may be grouped into three regimes: dilute, semi-dilute and concentrated [20, 23, 27]. In dilute polymer solutions, the polymer chains are hydrodynamically isolated from each other and instead of sieving they act as physical obstacles to biomolecule movement. Separation between larger fragments can occur, but the resolution between fragments of similar molecular weight is suboptimal. In a semi-dilute regime, increasing the amount of polymer in solution leads to a critical concentration at which the individual chains become entangled with one another and form a temporary network. In contrast to cross-linked gels, no irreversible chemical bonding occurs between the interacting chains. The polymer concentration at which this entanglement takes place is known as the entanglement threshold. The lifetime of this network is dictated by the mobility of the chains forming the mesh, the network integrity and the length and concentration of the polymer forming the network. The mobility of an analyte in such a polymer network is controlled by its size and electrophoretic mobility, the 'pore size' of the network and the applied electric field strength. While the entanglement threshold is dependent on the molecular mass of the polymer, the average pore size is solely dependent on concentration. Accordingly, two solutions of the same polymer at the same concentration, but of different molecular mass should yield the same average pore size as long as they are entangled. The entanglement thresholds for 300 kDa polyethylene oxide (PEO) and 8 MDa PEO are 0.25% and 0.07% respectively, based on the analysis provided by Heller [28], so solutions of PEO of molecular weight 300 kDa and 8 MDa above 1% concentration will be entangled and exhibit the same average pore size. High polymer concentrations show good resolution with long and short polymer chains, however wider size range separations are best performed using longer polymer chains.

The mechanism of DNA separation through a porous sieving matrix under an applied electric field has been studied intensively [20, 23, 26, 29-31]. Several theories have been proposed, the earliest of which is the Ogston model [32] which treats the eluting analyte as a non-deformable sphere. Here the sieving mechanism is likened to filtration driven by an applied field. This sieving model is relevant for DNA molecules whose radius of gyration is smaller than or equal to the pore size of the sieving media. Nevertheless, larger DNA molecules are separated and the reptation concept [33], where larger DNA molecules thread their way through 'tubes' formed by pores in the polymer network, was developed to explain this behaviour. Fragments conforming to this separation mechanism show mobility values that are inversely proportional to their molecular

weight. However, it has been observed that fragment resolution decreases with increasing fragment size. Alignment of larger fragments of DNA to the applied electric field in a size dependent manner reduces the dependence of mobility on fragment size and is known as biased reptation [34]. In a given entangled sieving matrix all three mechanisms of separation occur and fragments fall into one or other category depending on the pore size of the matrix, the radius of gyration of the DNA molecule and the applied field.

An ideal sieving matrix for capillary electrophoresis of dsDNA fragments would have to meet several requirements. It should have good separation properties over a wide range of fragment sizes, it should be easily loaded and removed from the capillary and should not require a pre-coated capillary or absorb at the detection wavelength. The matrix should also have a reasonable shelf-life, as degradation leads to irreproducible results. The translation of CE separation protocols to chip-based CE devices is not straightforward. To accomplish size selection, the sieving matrices applied should show similar resolution values as those obtained on a conventional CE apparatus. The shorter separation distances employed on-chip compared to those used in capillary systems represents a challenge when optimising electrophoretic separations. Another consideration is the viscosity of the sieving matrix which should be easily loaded and removed from the microchannel. Plastic and glass microchips can generally withstand pressures up to approximately 50 psi, and 200 psi respectively [35], compared to the 200 to 300 psi pressure regularly applied in CE systems used for DNA sequencing. The surface chemistry of most plastic microchannels differs considerably from the well characterised silica surface of capillaries used in CE. The sieving matrix should also provide adequate EOF suppression during a dsDNA separation. Numerous polymers have been trialled using both formats of electrophoresis by various groups. These include linear polyacrylamide [36, 37] and its derivatives, hydroxyethyl cellulose [38] and other cellulose derivatives and polyethylene oxide [39]. Recently, thermoresponsive copolymers like pluronics have shown promising results on-chip [40, 41]. However, replaceable low viscosity sieving matrices have been more successfully applied to both formats, than permanent acrylamide gels and other novel polymers.

Several PEO-based sieving matrices are investigated and discussed in this study. The properties of each matrix were assessed to determine if they met some or all of the requirements of an ideal matrix. Through this process a novel, low viscosity, low polymer length matrix was developed. Transfer of separation protocols from capillary to microchip necessitated the use of fluorescent

labels. A novel study on the effect of SYBR Green I on the migration dynamics of dsDNA fragments was carried out and a comparison between the separation of labelled and unlabelled fragments was achieved using UV detection. The presence of dye in excess quantities was shown to affect mobility, resolution and quantification of dsDNA fragments. Microchip separations employing labelled samples were prepared at specific dye to base pair ratios.

4.2 Experimental

4.2.1 Reagents and Separation Buffers

Rinse solution reagents (sodium hydroxide, hydrochloric acid and x10 solution of TBE) were purchased from Sigma-Aldrich (Poole, UK). Methyl cellulose (MC, viscosity 4,000 cP, 2 % in H₂O at 20 °C), hydroxy(propyl) methyl cellulose (HPMC, $M_n \sim 120,000$), 2-hydroxy-ethylcellulose (HEC, $M_w \sim 90,000$), and polyvinyl pyrrolidone (PVP, $M_n \sim 360,000$) were obtained from Sigma Aldrich (Poole, UK). Polyethylene oxide (PEO, $M_n \sim 100,000$, $M_n \sim 300,000$, $M_n \sim 600,000$ and $M_n \sim 1,000,000$) and polyethylene glycol (PEG, $M_n \sim 10,000$) were obtained from Alfa Aesar (Lancashire, UK). Longer polymer lengths of PEO ($M_n \sim 5,000,000$) were obtained from Avocado Research Chemicals (Heysham, UK). PEO, PEG and poly(oxyethylene) [POE] are chemically identical, with PEG typically defining polymers with a molecular mass below 20,000 g/mol, PEO defining polymers with a molecular mass above 20,000 g/mol, and POE defining a polymer of any molecular mass. Polymeric solutions tested using gel permeation chromatography confirm that the PEO polymers supplied are polydisperse. A 30% solution of acrylamide was prepared from the powder (Sigma Aldrich, UK). Linear polyacrylamide (LPA) solutions were prepared as detailed in [36], the 6% stock solution was diluted down to 3% before use. All polymer solutions employed in CE separations were prepared in 89 mM Tris, 89 mM Borate and 2 mM EDTA (x1TBE) except for PVP which was prepared at 2% and 10% in deionised water. Background electrolyte, x1 TBE, was diluted to one tenth of its stock concentration in deionised water prior to use in chip-based separations. Buffers were prepared by adding the appropriate amounts of polymer to the x1 TBE or x0.1 TBE and mixed for 24 hours at 25°C until the solution is homogeneous. As detailed subsequently some polymer solutions were filtered using a 5µm polyethylenesulfone (PES) syringe filter (VWR, UK). All polymer solutions were degassed prior to use.

4.2.2 Samples

A variety of double stranded DNA (dsDNA) ladders were employed to assess the sieving matrices developed herein. These included 25 bp, 50 bp and 100 bp dsDNA size ladders and Φ X174 RF DNA/*HaeIII* fragments which were obtained from Promega Corporation (Madison, USA). Another, wide range 25 bp size ladder was obtained from Invitrogen (Paisley, UK). The pBR322 *HaeIII* digest was obtained from Sigma (Poole, UK) while a 50 bp dsDNA size ladder and a DNA molecular weight marker, Roche Marker VIII (19-1114 bp) were obtained from Hoffman La Roche (Basel, Switzerland). Most samples were supplied in a 10mM Tris-HCL, pH 7.5, 1 mM EDTA solution unless specified. Table 4.1 lists the size in base pairs (bp) for fragments contained in each size ladder. Peak numbers were assigned to each fragment in a ladder. Separation profiles were labelled with the assigned number to aid peak identification. The molecular weight size markers were prepared for capillary electrophoresis by diluting to one tenth their stock concentration in deionised water. This procedure was followed for all dsDNA ladders (unless specified), with the exception of pBR322 *HaeIII* digest which was diluted to one fifth of its stock concentration. SYBR Green I was obtained from Invitrogen (Paisley, UK) at a x10,000 concentration. DNA samples were labelled with the dye by mixing at appropriate ratios in x1 TBE or x0.1 TBE. Samples for microchip separations were diluted to one fifth of their stock concentration in x0.1 TBE and labelled with SYBR Green I at a final concentration of 50 μ g/ml.

The Promega 25 bp DNA size Ladder consisted of 12 dsDNA fragments between 25 bp to 300 bp in 25 bp increments and was provided at a concentration of 0.36 μ g/ μ l. The 50 bp dsDNA size ladder (Promega, UK) had 16 dsDNA fragments between 50 bp and 800 bp and was provided at a concentration of 0.34 μ g/ μ l. Both size ladders had an additional 1,800 bp “backbone” fragment. The 100 bp dsDNA size ladder, also from Promega, contained 11 fragments, from 100 bp to 1000 bp in 100 bp increments, along with a 1500 bp fragment. The total DNA concentration in the sample provided was 0.13 μ g/ μ l. The Invitrogen 25bp DNA ladder consisted of 18 dsDNA fragments between 25 and 450bp in 25bp increments plus fragments at 500 bp and 2652 bp. This size ladder was supplied at a concentration of 1 μ g/ μ l. The Φ X174 RF DNA/*HaeIII* digest consists of eleven linear dsDNA fragments from 72 to 1,353 bp. Φ X174 RF DNA/*HaeIII* fragments are supplied at 0.5 μ g/ μ l concentration in 10 mM Tris-HCl (pH7.4), 5 mM NaCl, 0.1 mM EDTA.

Chapter IV

DNA Ladder	Peaks numbers and their associated fragment sizes (in bp) for each Ladder																	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
25 bp size ladder (Invitrogen)	25	50	75	100	125	150	175	200	225	250	275	300	325	350	375	400	425	450
25 bp size ladder (Promega)	25	50	75	100	125	150	175	200	225	250	275	300						
50 bp size ladder (Roche)	50	100	150	200	250	300	350	400	450	500	550	600	650	700	750	2642		
50 bp size ladder (Promega)	50	100	150	200	250	300	350	400	450	500	550	600	650	700	750	800		
100 bp size ladder	100	200	300	400	500	600	700	800	900	1000	1500							
pBR322 <i>HaeIII</i>	80	89	104	123	124	184	192	213	234	267	434	458	504	540	587			
Φ X174 RF DNA/ <i>HaeIII</i>	72	118	194	234	271	281	310	603	872	1078	1353							
Roche Marker VIII	67	110	124	147	190	242	320	404	489	501	692	900	1114					

Table 4.1: dsDNA molecular size markers used in this report. Each fragment is assigned a peak number which identifies the fragment on a separation profile.

The 50 bp ladder from Roche contained 15 double stranded DNA fragments between 50 and 750 bp and an additional 2642 bp fragment. Marker VIII contained 18 dsDNA fragments; fragment lengths not listed in Table 4.1 include 37, 34, 34, 26 and 19. The 50bp ladder and Marker VIII were provided at a concentration of 250 µg/ml in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0. pBR322 HaeIII digest contains 22 fragments between 8-587bp provided in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA. Fragment lengths not listed in Table 4.1 include 64 bp, 57 bp, 51 bp, 21 bp, 18 bp, 11 bp and 8 bp. This sample was supplied at a concentration of 50 µg/ml.

4.2.3 Capillary Electrophoresis: Procedures and Conditions

The PEREGRINE CE instrument described in Chapter 2 was run in reverse polarity mode. The capillary cartridge temperature was set to 25°C and absorbance was monitored at 254 nm. Bare fused silica capillaries (Composite Metal Services, UK) of 75 µm internal diameter and 375 µm outer diameter were used for all experiments. A variety of effective lengths were used and are detailed in each experiment. The PVA coated silica capillaries were obtained from Beckman Coulter (Buckinghamshire, UK) and between separations buffer was rinsed out using deionised water. The bare fused silica capillaries were initially cleaned by washing with methanol, 0.1 M NaOH and 0.1 M HCl for 3 minutes each. These flushes were interspersed by rinses with deionised water. Between separations the capillary was rinsed with 0.1M HCL for 5 minutes. When a pre-coat was employed the capillary was flushed with 10% PVP for 5 minutes prior to filling with a separation matrix. The samples were introduced into the capillary via electrokinetic injection and the separation voltages varied between experiments.

4.2.4 Miniaturised Capillary Electrophoresis: Procedures and Conditions

The microdevice was mounted on a platform specifically designed to allow application of high voltage. The platform has 12 electrode ports placed around the device (Figure 4.1). Electrodes could be placed at any port permitting flexibility in injector design, for the present work only four electrode ports were in use. Each electrode port holds a single platinum electrode, which was connected to a high voltage power supply (HVS448 3000V, Labsmith). The chip is held in position in a sunken section of the stage preventing any movement during optical measurements

and the MCE stage is mounted on to a microscope stage to allow optical detection. The aluminium base could also be used for temperature control during the separation.

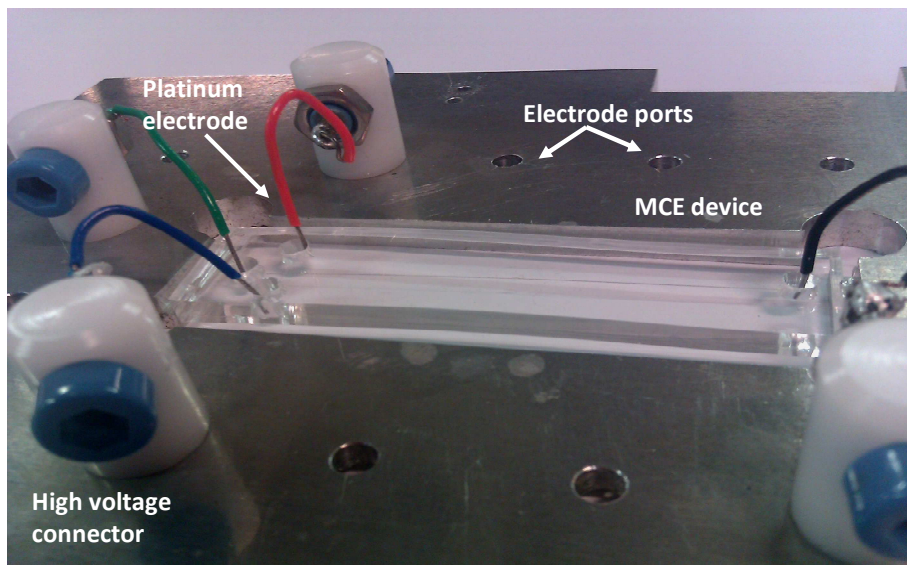


Figure 4.1: Microchip capillary electrophoresis platform. The aluminium MCE device holder was designed to be mounted on a microscope stage. There are 12 electrode ports surrounding the MCE device. High voltage connectors were screwed into appropriate ports; depending on the MCE device layout. Platinum electrodes were soldered to the high voltage connectors. A ledge was constructed (3mm wide and 3 mm down) from the top surface on the holder. In operation the MCE device is mounted on the ledge to off-set the change in focal length due to the thickness of the aluminium platform.

4.3 Results and Discussion

4.3.1 Sieving Matrix Development and Characterisation

4.3.1.1 Assessment of UV Absorption Characteristics of Separation Matrices

The UV absorption characteristics of potential polymers were initially established, since significant absorption in the 240-260 nm region would preclude their ability to act as a sieving matrix when combined with label-free detection at 256 nm. The absorbance values obtained for 0.5% solutions of PEO (300kDa), LPA, MC, HPMC, HEC and PVP in x1 TBE at 254 nm were 0.0061, 0.1138, 0.0115, 0.0114, 0.0127 and 0.0123 respectively. UV spectra were measured using a 1 mm path length quartz cuvette. A reference sample of x1 TBE was used in all measurements. LPA was seen to absorb strongly between 240 nm and 260 nm, leaving the cellulose derivatives and PEO as

possible candidates for a UV transparent sieving matrix. Minimum absorption was observed for the 0.5% solution of PEO (300 kDa) which exhibited an absorbance value two orders of magnitude lower than that observed for a 0.5% solution of LPA. The absorption values for 3% LPA and 4% PEO (300kDa) were 0.6977 and 0.0779 respectively. Additionally, the absorbance of PVP was observed to be significant at 254 nm. Nevertheless, since PVP is used as a pre-coating only a very thin layer is incorporated, which will have a negligible effect on light transmission.

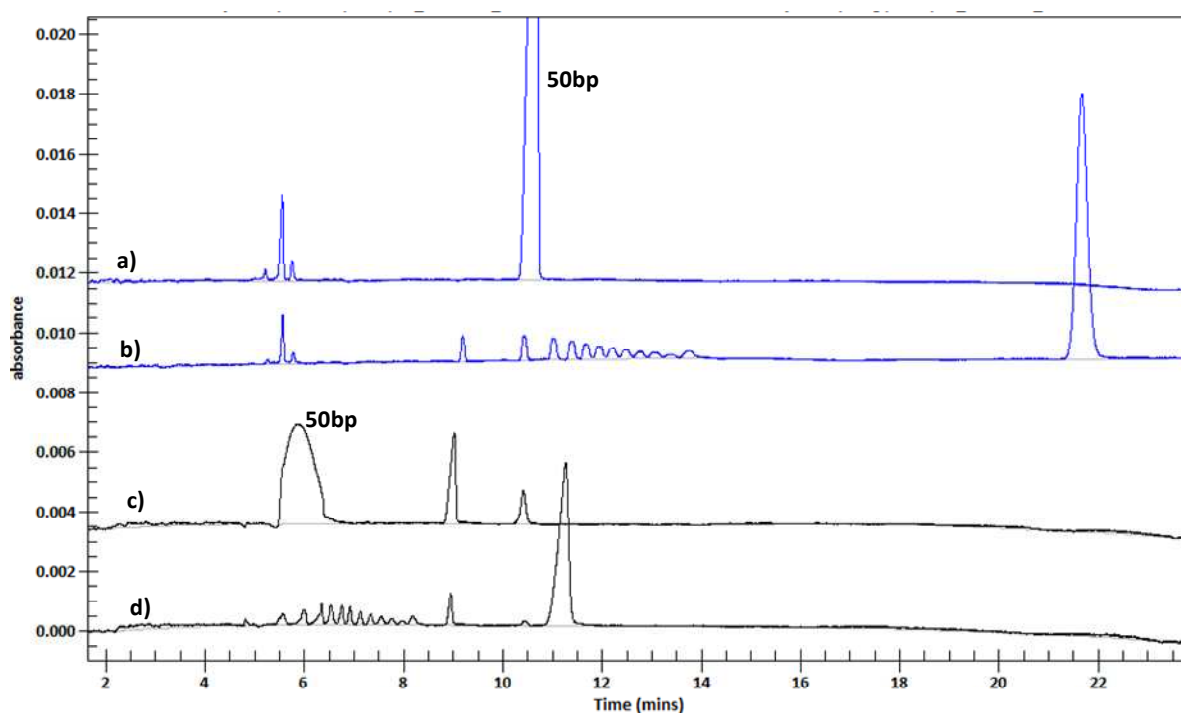


Figure 4.2: GST processed electropherograms of the 25 bp ladder (3.6 μ g/ml in deionised water, Promega) and a 50 bp fragment (10 μ g/ml in deionised water, Fermentas). (a) GST processed electropherogram of the 50 bp fragment performed in 4% PEO (300kDa) in x1TBE. (b) GST processed electropherogram of the 25 bp ladder performed in 4% PEO (300kDa) in x1TBE. (c) GST processed electropherogram of the 50 bp fragment performed in 3% T LPA in x1 TBE. (d) GST processed electropherogram of the 25 bp ladder performed in 3% T LPA in x1TBE. All four separations were performed using a PVA coated 34 cm long capillary of effective length 20 cm, at 25°C and using an electric field strength of 235 V/cm.

A comparison between 3% LPA and 4% PEO (300kDa) (Figure 4.2) matrices employed under identical separation conditions was performed using a PVA coated capillary (Agilent, Cheshire, UK). The results show that both matrices enable complete resolution of the fragments of a 25 bp dsDNA ladder. Additionally, the 50 bp fragment could be satisfactorily sized in each buffer. However, peak width in the PEO matrix was smaller indicating that greater resolution and sizing

accuracy could be achieved using this matrix. The resolution between the 125 and 150 bp fragments was 0.6 and 0.615 for LPA and PEO respectively. Under optimal separation conditions, using a 42 cm long bare fused silica capillary coated with 10 % PVP, a resolution value of 1.3 was obtained between the same fragments in the low viscosity PEO sieving matrix (Figure 4.2). Employing higher percentages of LPA will improve resolution, however, this increase will be accompanied by an increase in viscosity and UV absorbance, thus requiring higher loading pressures and fluorescent labels to aid detection. Significant differences in peak shape, migration time and signal intensity were also observed between the separations (results not shown) which may be attributed to the differences in pore size and UV transmission of both polymers.

4.3.1.2 Self Coating

Traditional DNA separation buffers exhibit a pH of 8–9. Under these conditions the negatively charged capillary wall develops an electrical double layer which leads to the generation of electro-osmotic flow (EOF). Standard DNA separations are performed under reversed polarity, i.e. against the EOF. A variation in the strength of this EOF will lead to a variation in the mobility of fragments migrating in the opposite direction. To prevent this, the capillary may be covalently or dynamically coated. Certain polymers including PVP and poly(dimethylacrylamide) can provide the dual functionality of being both a dynamic capillary coating as well as a sieving matrix [42]. The polymers adhere to the internal surface of the capillary providing a dynamic coating that can be removed and refreshed after each separation. Capillaries covalently coated with polyacrylamide are efficient at arresting EOF [21, 37], however these are expensive to manufacture, complicated to make and absorb in the UV. The self-coating ability of methyl cellulose, hydroxy(propyl) methyl cellulose (HPMC) and polyethylene oxide were tested, to determine if they could be used as a sieving matrix in the absence of additional surface conditioners. A 10% PVP solution was used as a positive control in these experiments and the capillary was activated using 1M NaOH to generate a strong EOF.

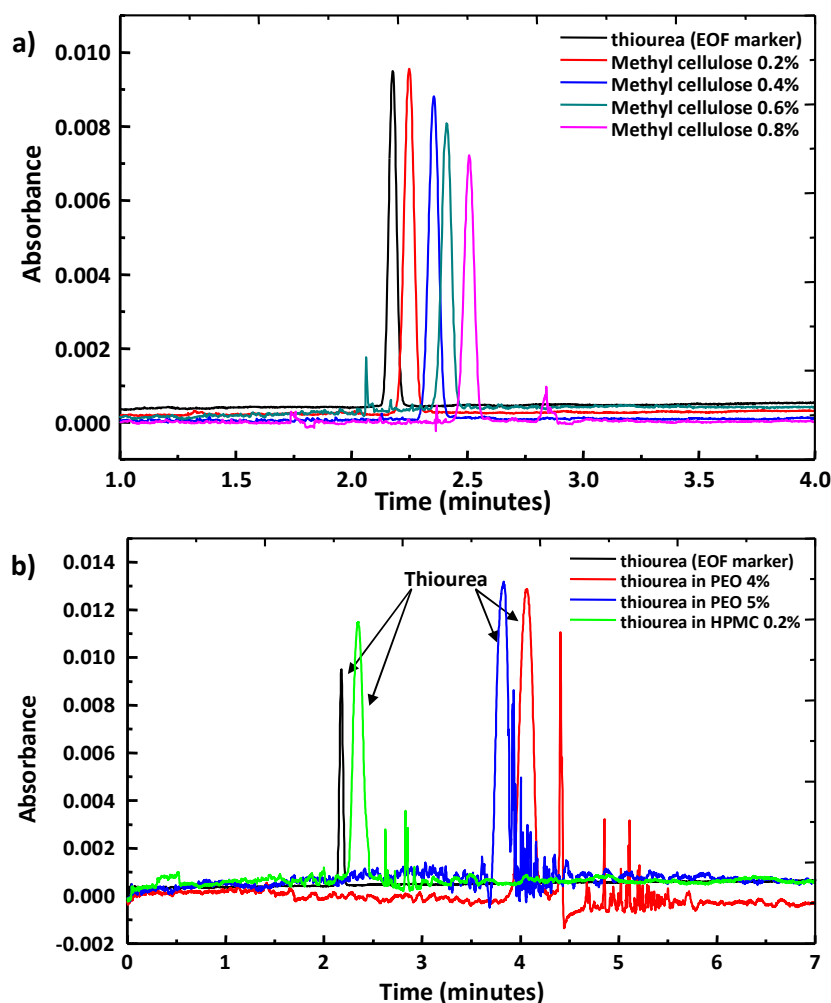


Figure 4.3: GST processed electropherograms of the EOF marker thiourea (100 $\mu\text{g}/\text{ml}$ in deionised water). (a) Separations performed after the addition of 0.2, 0.4, 0.6 and 0.8% methyl cellulose, (b) Separations performed after the addition of 4% PEO (300 kDa), 5% PEO (300kDa) and 0.2% HPMC. All separations were performed using a 24 cm long capillary of effective length 10 cm, at 25°C and using an electric field strength of 333.3 V/cm. The low intensity, short duration peaks observed in all electropherograms are a result of bubbles and BHT particulates.

Various concentrations of each polymer were made up in x1 TBE (pH 8.3), loaded into the capillary and an EOF check performed. Between separations the capillary was rinsed with 0.1M NaOH to remove the previous polymer solution and recondition the capillary. The results obtained for methyl cellulose, hydroxy(propyl) methyl cellulose and PEO are detailed in Figure 4.3. The results were compared to the strength of the EOF in the absence of polymer. For methyl cellulose a decrease in the strength of the EOF as a function of polymer concentration was observed (Figure 4.3a). This variation was reproducible over multiple sets of data. Higher concentrations ($> 0.8\%$) of methyl cellulose were not investigated due to excessive viscosities

making introduction into the capillary ill-defined. Similar experiments for HPMC and PEO (300 kDa) demonstrate that PEO is most effective at reducing EOF. Specifically, an initial EOF of 2.75×10^{-4} (cm^2/Vs) in the absence of polymer was reduced to 2.39×10^{-4} (cm^2/Vs), 1.7×10^{-4} (cm^2/Vs) and 1.046×10^{-4} (cm^2/Vs) in the presence of 0.8% MC, 0.2% HPMC (120 kDa) and 5% PEO (300 kDa) respectively. It should be noted that the small, sharp peaks observed in all electropherograms are a result of bubbles and butylated hydroxytoluene (BHT) particulates. Polymers at different concentrations were appraised to assess the effect of increasing concentration on the EOF. Results indicate that the strength of the EOF was inversely proportional to polymer concentration.

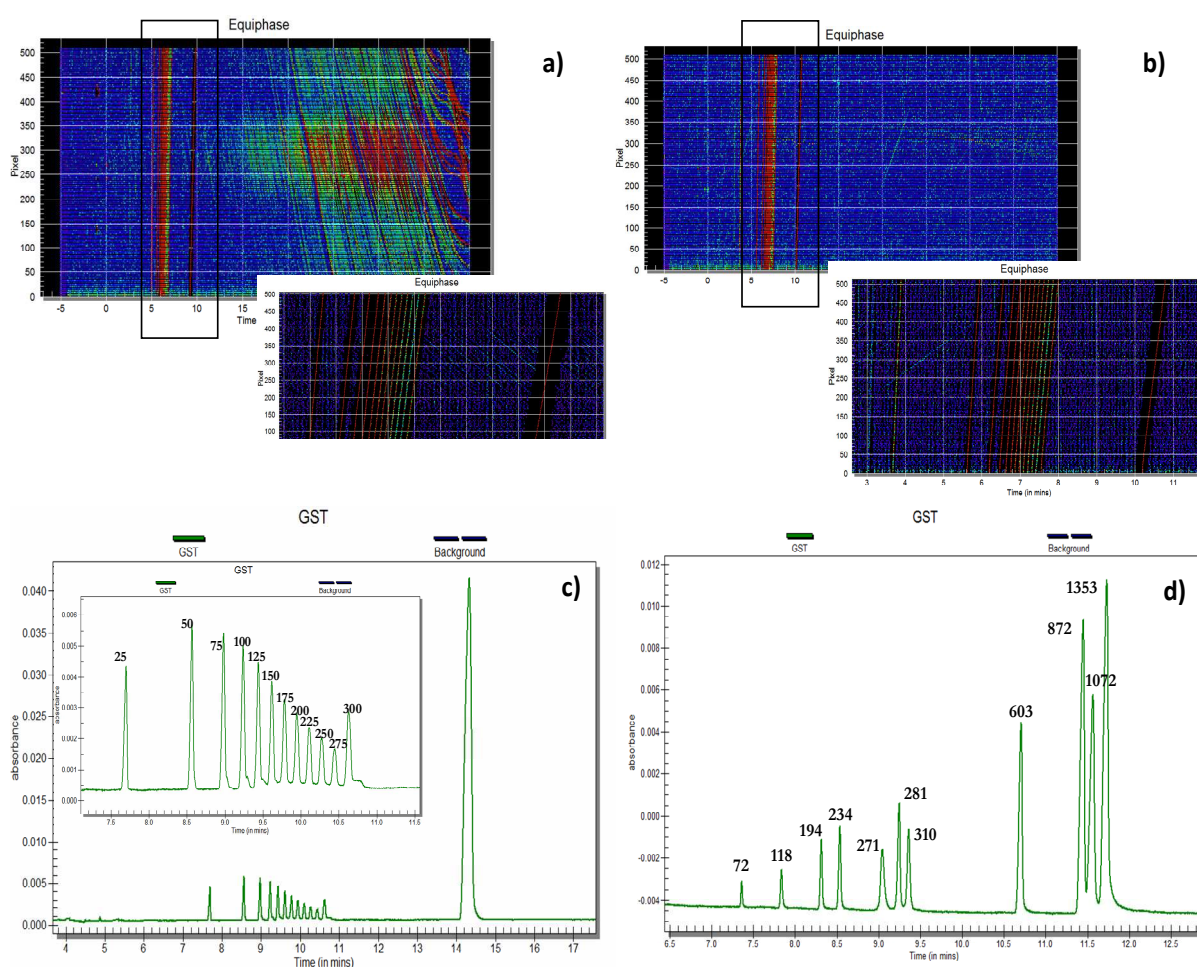


Figure 4.4: GST processed electropherograms and equiphase maps of the 25 bp ladder (36 $\mu\text{g}/\text{ml}$ in deionised water, Promega). (a) Equiphase map of a separation performed without a 10% PVP pre-coat, (b) Equiphase map of a separation performed after the use of a 10% PVP pre-coat. The inset images in both (a) and (b) are expanded views of the equiphase map of the 25 bp dsDNA ladder. The component bands of the ladder can be clearly seen and identified based on their characteristic slope. (c) GST processed electropherogram of a separation in (b). (d) GST processed electropherogram of the separation of ΦX174 RF DNA/HaeIII digest fragments under similar conditions to (b). All separations were performed using a 34 cm long capillary of effective length 20 cm, at 25°C and using an electric field strength of 353 V/cm.

Separations were performed with and without a dynamic coating of 10% PVP to assess the self-coating ability of PEO and separation stability. Figure 4.4a and b illustrate equiphase maps generated from the separation of a 25bp ladder (Promega, UK) in 4% PEO (M_n 300,000) in the absence and presence of 10% PVP (M_n 360,000) respectively. Separation between the 25bp fragments occurs in both cases, however, in the absence of PVP an EOF is clearly observed (bands migrating in the opposite direction to the dsDNA ladder). The particulates migrating in the direction of the EOF are insoluble contaminants and stabilizers present in PEO (most likely butylated hydroxytoluene). Filtration of these buffers with 5 μ m PES syringe filters reduces turbidity and removes the majority of particulates. However, viscosity and refractive index differences exist between the filtered and unfiltered buffer solutions and are due to the loss of polymer during filtration. Figure 4.4c and d illustrate the GST processed electropherograms obtained from the separation of the 25 bp dsDNA step ladder (Promega, USA) and the of Φ X174 RF DNA/HaeIII digest fragments respectively in the filtered 4% PEO sieving matrix using a 10% PVP pre-coat.

Separations incorporating a PVP pre-coat run under otherwise identical conditions showed a higher mobility for the dsDNA fragments. The log-log plot of electrophoretic mobility versus DNA fragment size in Figure 4.5 indicates that the resolving power of the matrix is superior in the absence of the PVP. However, this is due to the fact that the presence of an EOF in the absence of PVP retards migration, and thus improves resolution. To exploit this advantage requires a stable, consistent EOF on a run-to-run basis, which is almost impossible to achieve. A pragmatic option is to use a PVP pre-coat to remove EOF as a variable, and thus allow enhanced control over the separation process. Resolution may be improved by increasing the polymer length and concentration, the separation length and the use of additives. Changes in these parameters were investigated and are described in the following sections. Kang *et al.* [38] have compared the effectiveness of PEO (8 MDa) and HPMC (Hydroxy(propyl)methylcellulose) as sieving matrices and dynamic coatings. Results indicated that PEO is a better dynamic coating and sieving matrix than HPMC. This was attributed to the fact that PEO adsorbs more strongly to the capillary wall. However, the longer separation times observed when using PEO instead of polyacrylamide as the wall coating indicate the presence of an EOF. Polyethylene oxide functions well as a dynamic coating as long as it is continuously refreshed from the sieving matrix reservoir. Furthermore, it does not completely arrest the EOF, but rather diminishes its strength. Conditioning capillaries with PVP prior to separation enables UV detection and impedes EOF. In the absence of a

capillary wall coating, buffer additives may also be used to reduce the EOF. Shihabi [43] and Lin *et al.* [44] have used N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) and hexadecyltrimethylammonium bromide (CTAB) respectively as buffer additives to reduce EOF during separation. HEPES is a common additive which prevents sample adsorption to the capillary wall, however it absorbs strongly at 254 nm and sample stacking must be employed to improve sensitivity. Lin *et al.* demonstrate the use of CTAB as a buffer additive and capillary coating allows complete resolution of all Φ X174 RF DNA/*Hae*III fragments. In addition, their studies indicate that the use of CTAB improves both reproducibility and resolution. However, as noted by the authors CTAB interacts with both PEO and DNA molecules and affects the binding of ethidium bromide to DNA, necessitating a set of optimum conditions to be established and maintained before signals can be analysed.

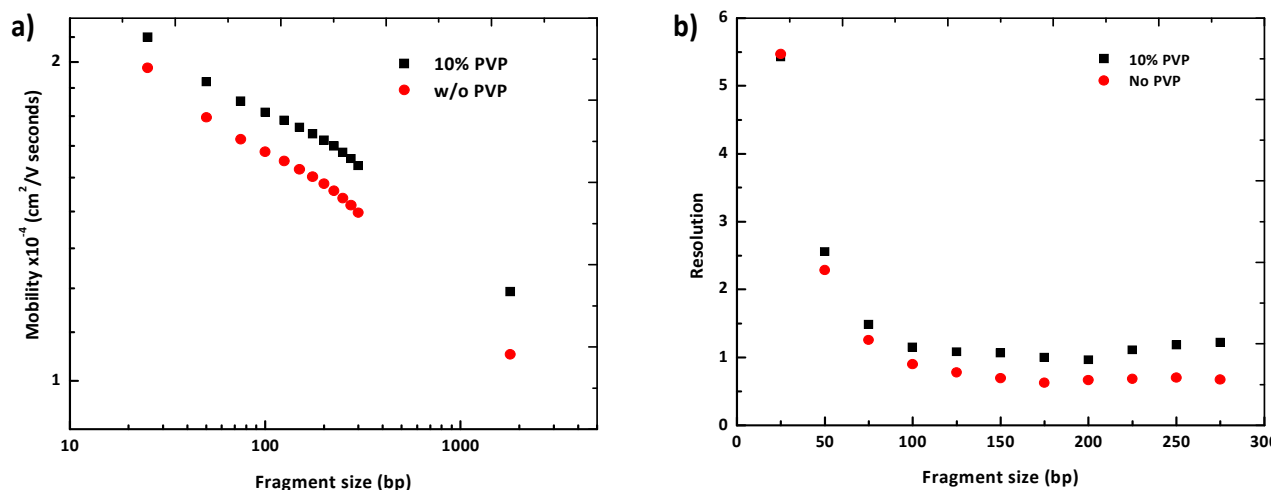


Figure 4.5: Monitoring the effect of PVP on the resolution and mobility of the fragments of a 25 bp dsDNA step ladder (Promega). (a) A log-log plot of mobility versus dsDNA fragment size. (b) A resolution versus fragment size plot.

4.3.1.3 DNA Separations in Low Viscosity Polymer Solutions

The separation of a 25 bp ladder was performed in semi-diluted solutions of PEO, to determine the concentration at which baseline resolution could be achieved. Similar separations performed by Chang *et al.* [39] using 3% PEO (300kDa) buffer and a DB-1 coated capillary, showed complete resolution of all fragments. Separations were performed in a 4% and 5% solution of PEO (300kDa) employing a PVP pre-coat. A comparison of the results obtained is shown in Figure 4.6a, employing a resolution versus fragment size plot. Higher resolution values were obtained

between all fragments separated in the 5% PEO (300 kDa) matrix. Notably, the most significant increases in resolution were observed for the longer fragment lengths.

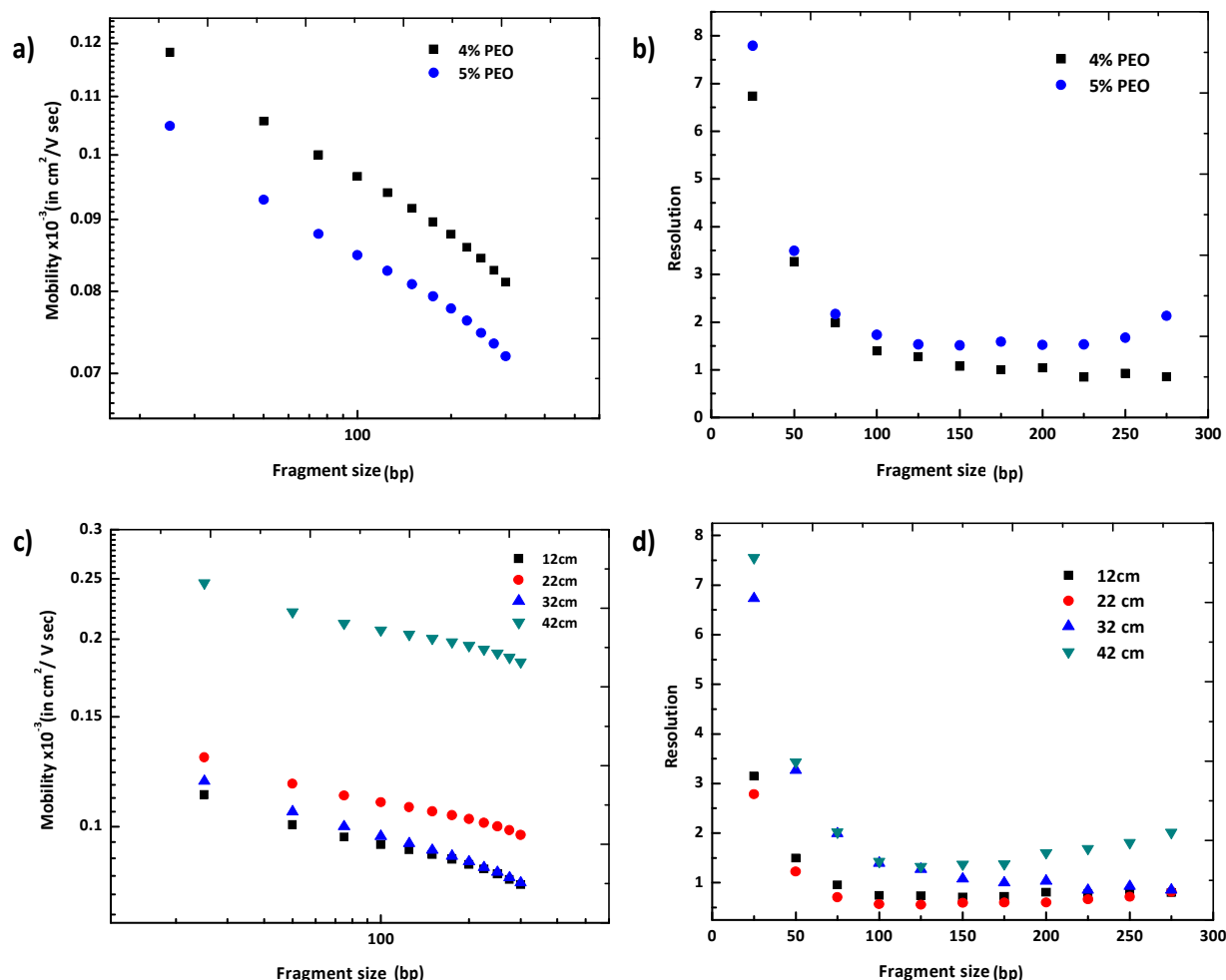


Figure 4.6: Effect of polymer concentration and effective separation length on the mobility and resolution of a 25 bp ladder (36 $\mu\text{g}/\text{ml}$ in deionised water, Promega). (a) and (c) are log-log plots of mobility as a function of fragment size while (b) and (d) are plots of resolution versus fragment size. In (a) and (b) separations were performed in 4% PEO (300kDa) and in 5% PEO (300kDa). Both separations were performed using a 44 cm long capillary of effective length 30 cm, at 25°C and using an electric field strength of 363.6 V/cm. In (c) and (d) separations were performed in 12 cm, 22 cm, 32 cm and 42 cm effective length capillaries. All four separations were performed using the 4% PEO (300kDa) sieving buffer. Additionally, both sets of analysis were performed at 25°C and using an electric field strength of 330 V/cm. For both experiments the capillaries were pre-coated with 10% PVP.

The effect of capillary length on separation efficiency was also studied using the 4% PEO sieving matrix. The 25 bp ladder (Promega, UK) was separated on a 12 cm, 22 cm, 32 cm and 42 cm effective length capillary with the field strength maintained at $\sim 330\text{V}/\text{cm}$ in all cases. As expected, resolution improves with an increase in capillary length under a constant electric field (Figure

4.6b). Resolution is proportional to peak spacing and inversely proportional to peak width. Consequently, an increase in peak width in tandem with an increase in peak spacing would result in an insignificant gain in resolution. Increasing capillary length increases the distance between peaks however peak width also increases due to diffusion occurring over a longer period of time. Therefore from Figure 4.6b the largest percentage gain in resolution was observed for the smaller fragments. These fragments move through the capillary more rapidly reducing the effect of diffusion on their peak width. Applying higher field strengths when increasing capillary lengths will counteract the effect of peak dispersion, improving resolution over a wider fragment size range. However, it must be noted that it is impossible to optimally separate all sizes of DNA under the same electric field by simply increasing capillary length or indeed by then further increasing the field strength. A compromise must be met between the separation length, field strength, capillary internal diameter and polymer concentration that minimises peak diffusion and peak dispersion due to Joule heating, allowing an optimum set of parameters to be designed for a given size range.

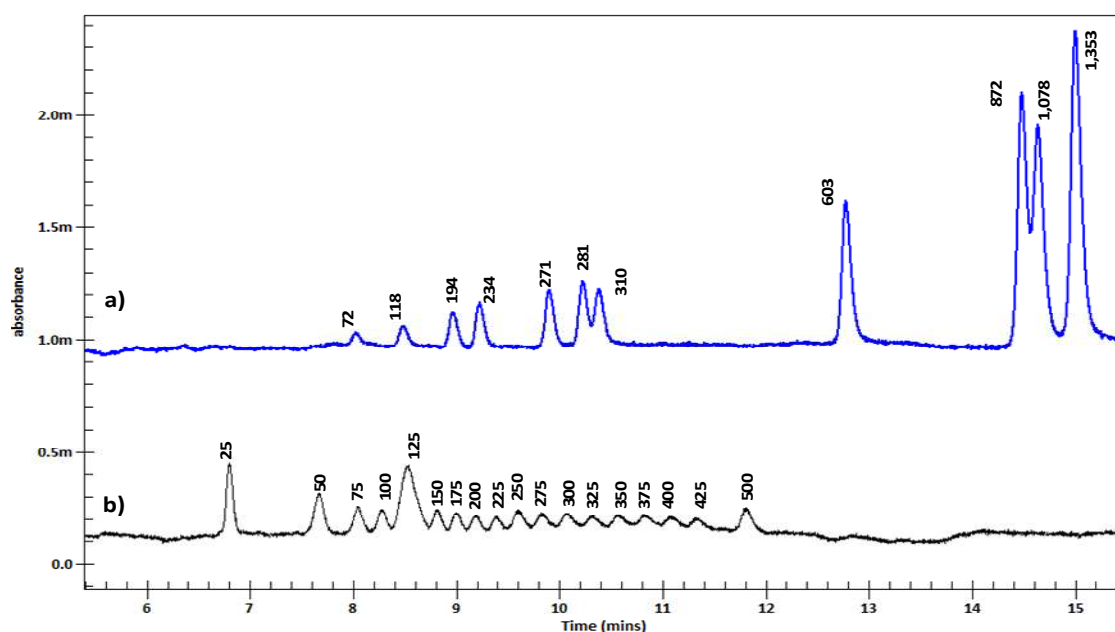


Figure 4.7: (a) Separation of a 25 bp ladder from Promega (36 $\mu\text{g}/\text{ml}$ in deionised water) (b) Separations of ΦX 174 RF DNA/*Hae*III digest fragments (500 $\text{ng}/\mu\text{l}$ in deionised water, Invitrogen). (c) Separation of a 25 bp ladder from Invitrogen. All separations were performed using a 24 cm long capillary of effective length 10 cm, at 25°C and using an electric field strength of 230 V/cm. In both cases the capillary was pre-coated with 10% PVP and the separation was performed in 4% PEO (300kDa).

Separation of a 25 bp ladder (Invitrogen, Paisley, UK) and the ΦX 174 RF DNA/*Hae*III digest was achieved using a bare fused silica capillary of effective length 12 cm. The separation was

performed with a 10% PVP pre-coat and a 5% PEO (300kDa) buffer under an electric field strength of 220V/cm. The GST processed electropherograms for the *Hae*III digest and the 25 bp ladder are detailed in Figure 4.7a and b respectively, and indicate successful separation of all fragments. The baseline resolution obtained for all 18 fragments of the 25 bp ladder within such a short separation length indicates the potential of the low viscosity sieving matrix.

4.3.1.4 Effect of Filtration and Re-crystallisation on the PEO Sieving Matrix

Separations carried out using PEO of various molecular weights (100kDa, 300kDa, 1MDa, 5MDa and 8MDa) over a period of months showed that the sieving matrix degraded. A loss in solution viscosity and resolution between DNA fragments was recorded (results not shown). In the absence of PVP a large number of bands moving in the direction of the EOF were also observed. These bands originated in the buffer at all points in the capillary. PEO undergoes auto-oxidation due to free-radical generation and this process is accelerated on exposure to ultra-violet light, strong acids and some oxidising agents [45, 46].

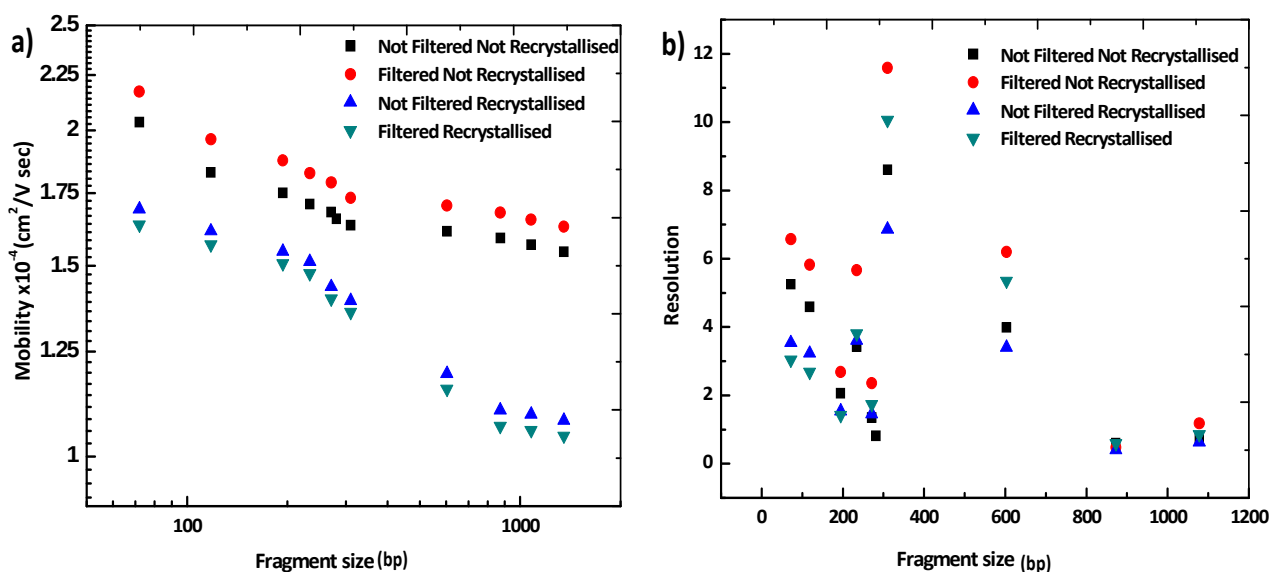


Figure 4.8: Separation of a Φ X174 HAEIII digest fragments (50 μ g/ml in deionised water, Invitrogen) in filtered or re-crystallised PEO sieving matrices. (a) A log-log plot of mobility versus fragment size for the digest in each sieving matrix. (b) A resolution versus fragment size plot of the digest in each sieving matrix. The four sieving matrices tested included a 4% PEO (300 kDa) buffer which was not filtered or re-crystallised (■), 4% PEO (300 kDa) buffer which was filtered using a 5 μ m PES syringe filter but not re-crystallised (●), 4% PEO (300 kDa) buffer which was not filtered but re-crystallised (▲) and 4% PEO (300 kDa) buffer which was filtered using a 5 μ m PES syringe filter and re-crystallised (▼) prior to use. All four separations were performed using a 54 cm long capillary of effective length 40 cm, at 25°C and using an electric field strength of 296.3 V/cm.

Typically PEO is mixed with a stabilizer, butylated hydroxytoluene (BHT) at concentrations between 200-500 ppm to retard degradation. BHT is a free-radical scavenger and it slows the degradation process down, but does not arrest it. However, BHT is insoluble in water and absorbs UV radiation at 254 nm. This contributed to the bands observed moving in the direction of the EOF (Figure 4.4a). To remove BHT the PEO was re-crystallised using acetone. This was carried out by loading a burette with PEO and passing acetone through it. As the acetone moves down the burette it solubilises BHT and washes it out of the burette. The re-crystallised PEO is air dried before use in the matrix preparation. Due to the high turbidity of the buffer solutions the sieving matrices were also filtered with a 5µm PES syringe filter (VWR, UK). Lower filter pore sizes were not used as viscosity measurements indicated a decrease in viscosity post filtration. This is due to the removal of some of the polymer during the filtration process. To ensure that these preventative measures did not effect the migration and resolution of DNA fragments, separations were carried out at every stage (Figure 4.8a and b). A 25bp ladder and Φ x174 HaeIII digest fragments were used to monitor the change in migration behaviour. Four sieving matrices were prepared using the standard recipe of 4% PEO (300 kDa) in x1TBE. The effects of re-crystallisation and filtration were tested both independently of each other and together. Figure 4.8a and b illustrate log-log plots of mobility versus fragment size and a resolution versus fragment size respectively for all four conditions. The mobility plots show that the highest mobility values were obtained for the sieving matrix that was filtered but not re-crystallised. Conversely, the lowest mobility values were observed for the filtered and re-crystallised matrix. Filtration lowers the polymer content of the buffer increasing the migration of fragments through it. Interestingly, the re-crystallisation process reduces the mobility of the fragments generating a steeper slope in both the filtered and unfiltered sieving matrices. The reasons behind this behaviour remain unclear. One possible explanation is that in the absence of BHT the polymer degrades faster, changing the length of the polymer and increasing its concentration in solution. These changes will alter the matrix pore size and increase its viscosity, reducing the mobility of the dsDNA fragments. Resolution values indicate that the highest values for all were obtained for the filtered sieving matrix that was not re-crystallised. However, the 271 bp and 281 bp fragments were only resolved by the matrix which was not filtered or re-crystallised. The loss of resolution observed with the re-crystallised matrices can be attributed to increases in peak width due to increased diffusion caused by the increase in viscosity of the solution.

The removal of BHT from PEO by re-crystallisation will lead to faster polymer degradation. UV imaging across the 1.2 cm photo diode array detection window may also accelerate this process. Plots (Figure 4.8a and b) show a decrease in mobility values and resolution between fragments in re-crystallised matrices. Addition of primary and secondary alcohols to PEO solutions has proven to be very effective in preventing the loss in viscosity [45]. The alcohols compete with the PEO for any available peroxides, preventing auto-oxidation. The addition of allyl alcohol, isopropanol, ethanol and methanol to PEO solutions exposed to ultraviolet light retarded the loss in viscosity observed in the absence of any stabilising agent. It is important to note that DNA is polar and insoluble in alcohols and that primary and secondary alcohols are often used to precipitate DNA. Here the effect of methanol, isopropanol and hexane on PEO viscosity and sieving behaviour were studied.

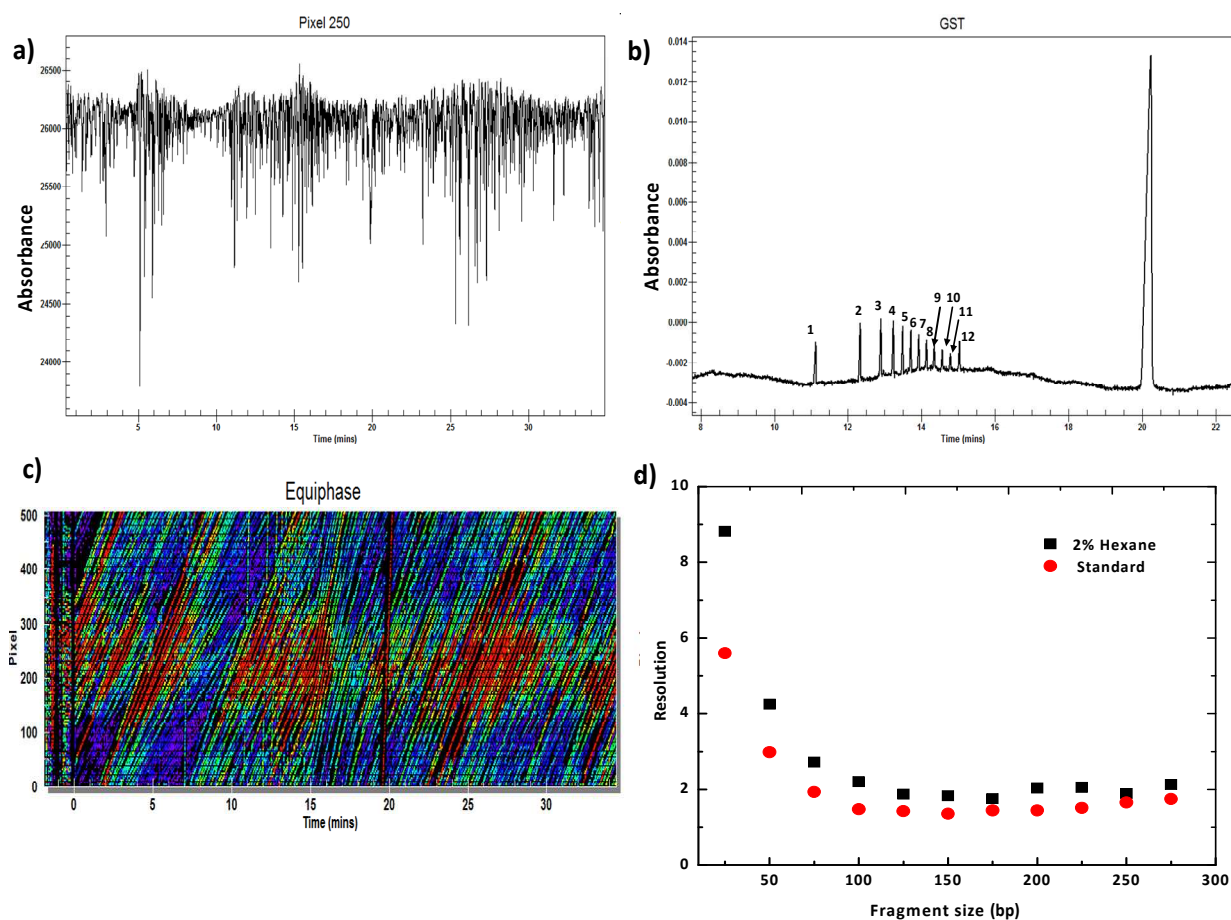


Figure 4.9: Separation of a 25 bp dsDNA ladder (36 μ g/ml in deionised water, Promega) in the presence of 2% hexane in the separation matrix. (a) and (b) show the single pixel electropherogram (Pixel 250) and GST processed electropherogram respectively. While (c) details the Equiphase map from while the GST profile was extract. (d) A plot of resolution versus fragment size. The sample was separated in a 4% PEO (300 kDa) buffer which was re-crystallized to remove BHT prior to use. 2% hexane was used as an alternative stabiliser to BHT. The separation was performed using a 54 cm long capillary of effective length 40 cm, coated with 2% PVP (360 kDa) at 25°C and using a field strength of 296.3 V/cm.

A 25 bp dsDNA ladder (Promega Corporation, Madison, UK) was used to assess the effect of the alcohol stabilisers. Separations were carried out in re-crystallised 4% PEO (300 kDa) in x1 TBE treated with either 2% methanol, 2% isopropanol, 2% hexane or 10% hexane. Separation of DNA fragments was observed in 2% hexane (Figure 4.9a and b) and 2% isopropanol (data not shown) but not in 10% hexane (data not shown) or 2% methanol (data not shown). That is the electropherogram did not contain any peaks for the separations performed in 10% hexane and 2% methanol. In all cases the single pixel electropherogram and equiphase map, Figure 4.9a and c respectively, showed high levels of background noise which is not usually observed.

A decrease in mobility was observed for the fragments separated with 2% hexane and 2% isopropanol. Figure 4.9d illustrates the effect of 2% hexane on the resolution between dsDNA fragments. An increase in resolution was observed for all fragments in the presence of 2% hexane compared to the standard 4% PEO (300 kDa) matrix. Organic solvents are known to increase the viscosity of electrolyte buffers. In addition, a change in the overall distribution of water molecules between the polymer matrix and DNA must occur. This change effects the latter's radius of hydration which in turn alters its migration behaviour. The results in all cases however were not reproducible; precipitation of the DNA at higher solvent concentrations or after prolonged exposure to solvent may have occurred. To prevent auto-oxidation of the PEO sieving matrix a water soluble UV transparent reagent is required which will not interact with the DNA molecule. The results obtained so far show that most conventional stabilisers do not match these requirements and the separation process is affected.

4.3.1.5 Separations Performed Using a 1.75% PEO (5MDa) Buffer

Longer chain length polymers would require higher levels of auto-oxidation to show similar losses in resolution. Consequently, a lower level of polymer degradation was observed in the 5MDa PEO compared to the 300 kDa polymer. Separations were performed using an increasing percentage of re-crystallised PEO (5MDa) from 0.75% to 1.75%. Two samples, a 20 bp and a 100 bp size ladder, were used for this set of experiments. Similar injection and separation parameters were employed to allow comparison between results. Increasing concentrations of PEO (5MDa) led to a decrease in fragment mobility. The electrophoretic mobility versus fragment size plot for the 100 bp ladder, analysed in each sieving matrix, is shown in Figure 4.11a. All the fragments of the dsDNA ladder from 100 bp to 1200 bp were resolved for all percentages of 5MDa PEO tested.

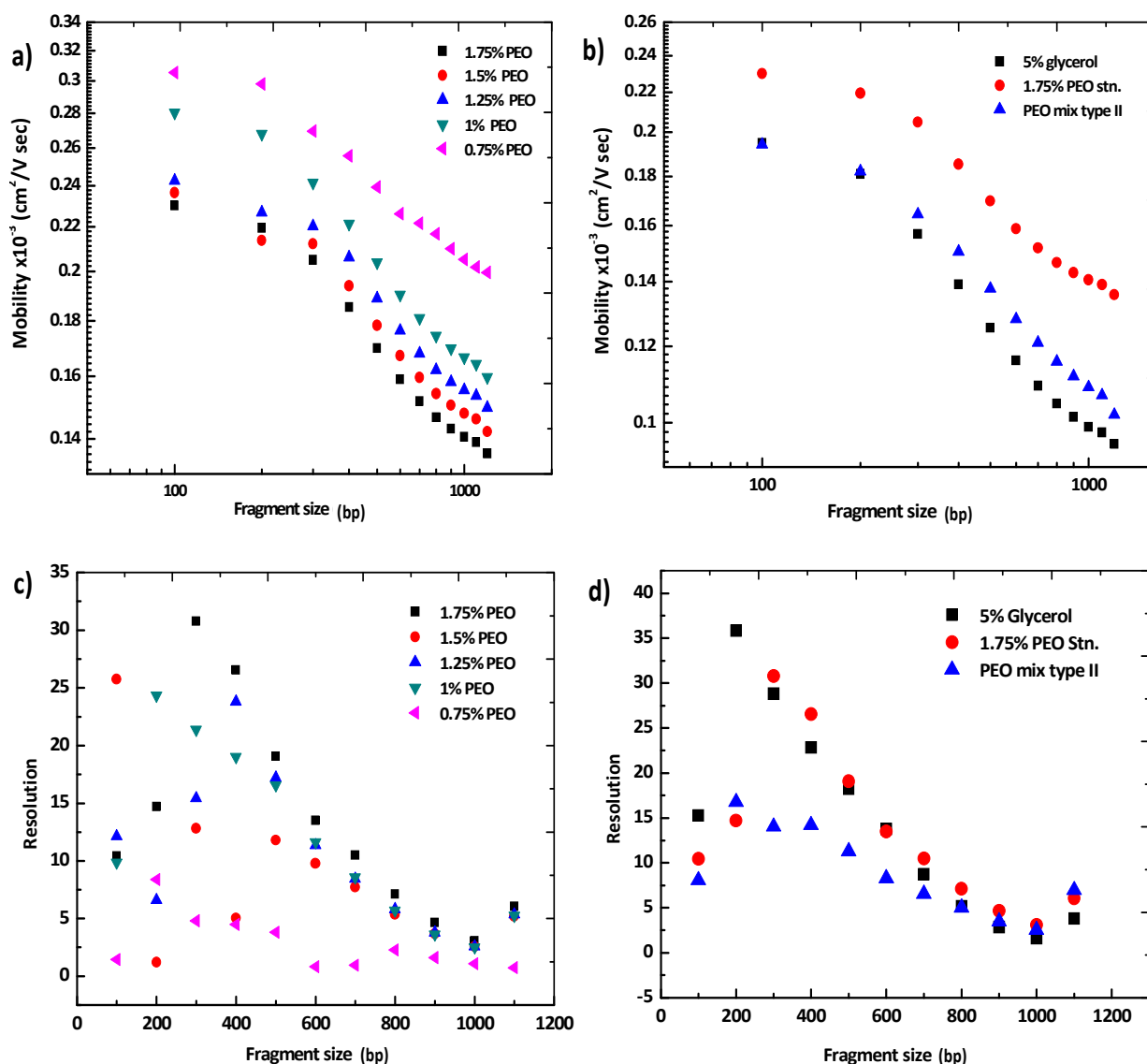


Figure 4.11: Sieving matrix effect on dsDNA velocity and resolution. (a) and (b) are mobility versus fragment size plots while the (c) and (d) are resolution versus fragment size plots. Both sets illustrate the effect of changing sieving matrix composition on the migration behaviour and resolution between dsDNA fragments. (a) and (c) show the effect of increasing polyethylene oxide (5 MDa) concentration from 0.75 % to 1.75% in the sieving matrix. (b) and (d) compare three different sieving matrices, 1.75% PEO (5MDa), 1.75% PEO (5MDa) with 5% glycerol and a mixture of several polymer lengths of PEO. A 100 bp dsDNA size ladder at $\mu\text{g/ml}$ was employed to demonstrate these changes. All the buffers were prepared in $\times 1$ TBE (pH 8.3). Separations were performed using a 54 cm long capillary of effective length 40 cm, at 25°C and using a field strength of 222.2 V/cm.

The log-log plots for all five sieving matrices (Figure 4.11a) show the signature sigmoidal shape observed by other authors [20]. Increasing polymer concentrations reduces the mobility of the fragments. Analogous to the mobility traces, the resolution plots can also be divided into sections corresponding to each sieving regime. For all polymer concentrations except 0.75 % PEO (5

MDa), a lower resolution value was observed for the lower molecular weight fragments. The Ogston sieving regime can be associated with this behaviour, where the fragment's radius of gyration is less than or equal to the pore size. Above 200 bp to 400 bp, the fragment size varies with PEO concentration and a steep increase in resolution was observed. The fragments are most likely migrating as described by the reptation model discussed previously. Here, mobility values are inversely proportional to fragment size and it would appear that resolution values behave similarly. This is corroborated by the steep slope obtained between fragment sizes 200 bp/300 bp to 900 bp. Longer fragment lengths show a slight increase in resolution compared to the 900 bp and 1000 bp fragments, signifying entry into the biased reptation regime. Comparatively, a very small change in resolution values was observed for all fragment sizes when analysed in the 0.75% PEO matrix. Separation observed here can be explained by the 'transient entangled coupling' mechanism proposed by Baron *et al.* [22] in 1993. This matrix is a dilute solution of non-entangled polymer chains. In transit through the capillary, DNA fragments collide with matrix polymers which are dragged along until they disengage and slide away. Larger DNA fragments have a higher probability of entangling with the polymer matrix and will be more retarded than smaller ones. Taken together both, plots (mobility and resolution) reveal that true sieving begins above 1% concentration for this PEO polymer length. Traces from the resolution plot (Figure 4.11b) indicate that the highest resolution was obtained at 1.75% PEO (5 MDa).

A standard separation protocol was developed for the analysis of dsDNA fragments in the 1.75% PEO (5MDa) sieving matrix. Separations were performed in bare fused silica capillaries (75 μ m ID) of total length 54 cm and effective length 42 cm. The capillary was pre-coated with 10% PVP prior to loading of the sieving matrix. Samples were injected electrokinetically at a voltage of 5kV for 20 seconds and separated under a voltage of 12 kV. Between separations the capillary was rinsed with 1M HCl followed by deionised water. Figure 4.10a, b, c and d show the separation of a 25 bp size ladder (Promega, 25-300 bp), 50 bp size ladder (Promega, 50-800 bp), Roche dsDNA marker VIII (Roche, 19-1114 bp) and pBR322 dsDNA size ladder (8-587 bp) in a 1.75% PEO (5 MDa) under these conditions.

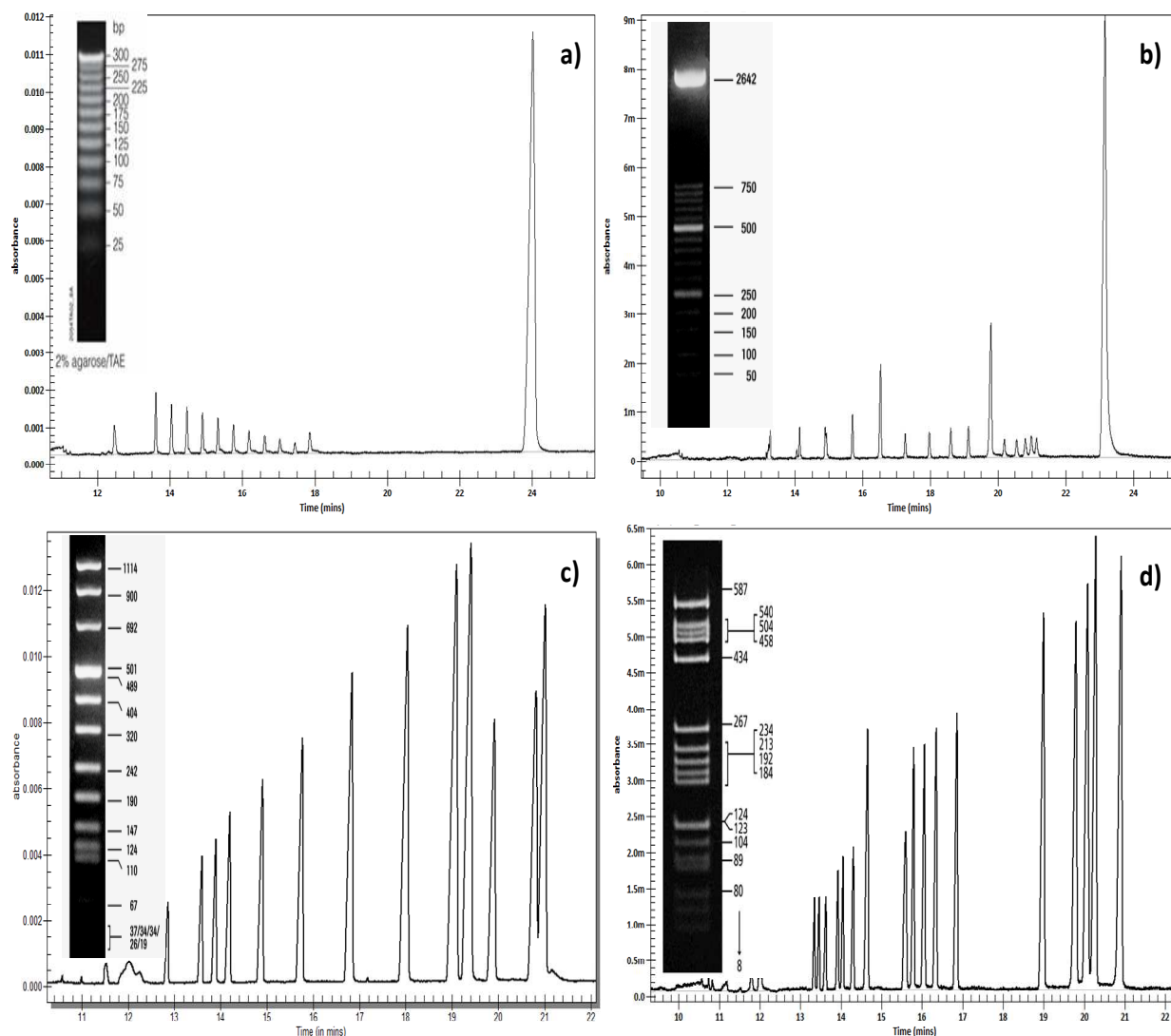


Figure 4.10: Separation of dsDNA ladders in a 1.75% PEO (5 MDa) buffer. (a) 25 bp dsDNA ladder (36 μ g/ml in deionised water, Promega). (b) 50 bp dsDNA ladder (25 μ g/ml in deionised water, Roche). (c) DNA marker VIII dsDNA ladder (25 μ g/ml in deionised water, Roche). (d) pBR322 dsDNA ladder (10 μ g/ml in deionised water). All four separations were performed using a 54 cm long capillary of effective length 40 cm, at 25°C and using an electric field strength of 222.2 V/cm. The separation performed on a 2% agarose gel provided by the manufacturer is also shown in each panel.

The use of additives and mixtures of PEO to improve resolution was also investigated. Separation channels employed in chip based electrophoresis demand matrices with increased sieving capacity to allow resolution between fragments over a shorter length. Modifications to the 1.75% PEO matrix were examined to aid buffer development for chip-based electrophoresis. The resolution between DNA fragments is controlled by changes in two parameters, electrophoretic mobility and molecular diffusion [24]. The electrophoretic mobility and molecular diffusion co-efficient of an analyte are inversely proportional to viscosity of the surrounding medium [47]. Therefore an

increase in viscosity of the separation medium would lead to an increase in resolution due to decreases in both mobility and diffusion co-efficient of the analyte. The addition of 5% glycerol to the 1.75% PEO (5MDa) sieving matrix demonstrates this effect.

Figure 4.11b shows the change in mobility values for the fragments of a 100 bp size ladder when separated in 1.75% PEO (5 MDa) with and without glycerol. The presence of glycerol leads to a 15 to 30 percent decrease in mobility and an increase in the slope of the curve. Cheng and Mitchelson [47] have shown similar results to those presented here. They examined the effect of increasing concentration of glycerol in an HPMC sieving matrix, which showed a decreased mobility with an increase in the concentration of glycerol. However, they remained unable to resolve fragments 271 and 281 in the Φ X174 HAEIII digest employed to demonstrate their work. This anomalous migration due to a primary sequence induced secondary structure can only be rectified by performing the separation at an elevated temperature. Unexpectedly, this result was not reflected in the resolution versus fragment size plot shown in Figure 4.11d. Only a small increase in resolution was observed between smaller fragment sizes in the presence of 5% glycerol. The standard 1.75% PEO buffer was also compared to a modified PEO buffer referred to as PEO mix type II. The PEO mix contained 0.8% PEO 100 kDa, 0.9% PEO 300 kDa, 0.3% PEO 600 kDa and 0.6% PEO 1 MDa prepared in x1 TBE. Employing a combination of polymer lengths at low concentrations should result in a matrix with low viscosity [48] and a wide range of pore sizes. A mobility versus fragment size plot (Figure 4.11d) illustrates the effect of the mix PEO sieving matrix on the resolution between fragments. A steeper decrease in mobility was observed in comparison to the control PEO matrix. This sieving matrix offers a low viscosity and high resolution alternative to the 1.75% PEO (5MDa) matrix for use in the chip based electrophoresis studies.

4.3.1.6 Effect of Dye on dsDNA Mobility

To pursue DNA separations in chip-based systems it was necessary to fluorescently label samples. The effect of intercalating dyes on dsDNA has been intensely studied. Nath *et al.* [49] and Gudnason *et al.* [50] both demonstrate the adverse effect of intercalators on the polymerase chain reaction (PCR). Gudnason and co-workers [50] investigated 15 different intercalating dyes including SYBR Green I and found that all excluding SYTO-13 and SYTO-82 inhibited PCR and influenced the melting temperature of DNA in a concentration-dependent manner. Hahn *et al.* [51]

and others [52-56] have shown migration differences between DNA molecules of the same length, but with different dyes attached. Hahn and co-workers [51] demonstrated that mobility and in turn molecular weight differences were observed between identical fragments labelled with rhodamine and fluorescein derived dyes. However no differences were observed between these fragments when labelled with different derivatives of the same dye. They were unable to determine which dye if any did not change the mobility of the fragments as unlabelled separations were not possible using the acrylamide based separation media. The effect of these labels (at various concentrations) on the migration behaviour of the dsDNA fragments was evaluated using the PERERGRINE CE system. The separation behaviour could be compared directly to an unlabelled sample as the dsDNA fragments were all imaged at a wavelength of 254 nm. Several dyes were interrogated, but only SYBR Green I was analysed in detail. The use of SYBR Green I in the detection of dsDNA is common in many molecular biology methods, including DNA quantification and real time PCR.

The structure of SYBR Green I [2-[N-(3-dimethylaminopropyl)-N-propylamino]-4-[2,3-dihydro-3-methyl-(benzo-1,3-thiazol-2-yl)-methylidene]-1-phenyl-quinolinium] was elucidated using mass spectrometry and nuclear magnetic resonance (NMR) by Zipper *et al.* [57] in 2004. The same authors found it had a molecular mass of 509.273 gm/mol and is provided by Invitrogen (Paisley, UK) at a concentration of ~10 mg/ml. They also reported that SYBR Green I carries two positive charges under standard conditions and that it was this that contributes to the dyes highly efficient binding to DNA and its stability during electrophoresis. There are three basic forms of interaction between DNA and dye; intercalation, minor groove binding and sample stacking. Via hydrodynamic studies Suh and Chaires [58] confirmed that SYBR Green I intercalates with dsDNA at low concentrations, while with increasing concentration base-specific minor-groove binding is observed. The resulting DNA-dye-complex absorbs at a wavelength maximum of 488nm and emits at a wavelength maximum of 522nm.

A stock concentration of SYBR Green I x1000 was prepared in deionised water. The sample was further diluted to the appropriate concentration along with a standard concentration of DNA prior to analysis. The 25bp size ladder and 50bp size ladder (Promega, Madison, USA) were provided at stock concentrations of 0.36 µg/µl and 0.34 µg/µl respectively. They were diluted to one tenth of the stock value prior to analysis. Samples with a final total dsDNA concentration of 0.036 µg/µl (25 bp ladder), 0.034 µg/µl (50 bp ladder), 0.068 µg/µl (50 bp ladder) and SYBR Green I concentrations of x100 (100 µg/ml), x10 (10 µg/ml) and x1 (1 µg/ml) were prepared in deionised water. The samples were injected and run under similar conditions to unlabelled

samples as described earlier. The migration time for each fragment was used to determine its mobility value and this was plotted against the dye-to-base pair ratio (dbpr) for each fragment (Figure 4.13a and b). The dbpr can be defined as the moles of dye per mole of DNA base pair. The moles of dsDNA were determined based on the average molar mass for a base pair as 660 g/mol (Sigma) while the molar amount of SYBR Green I was calculated based on the molar mass, 509.27 g/mol, as estimated by Zipper *et al.* [57].

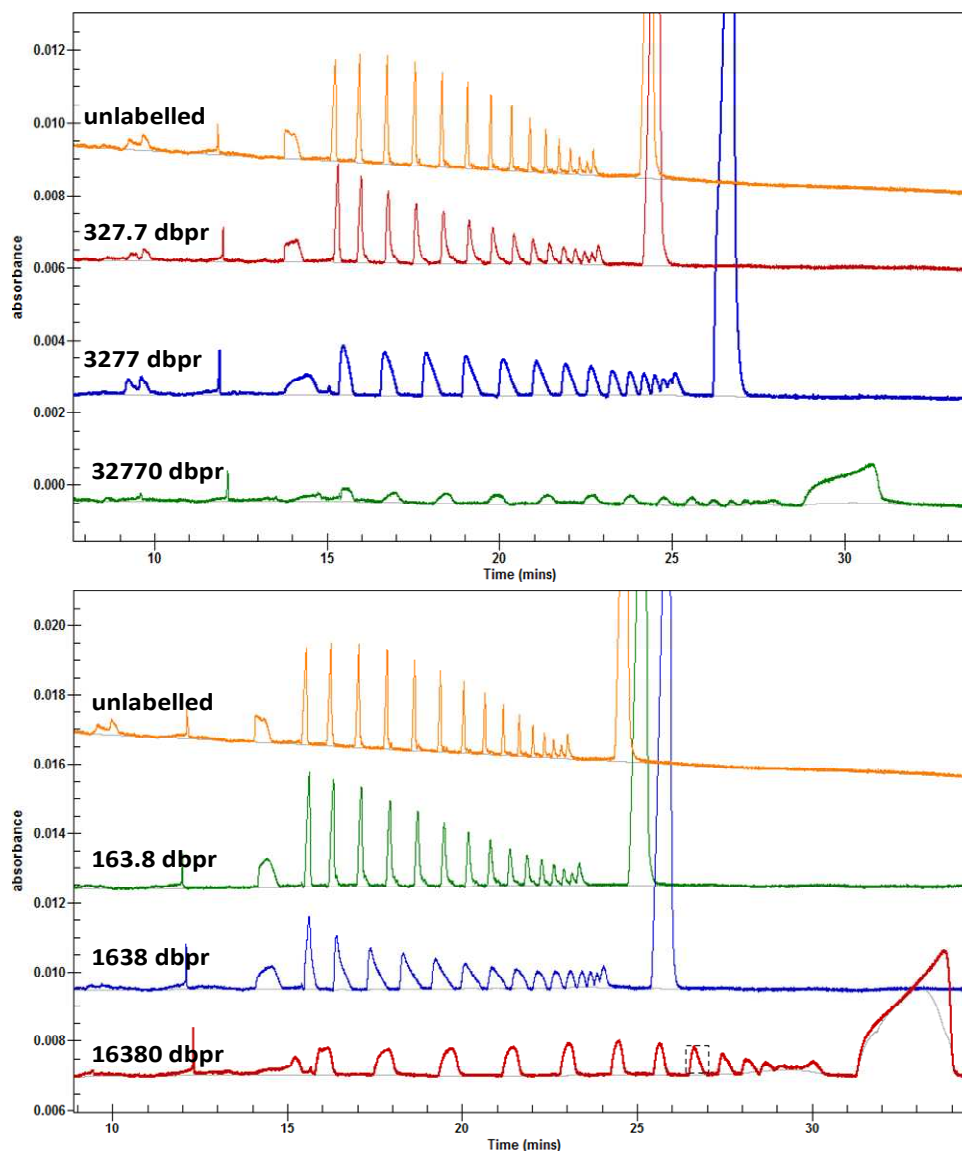


Figure 4.12: Overlay of GST processed electropherograms illustrating the effect of increasing concentration of SYBR Green I on electrophoretic migration of dsDNA fragments. Separation of 50 bp dsDNA size ladder (0.34 μ g/ μ l in deionised water, Promega) treated SYBR Green I with dye to base pair ratios of 163.8, 327.7, 1638, 3277, 16380 and 32770 compared to an unlabelled sample. All separations were performed using a 54cm long capillary of effective length 40cm, at 25°C and using a field strength of 203.7 V/cm. Separations were performed in the 1.75% PEO (5 MDa) sieving matrix.

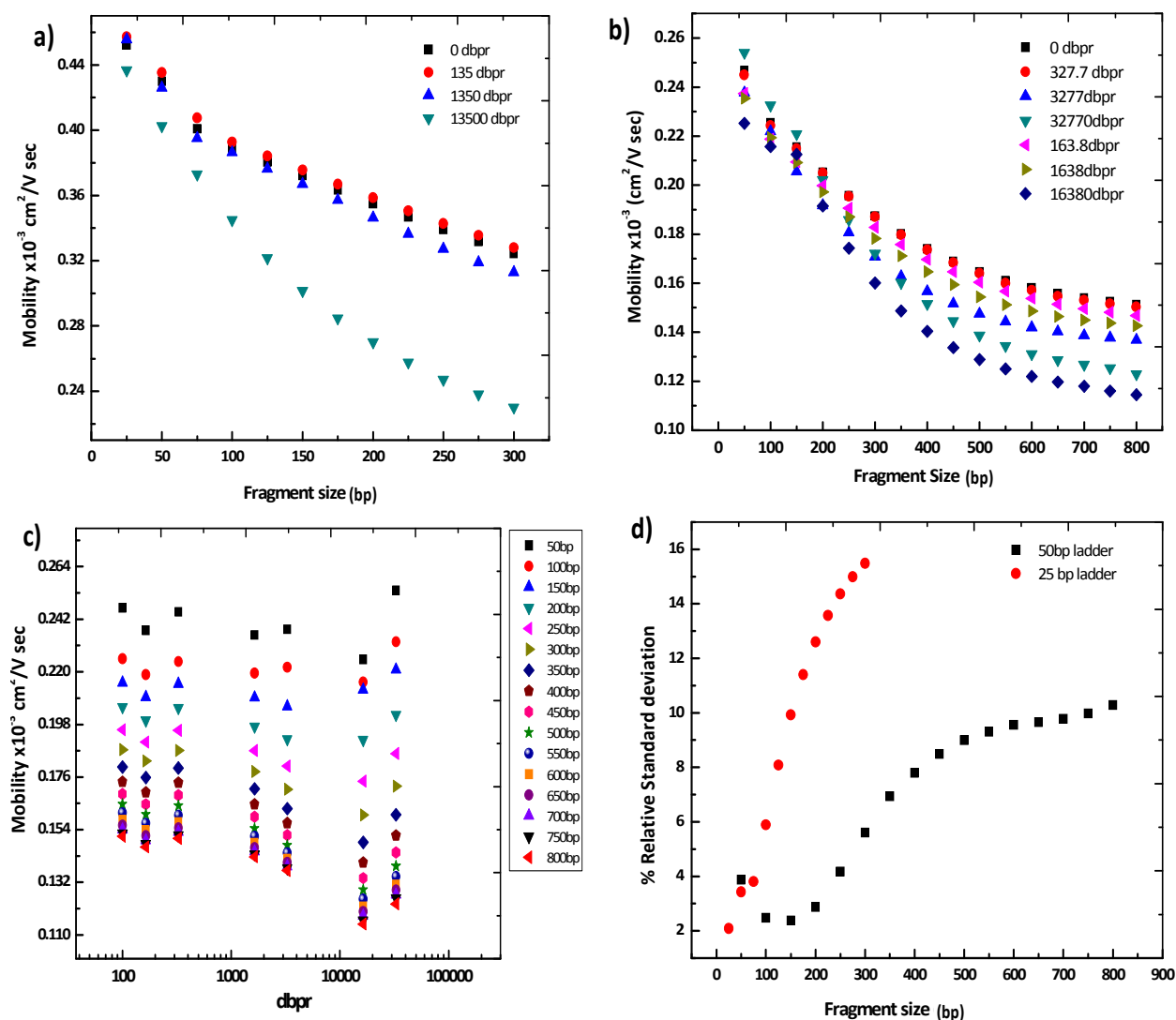


Figure 4.13: Plots illustrating the effect of increasing concentration of SYBR Green I on the mobility of dsDNA fragments. (a) and (b) are mobility versus fragment size plots of 25 bp and 50 bp dsDNA size ladders respectively, comparing increasing dye to base pair ratios to an unlabelled sample. The mobility versus dye to base pair ratio plot in (c) illustrates the effect of increasing levels of SYBR Green I on the mobility of individual fragments. (d) Plot of percentage RSD as a function of fragments size for both ladders. All separations were performed using a 54 cm long capillary of effective length 40 cm, at 25°C and using a field strength of 203.7 V/cm. Separations were performed in the 1.75% PEO (5 MDa) sieving matrix.

The GST processed electrophoretic profiles of SYBR Green I labelled 50 bp size ladder dsDNA fragments with increasing dye-to-base pair ratios are shown in Figure 4.12 along with an unlabelled sample. The profiles were aligned using the buffer fronts which would not be affected by the presence of SYBR Green I. The traces reveal distinct differences in migration profiles compared to an unlabelled sample. Increasing levels of SYBR Green I led to changes in peak shape and an

increase in migration time. Differences in the amount of dye bound to the DNA will lead to heterogeneity between fragments of the same size, increasing the width of peaks. Variations in charge and effective length will cause changes in mobility and band broadening. Therefore a single broad peak will contain fragments with an identical number of base pairs but with varying overall charge and effective length. In the presence of higher levels of dye longer injection times were used to obtain adequate signal. The reduction in conductivity between the sample and separation buffer, due to the presence of the dye, leads to lower amounts of DNA entering the capillary. Additionally, as DNA has a net negative charge due to the phosphate groups present in its structure, it has a constant mass-to-charge ratio as a single phosphate molecule is associated with one nucleotide. The addition of several positively charged dye molecules to it will reduce its net negative charge and change its mass-to-charge ratio, necessitating a longer electrokinetic injection time.

Mobility versus fragment length plots for labelled dsDNA fragments, from the 25 bp and 50 bp size ladder at various dye to base pair ratios, were compared to unlabelled samples (Figure 4.13 a and b). The signature sigmoidal curve is observed in all cases, however with increasing dye concentration the slope increases indicating a change in the sieving mechanism of dsDNA fragment and an apparent increase in resolution. The highest resolutions were observed at the highest dye-to-base pair ratios. Figure 4.13c plots the mobility versus dye-to-base pair ratio for each fragment of the 50 bp ladder. Here the percentage relative standard deviation (RSD) between mobility values for the same size fragment at different dye-to-base pair ratios increases with an increase in fragment size. The % RSD for the 50 bp fragments was 3.8 while the 800 bp fragment showed a %RSD of 10.3. A similar effect was observed with the 25 bp size ladder, with % RSD values starting at 2 for the 25 bp fragment and increasing to 15.4 for the 300 bp fragment. Figure 4.13d shows the plot of percentage RSD versus fragments size. An S-shaped curve is observed for the 50 bp size ladder, where a small increase in %RSD is observed for the shorter fragment lengths (50-200 bp) followed by an exponential increase for fragments between 250 and 400 bp. The RSD values plateau at approximately 10% for fragment sizes above 550 bp. The results from both size ladders indicate that with increasing SYBR Green I concentration larger mobility shifts are observed for higher molecular weight fragments. This is corroborated by the mobility data illustrated in Figure 4.13a and b. The process of intercalation involves insertion of the planar section of the dye molecule between adjacent base pairs, which necessitates a rotation in the helical structure of dsDNA and a lengthening effect to accommodate the dye [59, 60]. Intercalation of

other dimeric cyanine dyes typically follows the neighbourhood exclusion principle, where intercalation is not observed on either side of the base pairs that contain a dye molecule. Therefore, with increasing SYBR Green I concentration shorter fragments of dsDNA which accommodate less dye reach a steady state more rapidly in terms of charge and effective length compared to longer fragments. With increasing dye concentration larger fragments show a decrease in mobility until steady state is achieved and following which a slight increase in mobility is observed.

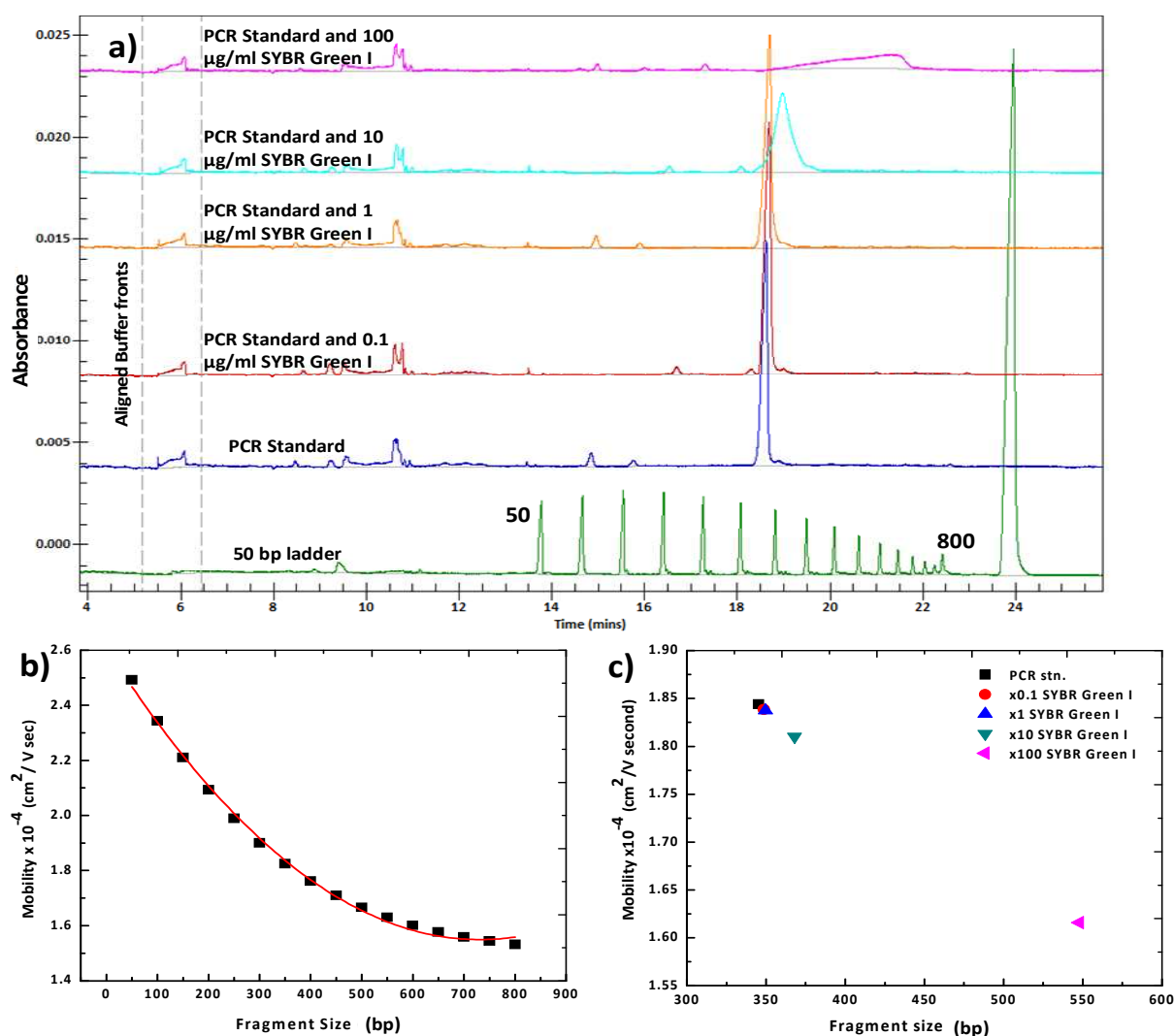


Figure 4.14: Plots illustrating the effect of increasing concentration of SYBR Green I on the mobility of PCR amplicons. (a) The electrophoretic profile of the 323bp amplicon with increasing concentrations of SYBR Green compared to an unlabelled sample and a 50 bp dsDNA ladder analysed under similar conditions. (b) The calibration curve generated from the 50 bp dsDNA ladder. This was used to determine the fragment size from the mobility values of each amplicon. (c) A plot of mobility versus fragment size illustrating the increase in estimated fragment size with increase in SG I concentration. All separations were performed using a 54 cm long capillary of effective length 40 cm, at 25°C and using a field strength of 203.7 V/cm. Separations were performed in the 1.75% PEO (5 MDa) sieving matrix.

A vast difference in the trend of %RSD values is observed between the two size ladders (25 bp and 50 bp). This is due to the disparity in total base pairs present and therefore the total number of intercalation positions available between them. Larger dsDNA fragments show greater variations in mobility values as they experience greater changes in conformation and flexibility. The results illustrate the effect of increasing concentration SYBR Green I on the mobility of dsDNA. As mobility values are used to generate molecular weight calibration curves for PCR, STR analysis etc. variation in mobility due to dye interaction can adversely affect mass estimation. Accurate mass assessment can only be performed if the concentration of amplified DNA is known and if the dye-to-base ratio is maintained below 100. A 323 bp PCR amplicon was used verify this assertion. Post amplification the sample was diluted by half and prepared with increasing concentrations of SYBR Green I (0.1, 1, 10 and 100 $\mu\text{g/ml}$). Samples were separated using the standard conditions described earlier; these are also listed in the figure legend.

Electrophoretic profiles obtained for each sample were plotted and compared to an unlabelled sample and a 50 bp dsDNA size ladder (Figure 4.14a). The 50 bp dsDNA size ladder was used to generate a calibration curve, by plotting mobility versus fragment size as shown in Figure 4.14b. Based on the polynomial function fitted to the calibration and mobility values of the unlabelled and labelled PCR amplicon, the fragment size for each sample was determined. As is seen from the electrophoretic profiles (Figure 4.14a), an increase in migration time and peak width is observed with increasing SYBR Green I concentration. At 100 $\mu\text{g/ml}$ (x100) SYBR Green I concentration a fragment size of 550 bp was obtained. Figure 4.14c plots the change in estimated fragment size with increasing dye concentration. Consequently, electrophoretic separations of dsDNA employing intercalating dyes should be set up and interpreted with care. Changes to the charge, effective length, conformation and flexibility of the biopolymer in the presence of dyes can lead to irreproducible and misleading results.

4.4 Chip-Based dsDNA Separations

Electrophoretic separations performed on chip are theoretically more efficient than those performed on capillary. The theoretical plate number (N) is directly proportional to the electric field strength (E) (Equation 1.13, Chapter 1). However, higher field strength will induce a higher current within the separation channel producing Joule heating. The power generated by Joule

heating is proportional to the conductivity of the buffer and electric field applied. Joule heating reduces the efficiency of electrophoresis by increasing the overall temperature of the buffer and by generating temperature gradients in the radial and axial directions. Consequently, the superior heat dissipation on-chip compared to a capillary made of the same material reduces Joule heating and enables higher voltages to be applied, increasing the efficiency of the separation. Petersen and co-workers [61] demonstrated this effect employing microchips made from different materials. They found that 3 times the electric field may be applied on glass and silica microchips than silica capillaries of the same dimension. While significantly lower fields could be applied to microdevices made from plastics or PDMS. However, fabrication of microchannels in glass or quartz requires harsh chemical treatment and takes between 2 to 3 days. Furthermore, aside from selecting device material based on its heat transfer coefficient Joule heating may also be reduced by reducing buffer conductivity, reducing channel radius and increasing the length of the separation channel. Therefore, polymer-based devices which have faster design translation times and are easier to make should be optimised to show minimum Joule heating for the substrate material employed.

4.4.1 Chip-Based Conditioning and Loading

Prior to plasma bonding (Chapter 2) the PDMS channel and bottom layer were sonicated in 0.1 M HCl, dried under nitrogen and then bonded. Following which the microchannels were loaded with 0.1 M HCL which was flushed out with water when the device was readied for use. The sieving matrix was loaded in to the sealed PDMS channel using a pressurised syringe. Matrices up to 2.5% PEO (5 MDa) in x0.1 TBE with 5% glycerol could be loaded without delamination of the bottom layer. Bottom layers below 1 mm thick could not be used due to stretching and delamination around the loading reservoir. Voltage was applied across the channels prior to injection and separation to equilibrate the buffer. The high voltage power supply, microscope and chip-holder are described in detail in Chapter 2.

4.4.2 Injection Formats

As with conventional CE controlled injection of a sample into the separation channel is critical for efficient analysis. Various injection geometries have been proposed for microchip electrophoresis devices. A recent review by Blas *et al.* [62] describes the electrokinetic-based injection modes

available for use in chip-based electrophoresis. These included the T-type injection [63], Cross-injection, T-cross injection [64] and multi-T injection [65]. The most widely used formats are the cross injector and the double-T injector initially proposed by Harrison *et al.* [15, 63, 66] and Effenhauser *et al.* respectively. Employing the cross-injector format samples can be loaded using floating, pinched [67], reversed [68], gated [69], gated with diffusion [70], double-L [71] and dynamic [72, 73] loading modes.

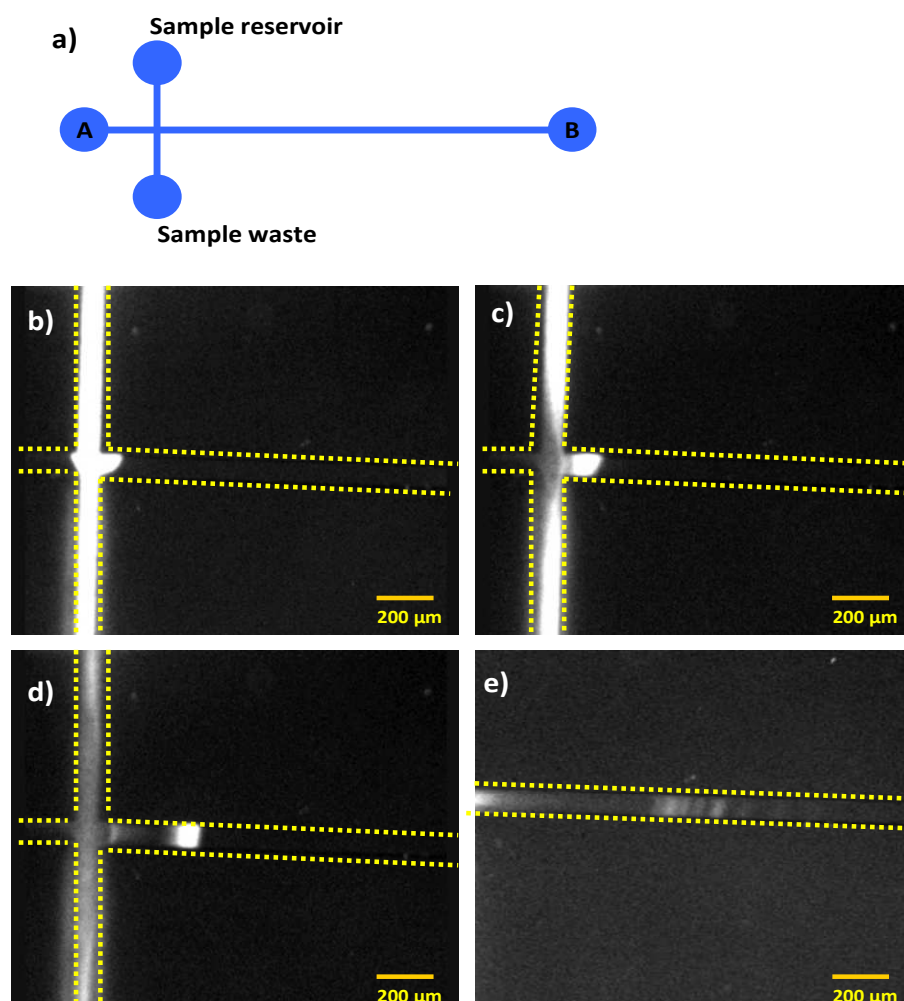


Figure 4.15: Injection and separation of dsDNA fragments on a microchip electrophoresis device fabricated in PDMS. (a) A schematic of the device with cross piece injector. The total length of the separation channel (from reservoir A to B) was 6 cm while its effective length was 5.5 cm. (b), (c), (d) and (e) illustrate the injection and separation mechanism using a 100 bp dsDNA size ladder labelled with SYBR Green I. Under an applied field of 200 V/cm. The negatively charged DNA is drawn down from the sample reservoir to the sample waste reservoir. The sample is prevented from diffusing into the separation channel using ‘pinching’ voltages (see text). The sample plug formed at the intersection is injected into the separation channel under an applied field. A discrete sample plug is maintained by applying pull back voltages at the sample and sample waste reservoirs (c) and (d). Separation between fragments occurs almost immediately. Sieving matrix employed was a 2.5% PEO prepared in 0.1x TBE.

The cross-injector is designed to deliver a discrete sample plug into the separation channel. Typically the injection procedure involves two basic steps, loading and dispensing. The sample is driven from the sample reservoir to the sample waste reservoir under an electric field when loading. Following which the sample plug contained at the intersection of the cross piece is moved into the separation channel under an applied field, i.e. dispensing. Figure 4.15a illustrates a schematic of the cross piece injection microdevice used to separate double stranded DNA in this study. The total length of the separation channel from buffer reservoir A to B was 6 cm, while the length from the point for injection to reservoir B was 5.5 cm. Each arm forming the cross-piece was 0.5 cm long. The height and width of all channels was 100 μm . The entire device was fabricated in PDMS as detailed in Chapter 2 and mounted on a glass slide for support. Figure 4.15b, c, d and e are images of the ‘pinched’ injection mode originally developed by Jacobson and co-workers [74]. During a ‘pinched’ injection the electric field is used to confine the sample during the loading and dispensing step. Here a fluorescently labelled dsDNA ladder is moved from the sample reservoir to the intersection of the cross-piece under an applied field. Voltages applied between reservoir A and B during the loading step confine the sample preventing it from leaking into the separation channel (Figure 4.15b). The plug formed at the intersection is then dispensed into the separation channel. Push back voltages are applied moving excess sample towards the sample and sample waste reservoirs (Figure 4.15c). Separation of the component bands of the ladder occurs almost immediately (Figure 4.15d) and when imaged 5 mm from the point of injection almost complete separation between fragments is achieved (Figure 4.15e). The plug size on a cross-piece injector using a pinched injection depends on the physical dimensions of the channel and the ratio of electric field strength between the sample and separation channel during loading and dispensing [75].

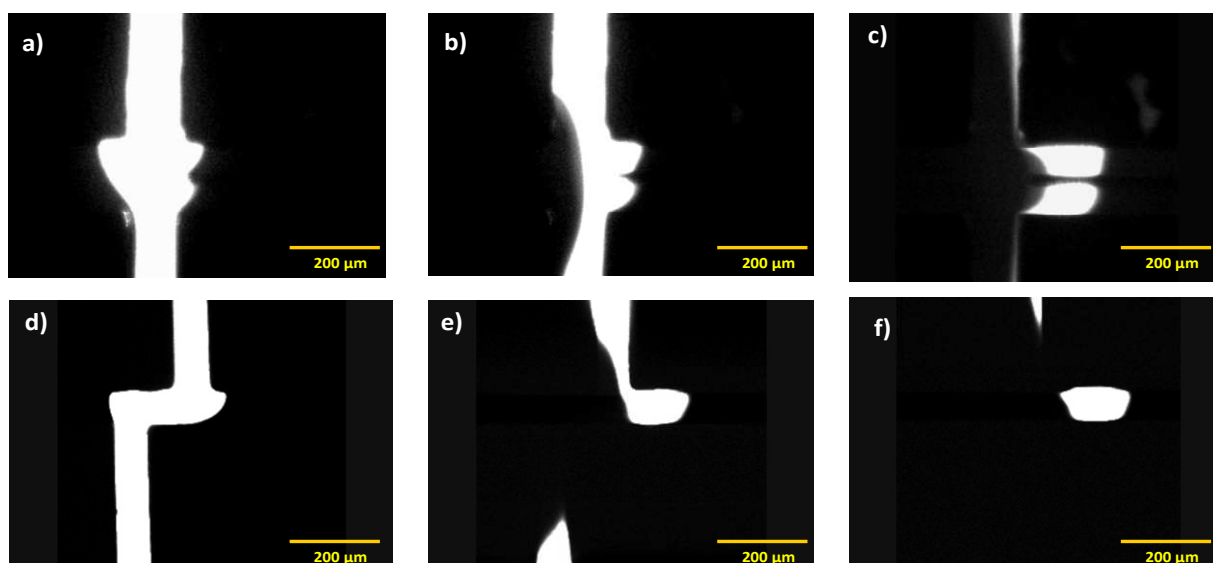


Figure 4.16: Injection mechanism on a dual channel cross-injector (a to c) and double-T injector (d to f) using a FITC.

Variations to the standard cross-piece design have been explored by other authors, usually to control the injection plug size. A six port injection format produces a smaller, streamlined plug while a double-T injector (Figure 4.16) produces a larger plug. A double-T and a cross-piece with one and two separation channels were also trialled in this study. The dual channel cross piece injector allowed simultaneous injection and separation of the sample into two separation channels as demonstrated in Figure 4.16a, b and c. Separation results obtained using this format showed analyte band mobility differences between the channels. Kim and co-workers [76] along with other authors have demonstrated the large variation in mobility and efficiency values obtained for a separation performed on PDMS. Kim *et al.* compared the EOF generated on separation channels of similar cross-sectional area made from quartz, glass and PDMS. They found that strength of the EOF was similar in all materials however the highest standard deviation in mobility was observed in PDMS devices. The authors attributed this deviation to the change in surface charge density of the PDMS as it aged. Therefore, variation in PDMS surface chemistry between each separation channel on the dual injector device is likely to be a major reason for the differences mobility observed.

The dsDNA separation results described here were all obtained using the double-T injection format. Compared to the standard cross-piece format a larger plug could be injected using the double-T injector. Figure 4.16d, e and f illustrate the loading and dispensing steps of an injection

performed on a device with such geometry. The approximate volume of the sample plugs were determined based on the volume of liquid contained at the intersection between the injection and separation channels. Approximately 3 nl was injected using the double-T format, while only 1 nl could be injected using the cross-injector. Peak width is directly proportional to initial plug size (Chapter 1). Therefore, smaller sample plugs will reduce peak widths and increase both efficiency and resolution. Given the shorter separation distances employed in microchip CE, injection formats are designed to provide small discrete sample plugs. However, the CCD camera used to monitor the separations was not sufficiently sensitive and a larger sample plug had to be injected.

4.4.3 Gel Separations on-chip

The capillary and microchip separation profiles for a 25 bp dsDNA size ladder and Roche marker VIII are shown in Figures 4.17 and 4.18. The chip-based separations were performed in 2.5% PEO (5MDa) in 0.1x TBE while the capillary separation was performed in 1.75% PEO in x1 TBE. After preliminary tests it was found that the higher concentration of PEO (2.5%) gave better resolution on the chip-based CE device. A lower concentration of TBE was used in the chip-based devices. The higher current values obtained at high electrolyte concentrations lead to Joule heating within microfluidic devices which in turn cause the sieving matrix to dehydrate and block the separation channels. Higher current values also lead to bubble formation, disrupting current flow and the separation.

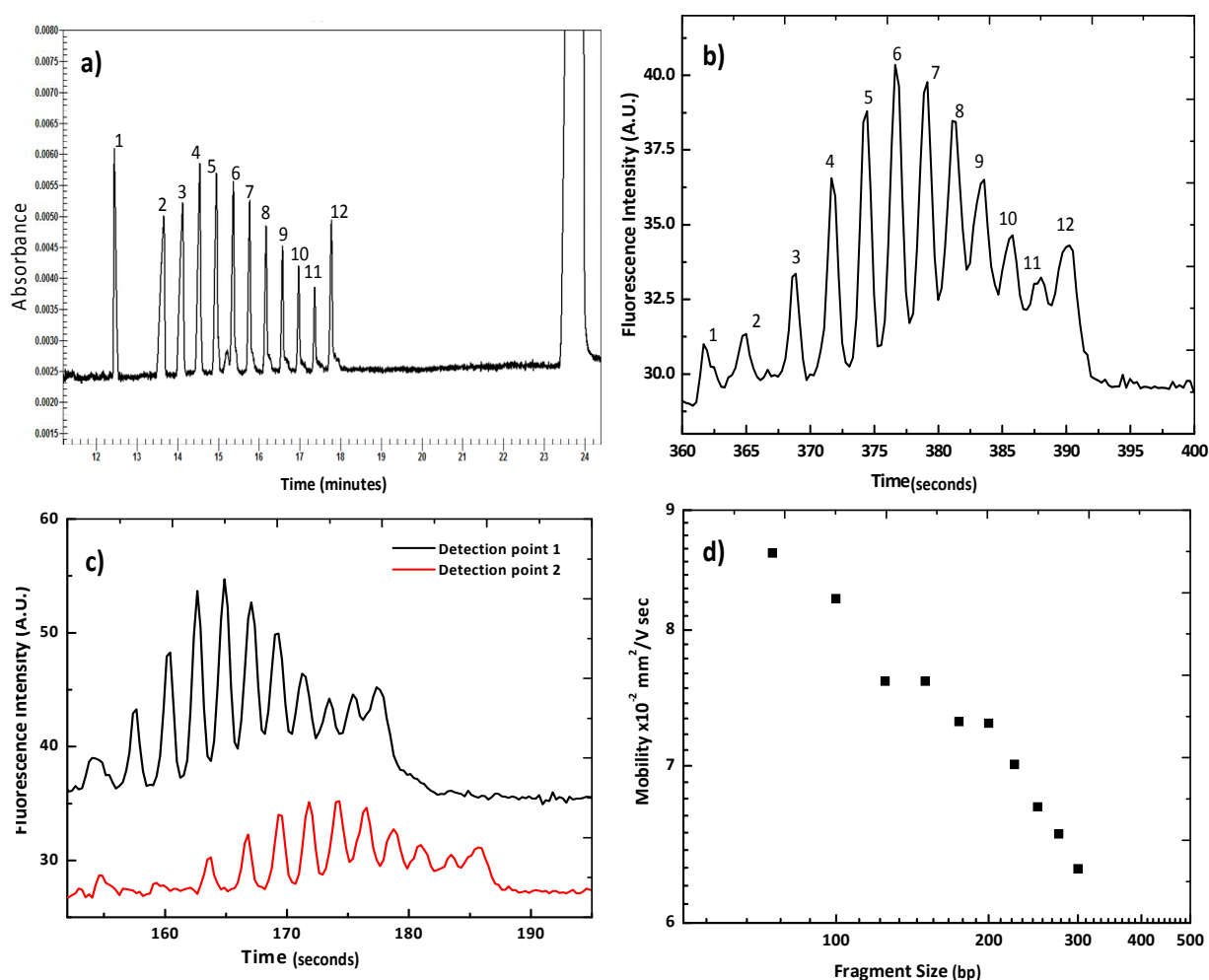


Figure 4.17: Electrophoretic separation of a 25 bp size ladder (Promega Corporation). (a) and (b) detail the separation of all 12 fragments of the 25bp dsDNA size ladder on the PEREGRINE CE instrument and PDMS microchip respectively. The 12 fragments extend from 25 bp to 300 bp in 25 bp increments. The microchip separation was performed in a 2.5% PEO (5MDa) in 0.1% TBE sieving matrix while the CE separation was performed in a 1.75% PEO (5MDa) in x1 TBE sieving matrix. (c) The separation of the 25 bp ladder at two points on the microchip, separated by a distance of 700 μm . The migration time values of the ladder peaks from these two points were used to determine the mobility of each band. (d) A plot of mobility versus fragment size for the microchip separation of the 25bp ladder.

The 25 bp ladder (Promega Corporation) and the Roche marker VIII (Hoffman La Roche) were separated on chip under a field strength of 166.7 V/cm as described in Figures 4.17 and 4.18 respectively. All twelve bands of the 25 bp size ladder were resolved while 11 of the 13 bands in the Roche marker were observed. As the time of the injection was not recorded the mobility values were determined by calculating their velocity across the observable length of the separation channel in the video recorded. Figure 4.17c illustrates the traces obtained at each end of the channel for the 25 bp ladder. Differences in corresponding peak times and the distance between the points of data collection were used to establish velocity values for each peak.

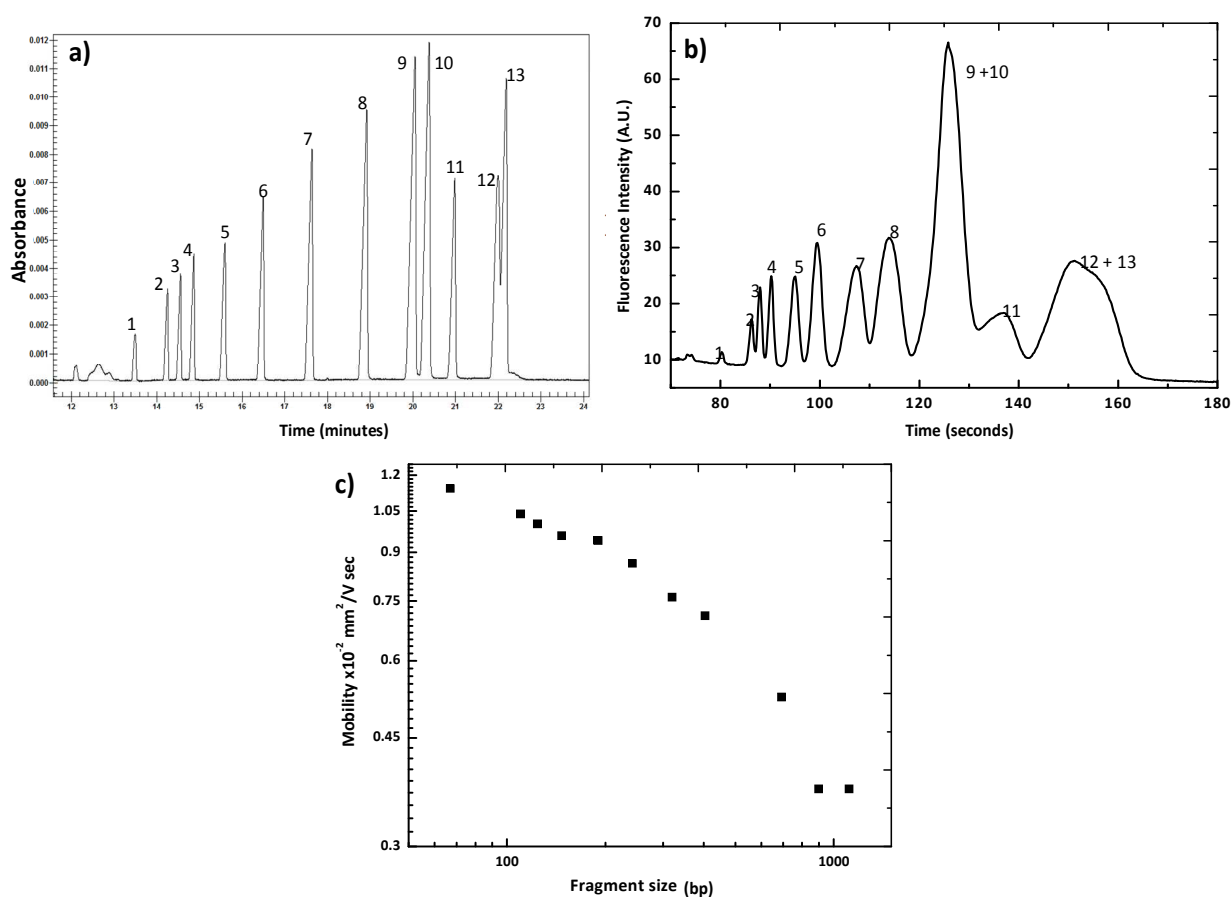


Figure 4.18: Electrophoretic separation of Roche Marker VIII. (a) and (b) detail the separation of 13 fragments of the restriction digest ladder on the PEREGRINE CE instrument and PDMS microchip respectively. The 16 fragments extend from 67 bp to 1114 bp and table lists fragment sizes and corresponding peak numbers. The microchip separation was performed in a 2.5% PEO (5MDa) in 0.1% TBE sieving matrix. (c) A plot of mobility versus fragment size for the microchip separation.

Mobility values were used to generate a log-log plot of mobility versus fragment size for both ladders (Figures 4.17d and 4.18c). Both curves showed shapes close to the expected sigmoid. However steeper slopes were obtained for the smaller fragments indicating higher resolution in this size range. While similar mobility values obtained for some larger fragments indicated little or no resolution was obtained between them on-chip. Furthermore, peaks 9 and 10 of the Roche Marker VIII ladder were resolved using capillary electrophoresis, but remain unresolved on microchip under the separation conditions employed. It should also be noted that these peaks remain unresolved on the agarose gel separation image provided by the supplier (Figure 4.10c inset). In addition, the 1,800 bp fragment of the 25 bp ladder was not observed in the results presented and was infrequently seen as part of the separation profile.

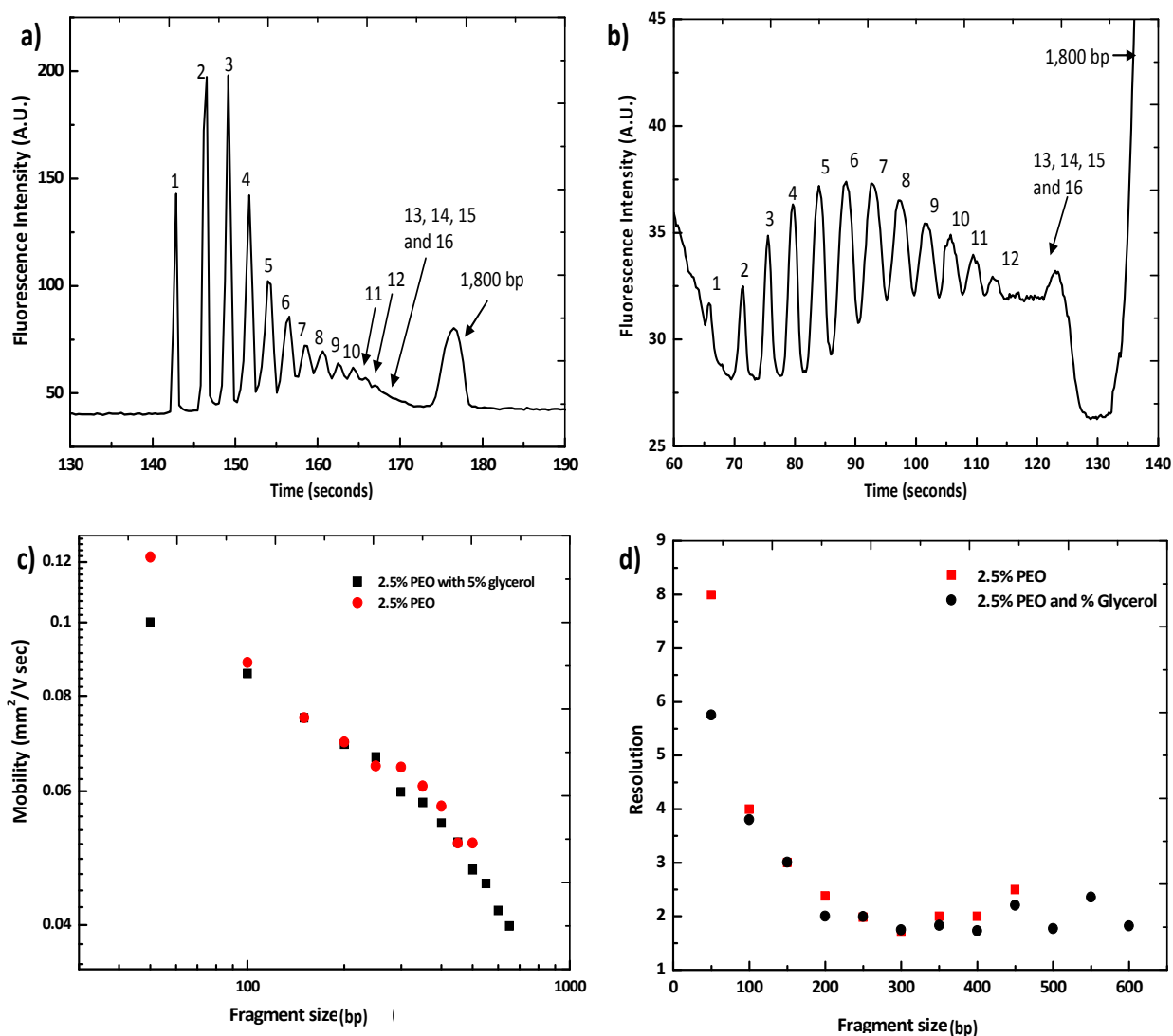


Figure 4.19: Electrophoretic separation of a 50 bp size ladder (Promega Corporation) in sieving matrices with and without glycerol. (a) and (b) detail the separation of dsDNA size ladder on a PDMS microchip using a 2.5% PEO (5MDa) in 0.1% TBE and 2.5% PEO (5MDa) with 5% glycerol in 0.1% TBE sieving matrix respectively. The 16 fragments extend from 50 bp to 800 bp in 50 bp increments. (c) A plot of mobility versus fragment size for the microchip separation of the 50bp ladder in both sieving matrices. (d) A plot of resolution as a function of fragment size for the microchip separation of the 50 bp ladder in both sieving matrices.

To aid analysis of a wider range of dsDNA fragment sizes glycerol was added to the microchip sieving buffer. Figure 4.19a and b show the electrophoretic profiles obtained for the separation of a 50 bp size ladder in the 2.5% PEO (5MDa) in x0.1 TBE sieving matrix with and without 5% glycerol respectively. Figure 4.19c shows a log-log plot of mobility as a function of fragment size for the 50 bp ladder in the presence and absence of 5% glycerol. Analogous to the effect observed in capillary separations, the presence of glycerol reduces the mobility value for each fragment due

to an increase in viscosity of the sieving matrix. Additionally, the shape of the mobility curve obtained in the presence of glycerol is more comparable to the traditional sigmoidal shape obtained when the ladder is separated using capillary electrophoresis.

A plot comparing resolution (Figure 4.19d) values for each fragment show that the presence of glycerol did not significantly improve resolution. However, the presence of glycerol appeared to affect the injection bias often observed on chip-based separations. The distribution of bands follows more closely the trend observed on capillary (Figure 4.14a). In the absence of glycerol, the peak height ratio between the 1,800 bp “backbone” fragment and the 50 bp fragment was 0.25 compared to a value of 5.08 in its presence. The peak height ratio observed on capillary between the same fragments was 7.17. Use of glycerol as an additive in the capillary separations indicated that its effect was more pronounced on the lower molecular weight fragments (Figure 4.11d). A similar effect is observed for the chip-based separations from the mobility versus fragment size plot (Figure 4.19c). Therefore, during injection on-chip, the speed of the smaller fragments is reduced while larger fragments move at a similar rate to that observed in the absence of glycerol. This has the effect of reducing the disparity between fragments of dissimilar molecular weight at the intersection of the crosspiece injector. Additionally, this effect will reduce the loss of lower molecular weight fragments to the sample waste reservoir.

Pinched electrokinetic injections have been demonstrated to be biased towards smaller, neutral, low molecular weight analytes. In 2001 Alarie and co-workers [75] found that the pinched injection strategy when employed on a cross injector exhibited a bias towards neutral molecules. They found that the bias was contributed to during both the loading and dispensing steps and up to a 27 percent difference in injected volumes was recorded. The degree of bias observed is dependent on the ratio of electric field strengths applied during each step. Jin and Luo [77] plotted the potential distribution in the cross-piece during the loading and dispensing step. These are illustrated in Figure 4.20a and b respectively. During the loading step the potential gradient acts to drive the sample towards the sample waste vial while preventing it from leaking into the separation channel as is indicated in the plot in Figure 4.20a. During dispensing and separation the potential distribution shifts to permit the plug at the intersection to enter the separation channel and the excess sample is “pushed back” to the sample and sample waste vials (Figure 4.20b). Consequently, the ratio of field strengths in the separation and sample channels control both the confinement of the sample at the intersection and the volume of each species injected.

Additionally, when such an injection is performed in a PDMS device in the presence of an EOF, the strength of the flows from each arm will contribute to the confinement of the sample. Variable surface conditions on each arm of the cross-piece will complicate optimisation of injection protocols. Other authors have demonstrated similar biasing using other modes of electrokinetic injection [78, 79] and although some geometries alleviate the problem they do not completely eliminate it. The only formats of injection that do not display partiality based on sample characteristics are those based on pressure differences [80-82].

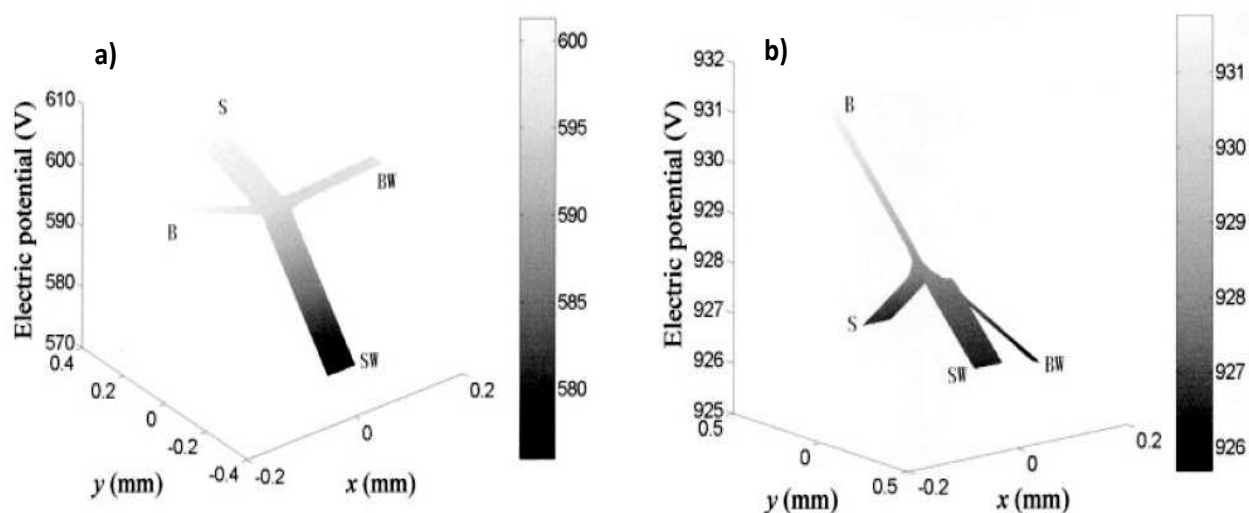


Figure 4.20: The calculated distribution of electric potential at the cross region during a pinched injection. (a) Potential distribution during loading. The voltages applied at sample (S), sample waste (SW), buffer (B) and buffer waste (BW) reservoirs were 990V, 0 V, 880 V and 1100 V respectively. (b) Potential difference during dispensing and separation. The voltages applied at sample (S), sample waste (SW), buffer (B) and buffer waste (BW) reservoirs were 880V, 880 V, 1100 V and 0 V respectively. Images reproduced from reference [77].

Under CGE conditions, the arms of the crosspiece injector are also filled with sieving matrix, dsDNA fragments are segregated during the injection process itself, as the larger fragments take longer to reach the intersection of the crosspiece. Therefore, depending on the injection time high mobility species are depleted from the sample reservoir or low mobility species do not enter the sample plug. Generally, the separation profiles obtained depend on the number of prior injections and may not reflect true analyte concentrations within the sample. The results obtained from the dsDNA ladder analysis on-chip reflect these observations, since the larger template strands were observed only occasionally. This is corroborated by the fact that the 1,800 bp fragment of the 25 bp ladder and peaks 12 and 13 of Roche Marker VIII were consistently underrepresented or

absent from the separation profiles obtained on-chip. Typically, it took several injections to equilibrate the sample at the intersection, as demonstrated in Figure 4.18b. After several injections with the advance and retreat of the DNA molecules, the pores of the matrix will stretch and provide sieving action to only the larger molecules. Consequently, lower peak areas were observed for the high molecular weight fragments in both ladders compared to the CE result. However, it should be noted that an electrokinetic bias is also observed using conventional CE, Figure in Chapter 5 compares the peak areas obtained using three injection techniques.

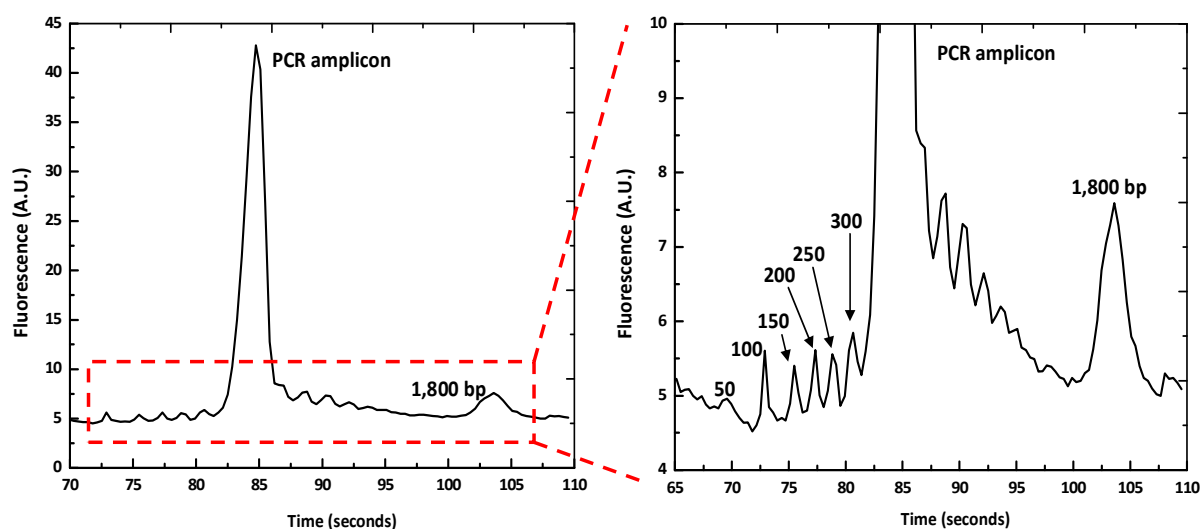


Figure 4.21: Electrophoretic separation of a 50 bp size ladder (Promega Corporation) spiked with 323 bp PCR amplicon on a PDMS microchip. Sample was prepared with x50 SYBR Green I concentration. (a) and (b) detail the migration of the amplicon fragment after the 300 bp of the dsDNA size ladder. The PCR amplicon co-elutes with several fragments, thus obscuring them. The microchip separation was performed in a 2.5% PEO (5MDa) in 0.1% TBE sieving matrix under an applied field of 183.3 V/cm.

Sizing of PCR amplicons using microchip electrophoresis would necessitate the separation of both amplicon and calibration ladder under identical conditions. As described earlier using the dual injector/channel device, surface chemistries in each channel even on the same device can differ significantly. Therefore molecular weight sizing of a PCR amplicon using a dsDNA ladder separated on another device or channel will lead to errors. To circumvent this problem PCR amplicon was added to the calibration ladder and analysed on the same device. The sample was prepared by diluting the 323 bp PCR amplicon, 50 bp dsDNA size ladder and SYBR Green I (x250) to one fifth their stock concentrations in x0.1 TBE. Sample was injected using the pinched mode and separated under an applied field of 183.3 V/cm in 2.5% PEO (5 MDa) in x0.1 TBE sieving matrix. The PCR amplicon migrates after the 300 bp fragment obscuring several bands of

the calibration ladder due to its high concentration (Figure 4.21). Further dilution of the PCR sample prior to analysis should result in a narrower peak, allowing calibration of the sample. Therefore the separation and sizing of PCR amplicons is achievable on chip-based devices, however quantification of the amplicon can be problematic due to the errors induced by the pinched injection strategy and the intercalating fluorescent label employed.

The resolution achieved between sample components when using gel electrophoresis is contingent on a number of factors, including sieving matrix concentration and pore size, initial injection volume, temperature, separation length, applied field and molecular diffusion. The resolution (R) between sample components can be determined using the formula $R = \Delta X / [1/2 (W_1 + W_2)]$. Where ΔX is the distance between the centroid of the two peaks and W is the width of the peak at half height. Therefore, one way to improve resolution is to reduce peak width. The width of the band in the presence of a sieving matrix is controlled by four basic factors; diffusion, temperature, detection volume and injection volume (Chapter 1). In chip-based CE streamlining the sample plug using a pinched injection or a six-port injector reduces the volume of the injection improving the resolution. However, this approach soon reaches its limitation with detector sensitivity and trace sample analysis.

An alternative approach to improving resolution is to increase peak spacing by increasing the velocity difference between peaks or by increasing the distance travelled by each fragment. Increasing the velocity is dependent on the separation mechanism employed – free solution electrophoresis or sieving electrophoresis and the physical characteristics of the analyte – its charge, size, shape and orientation. The sieving matrices described here vary in viscosity and pore size enabling separation over a wide range of dsDNA fragment sizes. However, manipulating the selectivity of the sieving media will only improve resolution up to a certain extent. Further increases can only be realised by optimising other separation parameters including electric field strength (E) and separation length (L). Recently, Ni *et al* [83] proposed a mathematical expression for resolution (R_s) using E and L . When plotted the expression yields a curved surface dependent on the values of E and L (Figure 4.22). All values of E and L standing on the same curve will produce the same resolution for a given analyte. The plot obtained indicates increasing the applied field indefinitely will not increase the resolution and that for field strengths between 50 and 300 V/cm increasing the length of the separation channel will increase resolution. Consequently, once all the

parameters controlling performance on a chip-based device have been optimised further improvement in resolution can only be achieved by increasing the channel length.

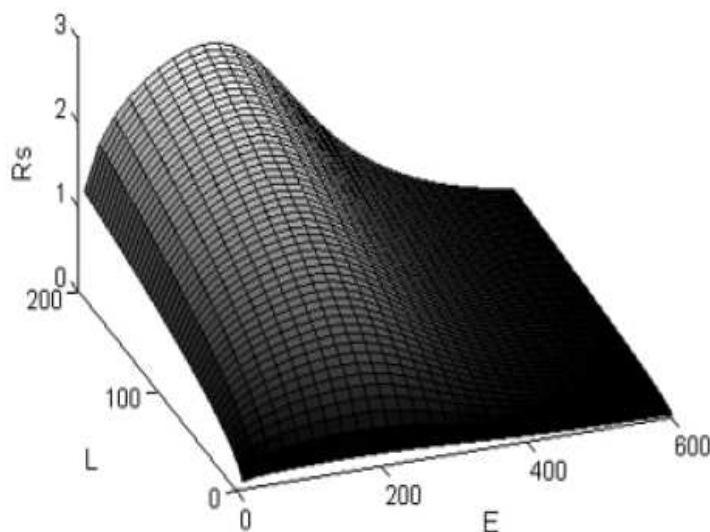


Figure 4.22: A three dimensional plot describing the effect of separation length (in centimetres) and electric field (in Volts/centimetre) on resolution. Image reproduced from reference [83].

Increasing the distance travelled to improve resolution is one of the simplest solutions, although it increases analysis times. Longer channels also increase the foot-print of the device; authors have tried to circumvent this problem by inserting turns into the channel geometry. Unfortunately, these bends can induce band broadening due to the ‘race-track’ effect reducing the resolution obtained. Paegel and co-workers [84] and Griffiths and Nilson [85] have proposed alterations to the geometry of the turn to alleviate this effect. Additionally, the longer channels are harder to fill with sieving matrix. The increased surface area on a PMDS device would require careful conditioning to reduce differences in surface chemistry throughout the separation length. Furthermore the most commonly used imaging modality is fluorescent detection, which can be integrated with most device substrates. In fact, the first microfabricated capillary array electrophoresis device which was demonstrated in 1997 by Woolley *et al.* [86] employed a Laser induced fluorescence (LIF) based detector. Fluorescence-based methods enjoy much popularity due to their high sensitivity and low detection limits. However, the use of extrinsic labels has been shown to affect the overall charge to mass ratio of the DNA molecule and thus they need to be used with care. Label-free detection methods, based on intrinsic UV absorption, are attractive since nucleotides absorb sufficiently at 260 nm due to the constituent aromatic rings of both purine and pyrimidine bases [87]. As PDMS and silicon are not transparent below 280 nm and

fabrication of microdevices in glass is tedious and time-consuming, a resolution to some of the issues discussed above would be to use a fused silica capillary instead of a microchannel. A hybrid cross injector chip with capillary separation channel was designed. Using this device the sample plug was generated using the pinch injection strategy and then separated in the capillary. The device uses the same fabrication procedure as standard PDMS devices with the added advantage of not specifying a separation length. The separation length is defined by the length of capillary attached to the device and can be altered depending on the application

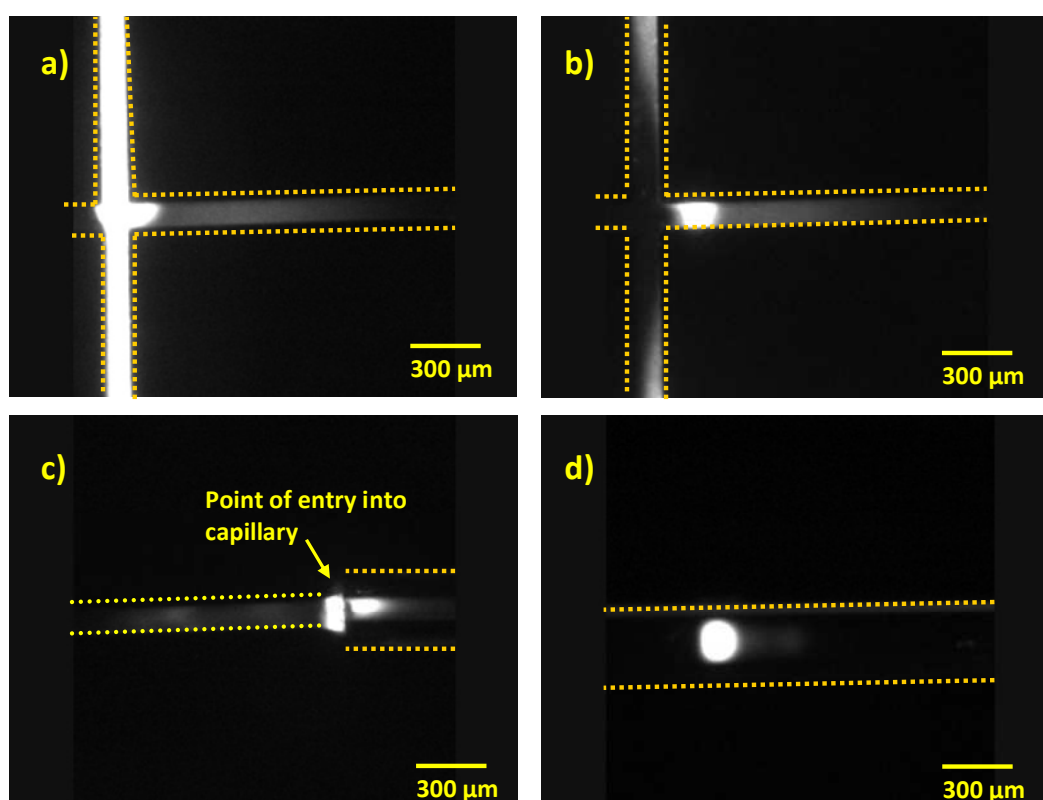


Figure 4.23: PDMS microchip with inserted bare fused silica capillary of 100 μm internal diameter and 375 μm outer diameter. (a) and (b) illustrate the pinched injection of a 100 $\mu\text{g}/\text{ml}$ FITC solution using a cross piece injector. Post injection the sample plug migrates down the separation channel and into the inserted capillary as shown in (c). (d) The sample band migrating within the capillary outside the PDMS device.

Figure 4.23a, b, c and d illustrates the injection of a sample plug from a cross piece injector into a capillary inserted into the PDMS microdevice. The separation channel was modified to include a wider section (300 μm) starting 0.5 cm from the point of injection. A 100 μm internal diameter capillary was chosen to match the width of the separation channel. A detection window was burnt into the capillary, following which it was conditioned and filled with buffer analogous to the

procedure followed with a CE instrument. The short section of channels forming the cross piece injector and separation channel was also loaded with sieving matrix following which the capillary was inserted into the PDMS device. The outlet end of the capillary was placed in a buffer reservoir and a conductivity check was performed to insure an electrical connection between the buffer in the capillary and the PDMS device. A 100 $\mu\text{g}/\text{ml}$ solution of FITC was used to test this set up. The injection and jumping of the separated bands into the capillary is illustrated in Figure 4.23.

Replacement of the microchannel with a capillary permits the use of any separation length without design modification and re-fabrication. The capillary surface chemistry is well understood allowing existing CE protocols to be transferred with minimal optimisation. The capillaries can be pre-loaded with buffer and then inserted permitting the use of high viscosity buffer typically used for high resolution separations in CE. All detection formats currently in use on CE instrumentation can be applied to this set up, including UV detection at 254 nm and 214 nm wavelengths. Conversely, PDMS is only transparent down to 280 nm and does not allow detection at lower wavelengths. Additionally, the cross piece can now be filled with a low conductivity gel free buffer enabling sample stacking at the channel-capillary interface. Nevertheless, a crosspiece injector will only allow single sample analysis thus reducing throughput. The following chapter describes a novel droplet based injection format employing the capillary interface described above. This will enable multiple sample analysis on a chip-based capillary electrophoresis device.

4.5 Conclusion

The performance of an electrophoresis system is defined by its efficiency, sensitivity and resolution. The highest performance can be achieved by optimisation of the parameters which control the efficiency, sensitivity and resolution of the system. In the case of a gel electrophoresis system these include the sieving matrix, separation length, applied field, initial sample volume, temperature and detection modality employed. Herein we have shown that a low viscosity, low molecular weight solution of PEO may be used in this respect to separate dsDNA fragments. The described PEO-based sieving matrix is UV transparent, has a low viscosity (allowing facile introduction into the capillary) and allows separation of a wide range of fragment sizes with sufficient resolution to enable mass calibration. The matrix is stable over a wide temperature range and allows accurate sizing of fragments. Aside from the low viscosity matrix a high molecular weight based PEO

sieving matrix was also demonstrated. The separation protocols were successfully transferred on to a chip-based device employing sieving matrices with longer polymer lengths. A 2.5% PEO (5 MDa) in x0.1 TBE sieving matrix was developed to separate 25bp and 50bp dsDNA size ladders on-chip. Increasing polymer length and viscosity using glycerol improved the resolution between fragments. Compared to capillary electrophoresis instruments microchip CE devices have shorter separation lengths, are harder to fill with high viscosity buffers due to de-lamination, have unusual surface chemistries and are not transparent below 280 nm limiting the optical detection formats to fluorescence based methods

In addition, the effect of SYBR Green I intercalating dye on the electrophoresis mobility of DNA fragments was investigated. It was established that the fluorescent label effect dsDNA mobility in a concentration dependent manner. Consequently, quantification and sizing of a sample of unknown concentration will lead to errors. Furthermore, treating the calibration ladder to a similar quantity of SYBR Green I will not alleviate the effect. The effect was substantiated using a 323 bp PCR amplicon which was sized at 550 bp in the presence of 100 µg/ml SYBR Green I. The sizing was performed using an unlabelled calibration dsDNA ladder.

A hybrid device connecting a chip-based cross injector to capillary separation channel was also demonstrated. Replacement of the microchannel with a capillary has several advantages including the use of any separation length without design modification and re-fabrication. Additionally, existing CE protocols can be translated to the hybrid device with minimal optimisation. Furthermore, from literature, the injection geometry allows greater control over the sample volume injected compared to conventional CE injection strategies. The capillary interface was used as part novel droplet based injection format described in the following chapter. This interface device has enabled multiple sample analysis using a single capillary and single point of injection with cross contamination between samples.

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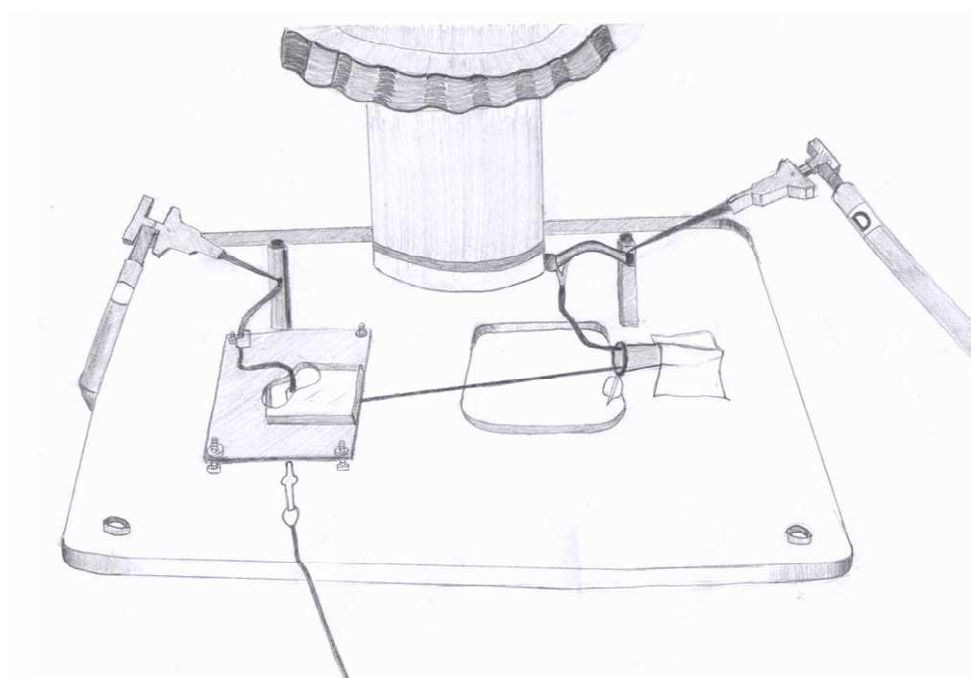
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Chapter V: Interfacing Micro-droplets to Continuous Flow Microchip Electrophoresis



Synopsis

Traditional on-chip electrokinetic injection is performed employing cross-piece geometry. This format is fraught with problems as detailed in Chapter 4. A novel droplet to CE interface has been described herein to circumvent these issues and make injection a controlled and reproducible function. The micro-device which acts as the interface between segmented and continuous flows employs a hydrophobic, oleophilic material to remove the oil carrier phase at the intersection of the two flows. Accordingly, the oil is absorbed passively into the membrane while the aqueous droplets emerge and enter the separation channel. The interface permits the segmented and continuous flows it is connecting to function independently of each other, permitting connection when both systems have stabilised. Injection volumes can be controlled by the size of droplet and the distance between the droplet delivery tubing and the separation channel. It is demonstrated here that employing a 200 μm I.D. droplet delivery tubing and a flow rate of 50 $\mu\text{m}/\text{second}$, a droplet with length between 200 - 500 μm can jump whole into the separation channel.

Both CZE and CGE separation modes are successfully demonstrated, proving the potential of the interface to be used in a wide range of applications such as small molecule separations, proteomics, genomics, metabolomics and other chemical and biological assays. Here the interface was assessed using fluorescent dyes and dsDNA. Fluorescein iso-thiocyanate and Eosin Y solutions at final concentrations of 100 μM and 20 μM respectively were analysed using CZE and both components were resolved. Moreover, the time interval between droplet injection and separation was maintained during the analysis. The variation in mobility values for Eosin Y and FITC over seven consecutive injections was determined as 7.9% and 8.9% respectively. A mixture of FITC and Eosin Y was also analysed using CGE and reproducibly yielded seven peaks of which at least six peaks can be confirmed to be part of the FITC electrophoretic profile. Two dsDNA molecular weight standards, Marker VIII (Hoffman La Roche) and 50bp step ladder (Promega Corporation), were used to assess the performance of the droplet interface device with the capillary separation channel using the 2.5% PEO (5 MDa) in x0.1 TBE sieving matrix. Separation of component bands was achieved in both cases along with multiple injection and separation of 50bp ladder. The log-log plots of mobility versus fragment size for each sample showed the expected sigmoidal shaped. However, analysis of the multiple injection results indicated a 2-10% variation in the mobility values between fragments. Larger variation

was observed for the smaller fragments, this can be attributed to stretching of matrix pores by the larger fragments of a prior injection. It is important to note that the droplet interface allows injection and separation of consecutive droplets without interrupting the separation voltage, this circumvents the traditional problems associated with this type of process, such as band broadening and sample degradation due to excessive UV exposure.

Another advantage of the interface is its ability to perform high throughput injection of controlled volumes with no bias, permitting CE separations to be used as a tool in quantitative analysis. To demonstrate this droplets were pre-generated with fluorescein concentration decreasing from 100 to 40 μM while that of Eosin increasing from 20 to 80 μM , both in 20 μM steps and analysed using CGE. Linear fitting of the curves generated by each dye showed R^2 values above 0.9 in both cases, indicating a good fit. Consequently, signal obtained from each droplet accurately reflects its concentration, permitting the generation calibration curves for quantification of samples with unknown concentration. In addition, the interface can be set up to allow injection to conventional CE instrumentation. Such a system not only has the potential to handle hundreds of samples with minimal user intervention but current protocols can be easily transferred with minimal optimisation.

5.1 Introduction

Electrophoresis is one of the most powerful tools in separation science. Capillary and microchip electrophoresis (CE/MCE) methods have been developed to provide automated analysis in a broad range of applications, such as DNA [1-3], protein [4-6], small molecule [7, 8], enzyme assay [9] and cell [10, 11] analysis. Benefits including lower sample and reagent volumes, facile automation and easy coupling with other analytical techniques, improved efficiency and high analytical throughput are inherent to these formats of electrophoresis [12-15]. While there have been extensive studies on separation conditions [16], surface chemistries and surface modifications [17-19], methods currently in use for sample injection and post-separation analyte fractionation and collection vary little from the original formats. The primary injection methods for CE and MCE are hydrodynamic and electrokinetic [20-23]. During hydrodynamic injection, a defined plug is driven into the capillary or crosspiece intersection using a pressure difference. In electrokinetic injection mode, the sample is driven into the capillary under an applied field. As demonstrated in Chapter 4 injections carried out under an applied field are typically biased towards smaller high mobility molecules [24]. Consequently, the separation profile obtained can misrepresent the sample content. Conversely chip-based pressure injections require external pumps and fabrication of on-chip valves. Hydrodynamic injections employed on CE instruments are generally more reproducible and permit accurate quantification. However, they require precise pressure control. Consequently, there is a need for alternative injection mechanisms that are simple to implement and enable reproducible delivery of specific sample volumes without bias.

Droplet microfluidics has been the subject of much attention over the past five years [25-28]. Here femtolitre to nanolitre volume droplets of one phase are encapsulated in a continuous immiscible phase [25]. Such small confined volumes overcome problems associated with diffusion in larger volumes and provide ideal reactors for molecules, cells [29, 30], and tissue [31-33]. Crucially, such encapsulation can be performed in a digital and high throughput manner [34]. Therefore, of late there has been a growing interest in coupling electrophoretic separation with droplet microfluidics, specifically for sample injection and post separation fraction collection. One of the first attempts to controllably introduce the contents of a droplet into a CE separation channel was described by Edgar *et al.* [35] in 2006. The authors used a mixture of FITC labelled amino acids to assess the device detailed in Figure 5.1b. The device consisted of a droplet generation region and a separation channel which were separated by an immiscible partition at the point of injection (Figure 5.1a).

Droplet injection was achieved by breaking and resealing the boundary. Although separation of droplet components was achieved the authors highlighted several disadvantages including precise pressure control, selective channel pre-treatment to generate hydrophobic and hydrophilic surfaces and oil contamination that results in a loss of electroosmotic flow (EOF).

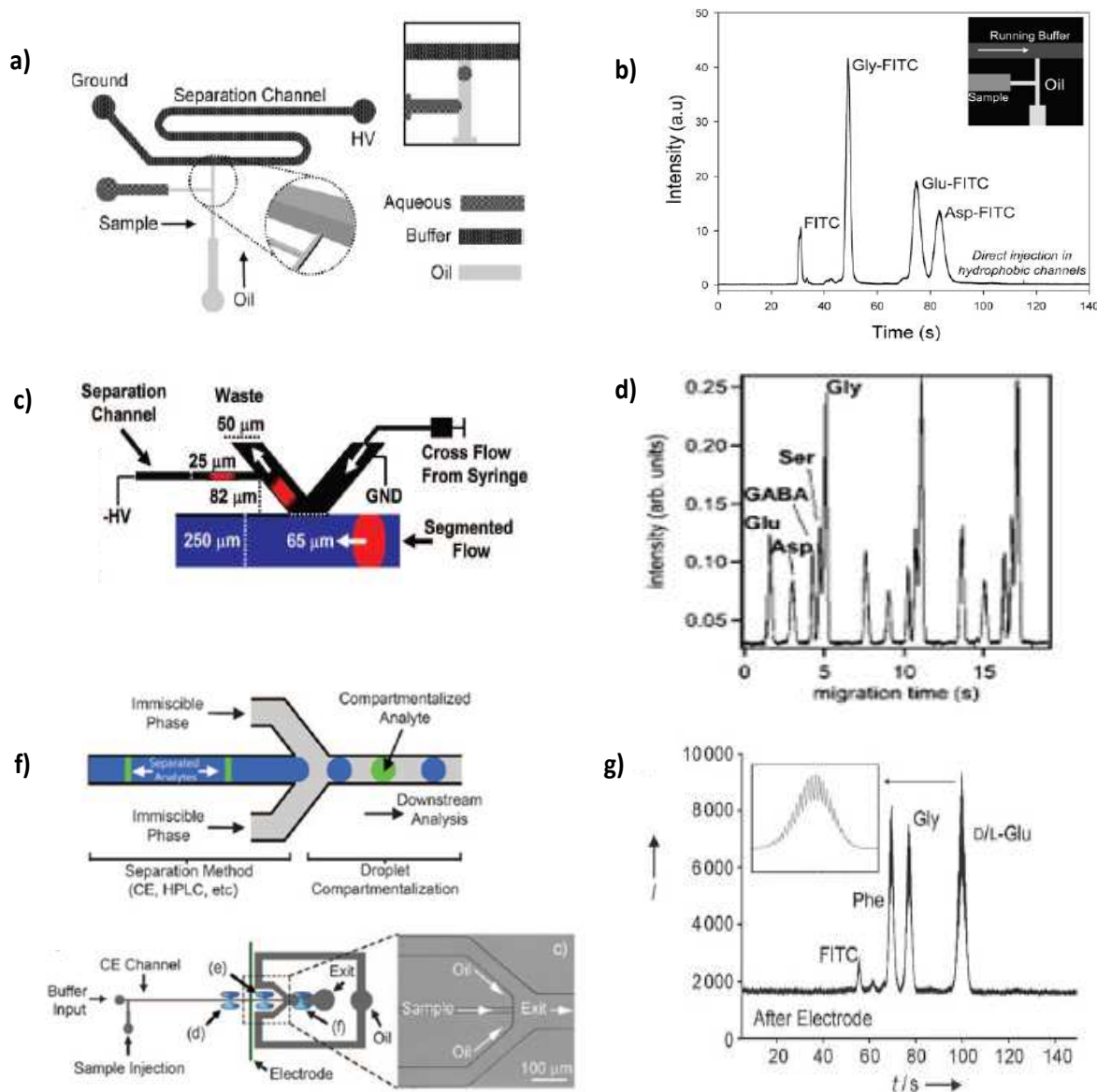


Figure 5.1: Droplet interfaces to CE separation microdevices. (a) Droplet interface for sample injection reproduced from reference [35]. The interface consisted of a droplet generation region and a separation channel which were separated by an immiscible partition at the point of injection. (c) K-shaped droplet interface for sample injection reproduced from reference [36]. The device consisted of three regions segmented flow channel, a V-shaped cross-flow channel and a separation channel. (e) EOF driven droplet interface for analyte fractionation reproduced from reference [38]. The interface consisted of a sample injection region, a separation channel and a droplet-generation region. (b), (d) and (f) A mixture of FITC labelled amino acids separated on the devices shown in panels (a), (c) and (e) respectively. The inset in (f) shows the droplet-formation induced band oscillation on the D/L-Glu peak.

In 2008 Roman *et al.* [36] described a microfluidic device employing a K-shaped element to transfer sample from segmented flows to an electrophoresis channel. Figure 5.1c shows a schematic of the device which consisted of a 250 μm wide segmented flow channel, a V-shaped cross-flow channel and a separation channel. The cross flow channel served two functions; it counterbalances the pressure exerted by the segmented flow channel at the K-shaped interface and it washes excess sample from the mouth of the separation channel effectively terminating the injection. The presence of oil at the K-shaped interface leads to the formation of a virtual wall which disappears when an aqueous droplet occupies the space, causing an injection into the cross-flow channel. The sample is then moved to the mouth of the separation channel and a plug is injected using an applied field. Using this device the authors demonstrated the serial injection and free solution separation of a mixture of amino-acids (Figure 5.1d). However, this format of droplet injection incurs sample wastage as only a small portion of the droplet is injected. In addition, the sample is further diluted in the cross-flow channel and the injection volume is difficult to control as it depends on several factors. Furthermore, the operation of such a device would require exquisite control over the pressure balance. The following year Niu *et al.* [37] also demonstrated a droplet interface which was used to connect nano-liquid chromatography (nano-LC) to a CE instrument. The interface employed a pillar array situated at the intersection of the droplet delivery channel and the separation channel was used to actively extract the oil phase. This enabled injection of the whole droplet into the separation channel improving on the device described by Roman *et al.* [36]. However this device also required precise pressure control making it difficult to operate and obtain reproducible results.

In 2009 Edgar *et al.* [38] demonstrated a novel device using droplets to spatially confine sample components separated during electrophoresis. Figure 5.1e shows a schematic of the device which consisted of three regions, a sample injection region, a separation channel and a droplet-generation region. The EOF generated in the separation channel was used to drive the aqueous phase to bud off into the continuous phase at the modified T-junction, thus inducing droplet formation. The authors estimated the minimum absolute pressure generated by EOF to be 11 kPa, which they described as sufficient to generate droplets at kHz frequencies. Detection was carried out at three points on the separation channel to assess the effect of the droplet-generation interface on the CZE separation. Although all sample components were resolved, each peak showed increased width and on closer inspection were found contain oscillations (Figure 5.1f). The frequency of the band oscillation was identical to the frequency of droplet

formation. In addition, the effect grew more pronounced further down the separation channel and closer to the droplet-formation interface. Although Edgar *et al.* [38] demonstrated an MCE to segmented flow interface device, in operation the results presented indicate a loss in efficiency of the interfaced separation. Similar approaches have also been used in droplet interfaced mass spectrometry. Droplet interfacing has opened new paradigms for chemical analysis of small and ultra small volume droplets which will have profound importance in proteomics, genomics, metabolomics and disease diagnoses once a robust interface is demonstrated.

Droplet interfaced separations require the ability to address single droplets with efficient oil removal. Oil removal is crucial to establish a stable connection between the discrete droplets and continuous microflows, especially for system automation and parallelization. Current approaches still suffer from complicated local surface treatment of channels, complex chip design and delicate pressure control. Furthermore current approaches have only demonstrated droplet interfacing to separation channels using free solution electrophoresis. The use of capillary gel electrophoresis, more relevant to fields of proteomics and genomics, introduces more challenges to the design of such an interface. Moreover the samples used in literature were simple mixtures of amino acids. Therefore, an efficient and simple design is required to allow the widespread adoption of this approach, specifically one which would facilitate the analysis of relevant samples.

The work presented here describes a droplet-to-CE interface employing a hydrophobic but oleophilic membrane surface to effect oil removal. This surface is located at the intersection of the droplet delivery tubing and separation channel. At the interface the oil phase is absorbed passively into the membrane and supporting tissue, while the aqueous droplets emerge and enter the separation channel easily. This design decouples the droplet generation region from the separation channel, permitting interfacing when both systems have stabilised. Such an approach is demonstrated employing chip-based zone electrophoresis and gel electrophoresis, to achieve high throughput and quantitative analysis of small volume samples. The interface may also be applied as a droplet interface to conventional CE instrumentation.

5.2 Oil Absorption

One of the critical functions of a segmented to continuous flow interface is efficient droplet extraction or oil removal. In 2004 Feng *et al.* [39] reported a novel material which was both super-hydrophobic and super-oleophilic. The authors demonstrated the characteristics of the film by separating diesel oil from water; the water remained on the film while the oil was absorbed and passed through as shown in Figure 5.2a.

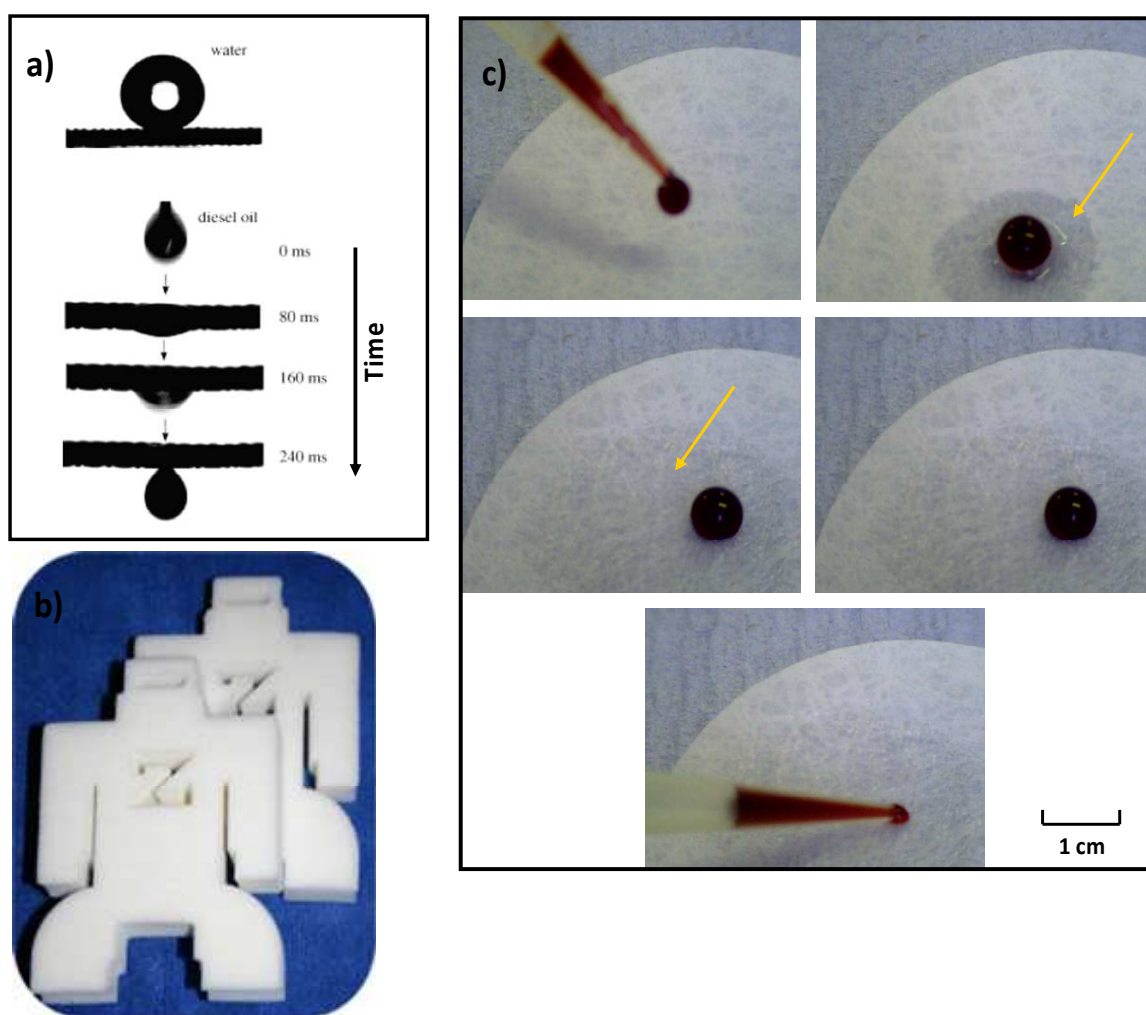


Figure 5.2: Oil absorbing materials examined for use in the droplet-MCE interface and LC-MALDI interface device (Chapter 6). (a) A super-hydrophobic and super-oleophilic mesh film developed for the separation of oil from water. Image reproduced from reference [39]. (b) Zorboid foam®, used in swimming pools as a scum remover; this is an oil absorbing hydrophobic foam. (c) The oil absorbing capability of the PTFE membrane employed in this work. An aqueous solution of red dye mixed with FC-40 oil is pipetted on to the film; the oil is absorbed by the film following which the aqueous droplet is pipetted off.

They demonstrated that on this material water had a contact angle of 150° while diesel oil presented a 0° angle. This film was prepared by coating a stainless-steel mesh with an emulsion containing fluorine. In the current studies several similar commercially available materials were trialled. These included a spa scum remover (Zorboid Foam©, Figure 5.2b) and a PTFE film (Whatman T-38, VWR, UK) used to filter acids. Both materials were super-hydrophobic and super-oleophilic. Preliminary studies performed with the Zorboid foam indicated that it could separate oil from aqueous droplets. However, the porous uneven surface of the foam posed a practical problem by preventing a stable connection between the droplet delivery tubing and the separation channel at their intersection. Consequently redesign of the droplet – CE interface necessitated confinement of the foam which altered the surface volume ratio and reduced the level of oil uptake.

In contrast, the PTFE film manufactured by Whatman T-38 (VWR, UK) is a thin film on a PTFE fibre support. The top surface of the film is flat, hydrophobic and oleophilic. To demonstrate its surface chemistry a mix of FC-40 oil and an aqueous solution of red food dye was applied to the film (Figure 5.2c). The oil is absorbed by the film leaving the dye loaded aqueous droplet on its surface. This droplet can be removed from the film with minimal sample loss and is oil free. To demonstrate the efficiency of oil removal PCR samples prepared with mineral oil were applied to the film post amplification, following which the sample was desalted using a desalting column (Pierce, UK) and analysed using CE. The electrophoretic results compared well with samples prepared using the standard oil removal procedures (data not shown). In fact using the film involved fewer and less tedious steps than the traditional method of centrifugation and oil extraction using a pipette.

This film suited the droplet interface assembly as it was flat and non-deformable under an applied pressure. Therefore it was used for all experiments detailed in this chapter. In operation, 2 or 3 layers of fibre free tissue were placed beneath the film to increase its oil absorbing capacity. The oil absorbed at the top surface of the PTFE film is transferred to the tissue below the film, increasing its net oil absorption capacity. The modified film's capacity for oil absorption was studied using a piece approximately 2 cm in diameter and 1 mm thick (including tissue). More than 200 μL of FC-40 can be completely absorbed. Such a volume of oil can carry 20 μL of aqueous phase in droplet format, with an oil/sample occupancy ratio of 10:1 in the tubing. Consequently, before a new piece of material is required, more than 10,000 sample droplets can

be injected; far more than needed in a typical CE analysis. Such an oil absorption ability is also crucial for the large scale integration of parallel separations.

5.3. Interface design

An ideal interface permits the techniques it is connecting to function independently of each other. This was a key premise behind the design of the segmented to continuous-flow-interface. Schematics of the interfaces developed are detailed in Figure 5.3. The main features include a separation channel, droplet delivery tubing and a PTFE membrane used for oil removal. The droplet delivery tubing transports droplets from their source to the separation channel. The PTFE oleophilic membrane placed at the intersection between the delivery tubing and the separation channel absorbs the carrier phase. Most of the devices used in this study were single channel devices. The separation channel was ‘closed’ over its entire length except at the intersection with the delivery tubing. At this point the channel was defined by the three walls formed as part of the PDMS mold. Its underside remained open to air providing a direct connection between the mouth of the droplet delivery tubing and the separation channel (Figure 5.3c). Droplets enter the channel at this point and transit into the separation channel under an applied field. The polarity of the applied electric field depends on the mode of capillary electrophoresis. Capillary zone electrophoresis was carried out in the direction of the EOF and the inlet reservoir was held at a positive voltage. Sieving matrix separations were performed in the reverse direction to EOF and the inlet reservoir was held at a negative voltage. It should be noted that the PDMS separation channel can be replaced with a fused silica capillary (Figure 5.3b). Consequently, this device may also be applied as a droplet interface to capillary electrophoresis instruments.

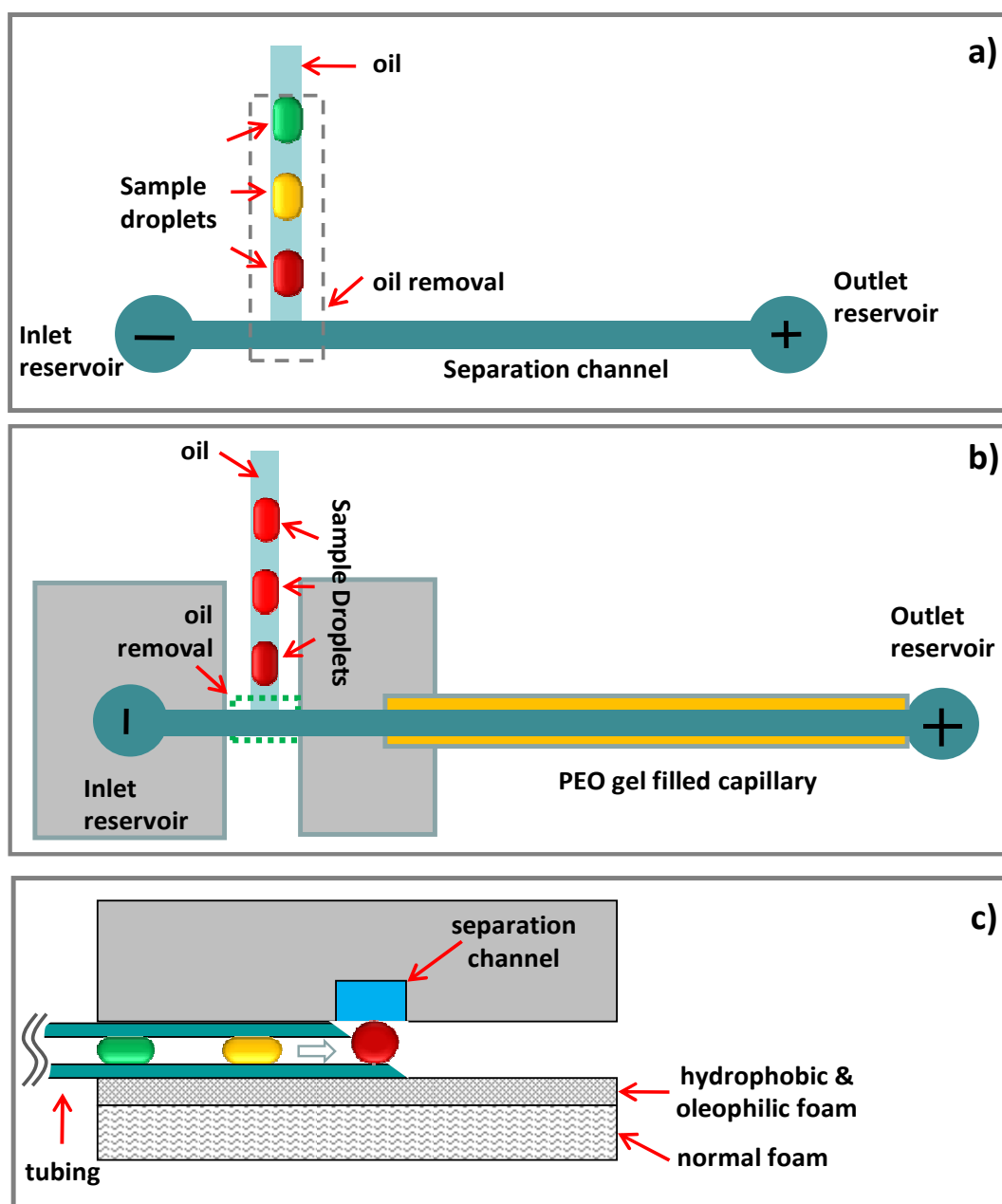


Figure 5.3: Schematics of droplet microchip interface devices. (a) and (b) detail the top view of the interface with chip-based and capillary based separation channel respectively (c) The side-on view of the droplet interface device at the intersection of the droplet delivery tubing and the separation channel. The aqueous droplets carried by the oil are delivered to the intersection between the open electrophoresis microchannel and the droplet delivery tubing. The oil flows out of the tubing and is absorbed by the PTFE membrane. The aqueous droplets arrive at the intersection and jump into the hydrophilic separation channel.

5.4 Experimental Set-up

5.4.1 Sample and Sieving Matrix Preparation

Rinse solution reagents (hydrochloric acid and x1 solution of TBE) and Polyvinyl pyrrolidone (PVP, $M_n \sim 360,000$) were purchased from Sigma-Aldrich (Poole, UK). Poly(ethylene) oxide (PEO, $M_n \sim 5,000,000$) was obtained from Avocado Research Chemicals (Heysham, UK). Fluorescent dyes Eosin Y, Fluorescein and Fluorescein iso-thiocyanate (FITC) were obtained from Sigma-Aldrich (Poole, UK), while dsDNA fluorescent label SYBR Green I was obtained from Invitrogen (Paisley, UK) at a x10,000 concentration (or 10 mg/ml, see Chapter 4). Two double stranded DNA (dsDNA) ladders were employed to assess the interface device. A 50 bp dsDNA step ladder was obtained from Promega Corporation (Madison, USA) and Marker VIII (19- 1114bp) was obtained from Hoffman La Roche Ltd. (Basel, Switzerland). Both samples were supplied in a 10mM Tris-HCL, pH 7.5, 1 mM EDTA solution. A stock of SYBR Green I (x500) was prepared in deionised water. The 50 bp step ladder and Marker VIII were diluted to one fifth of their stock concentrations in x0.1 TBE and labelled with SYBR Green I at a final concentration of x50. Fluorescein, FITC and Eosin Y were prepared at a stock concentration of 1.5, 1.8 and 6 mg/ml respectively in water. The samples were further diluted 1000 times in x0.1 TBE prior to droplet generation.

Background electrolyte, x1 TBE, was diluted to one tenth of its stock concentration in deionised water prior to use in MCE separations. The free solution separations of fluorescent dyes were carried out in this diluted buffer (x0.1 TBE). Molecular weight assessment of dsDNA ladder was achieved using the 2.5% PEO (5 MDa) matrix described in Chapter 4. The sieving matrix was prepared by dissolving 2.5% (w/v) PEO (5 MDa) in x0.1 TBE and stirring for 24 hours at 25°C until the solution is homogeneous. The polymer solution was filtered using a 5µm polyethylsulfone (PES) syringe filter (VWR, UK) and degassed prior to use. A 10% solution of PVP was prepared in deionised water and employed as a pre-coating agent.

5.4.2 Droplet Generation

Microdroplets were prepared using two generation formats. The first used PDMS devices with a T-junction design [26, 40]. These devices had channels 150 μm high and an outlet channel 300 μm wide. PTFE tubing of outer diameter 375 μm was inserted into the outlet channel and sealed with PDMS. PDMS sealing was carried out at 150°C and provided a water tight seal. The droplets once formed, flowed to the outlet and into the PTFE tubing. The PTFE tubing was cut to a total length of between 15 and 20 cm and as illustrated in Figure 5.2 the free end was used as the droplet delivery tubing.

Droplets were also generated using an automated droplet generation device (Droptech, London, UK). This instrument was used to generate droplets from multiple samples. The aqueous sample solution is placed above the carrier oil phase and one end of a PTFE tubing is moved between the two phases. The other end is connected to a syringe which is used to draw both phases in succession into the tubing, generating aqueous droplets in oil. A constant suction was provided by a syringe pump operating (Harvard Apparatus, PHD Programmable 2000) in refill mode. The size and frequency of the droplets is controlled by the ratio of time spent in each phase and suction force applied. The droplet generation rate was of particular importance when performing separations employing sieving matrices, since the presence of gel slows the motion of the analyte through the separation channel. Analyte bands from separate injections would co-elute if the droplets were too close to one another. When the required number of droplets was generated the inlet end was cleaned and cut at a 30° angle, ready to be situated at the interface. Of the two droplet generation formats, the automated version proved more successful. When using the traditional T-junction format of droplet production, the rate of droplet formation could not be absolutely controlled. Stable droplet generation was only achieved above 2-3 $\mu\text{l}/\text{minute}$ oil flow rate. Although droplets generated at this rate merged with the continuous flow, separation of their components was not achieved. Even at high field strengths (500 V/cm), the content of each droplet could not be moved into the separation channel before the next droplet arrived. Using the automated format, the droplets stored in the tubing could be delivered at the desired rate by controlling the applied flow rate. In addition, droplet formation is independent of the interface, permitting storage and incubation of droplets if required, and multiple sets of tubing to be prepared and analysed when ready. Furthermore, droplets can be generated from multiple

samples without cross contamination, which is not easily achievable using the traditional T-junction device.

5.4.3 Device Assembly

There are three basic parts forming the complete device; the electrophoretic separation channel, the droplet generation device and the PTFE tubing and membrane connecting them. The separation channel was fabricated in PDMS using standard photolithographical techniques detailed in Chapter 2. The separation channel was 6 cm long and 100 μm wide. It had two buffer reservoirs at either end. Initially two pieces of glass (zero thickness, Borosilicate glass, VWR) were used to seal the channels (Figure 5.4). However, these devices were difficult to assemble and fragile to handle during use. In addition the glass was coated with Duxback (PPG Industries, Pittsburgh, PA), to render the edges near the open channel hydrophobic. Duxback is a widely used commercially available treatment for glass in the automotive industry. Windscreens are treated with this solution to render them hydrophobic. The composition of this product and its method of action is undisclosed.

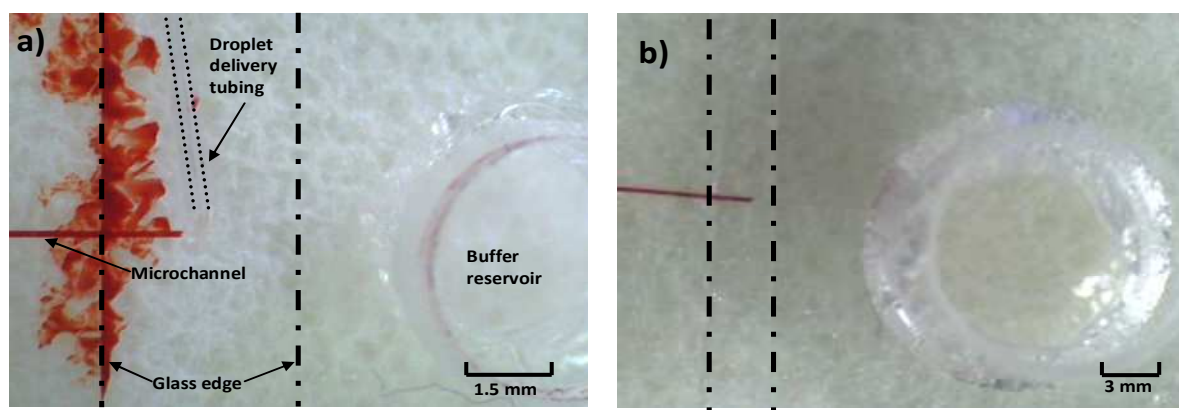


Figure 5.4: The effect of Duxback on the buffer loading process in the glass bottom droplet to CE interface device. Red food dye was added to the buffer to enable visual monitoring of the loading process. The edge of the glass slides and the droplet delivery tubing are highlighted with the aid of dotted lines in both images. (a) In the absence of Duxback the buffer leaks into the separation channel. (b) The buffer moves directly into the separation channel when the edges of the slides are coated with Duxback.

In the absence of this coating the inner edges of the glass would remain hydrophilic and buffer loading would not occur efficiently. In Figure 5.4 red food dye was used to demonstrate the problem. Dye loaded at the outlet reservoir travels down the separation channel under capillary

action. When it arrives at the open section of channel, if the glass is not treated with Duxback, the dye will run along the edge of the glass as shown in Figure 5.4a. The dye will continue to leak into the open region and under the device until the outlet reservoir is depleted. If the edge is coated with Duxback the dye will run into the microchannel (Figure 5.4b) and into the inlet reservoir without leaking into the surrounding device. PDMS, which is inherently hydrophobic, did not have to be treated in this way, reducing the number of assembly steps. Accordingly, glass was replaced with PDMS.

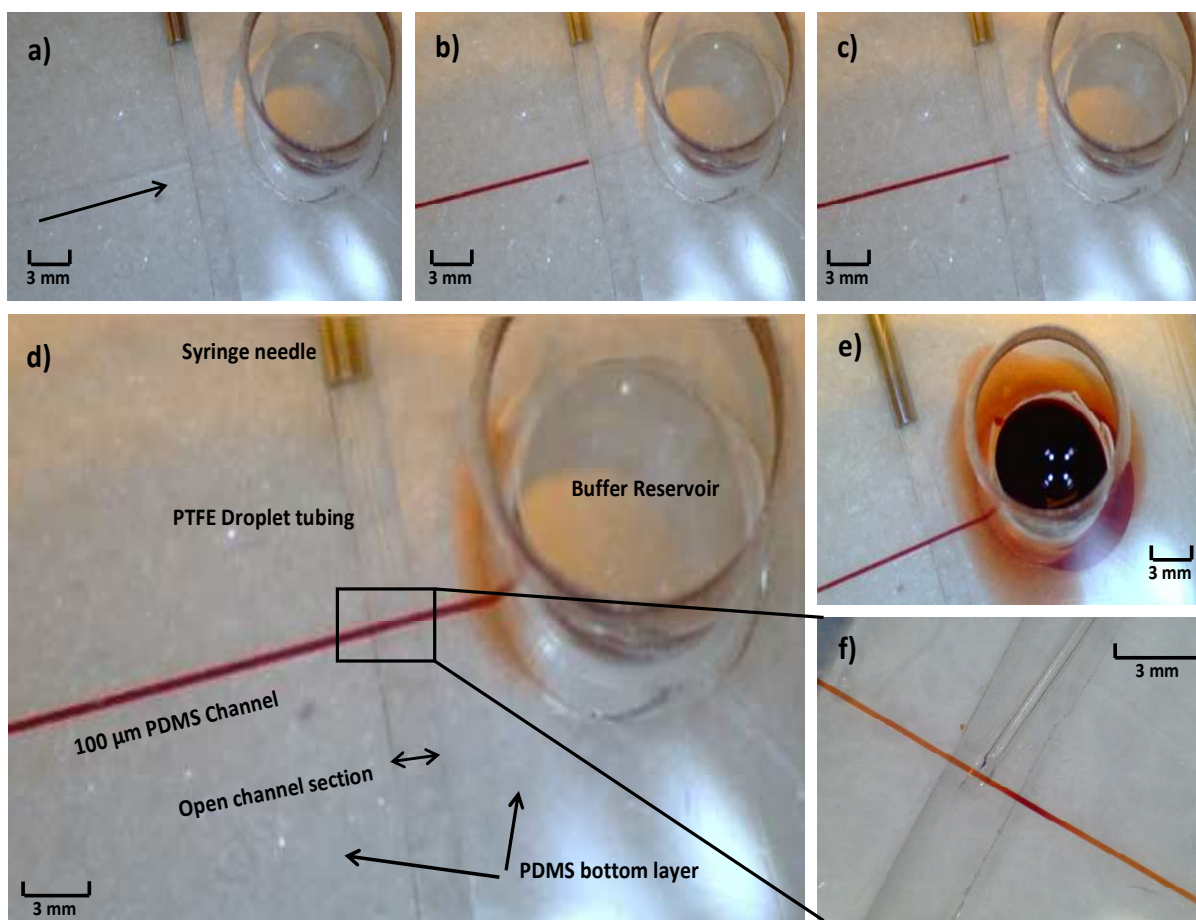


Figure 5.5: Loading of CZE running buffer into the droplet to CE interface. The device has two PDMS bottom layers, a single separation channel ($100\ \mu\text{m} \times 100\ \mu\text{m}$) top layer, with a 3 mm gap at the interface. The gap is situated near the inlet buffer reservoir and enables the droplet delivery tubing to access the separation channel. (a) The open section of the separation channel prior to buffer loading. A red dye is used to monitor buffer loading, and the buffer is loaded from the outlet end of the separation channel. (b) and (c) show the buffer approaching and crossing of the open section of the separation channel. (d) An expanded view of the set up, with the separation channel now completely filled with red dye. All components of the device are labelled. (f) Provides a clear view of the placement of the droplet delivery tubing under the separation channel while (e) shows the device once the inlet reservoir has been filled.

To assemble the separation device the PDMS channel mold and sealing layers were cleaned by sonicating in deionised water, then dried under nitrogen and treated with an oxygen plasma. The PDMS sealing layers were between 300-500 μm thick and were arranged as shown in Figure 5.5 with a 2-3 mm gap between. Prior to sealing, the edges of the two bottom layers facing each other were trimmed by 2 or 3 mm. The channel was then placed over the two sealing layers and bonded. With this arrangement the channel above the gap is not sealed. The gap was situated at one end of the channel, approximately 0.5 cm away from the inlet buffer reservoir. The edges of the sealing layers were cut after plasma treatment. Therefore the newly exposed PDMS surface forming the vertical edge of the bottom layer was now hydrophobic, while the top surface (making the seal) remains hydrophilic. The resulting device has a hydrophilic separation channel while the rest of the interface region is hydrophobic. A modified version of this device was prepared for use with a capillary based separation channel (Figure 5.6).

Buffer is loaded into the sealed device via the outlet end of the separation channel. The liquid is pulled through the microchannel under the force of capillarity. Filling of the open channel is demonstrated in Figure 5.5a to e using a red food dye for visualisation. When the buffer reaches the open channel it moves across it towards the inlet reservoir. Then the inlet reservoir is topped up with buffer. During buffer loading the exposed section of channel was placed over the PTFE membrane to provide a hydrophobic barrier and prevent the liquid from seeping out of the channel walls. Figure 5.5f shows the placement of the droplet delivery tubing in relation to the separation channel. Buffer loaded into the separation channel does not enter or interact with the delivery tubing. Manoeuvring the delivery tubing, once it was placed beneath the separation channel was complicated. Employing a metal jacket for the tubing, in the form of a flat headed syringe needle, eliminated this problem.

Free solution separations were carried out on the PDMS device as the x0.1 TBE separation buffer was easy to load, remove and replace. Gel electrophoresis of DNA fragments were carried out on similar PDMS devices. The channel was shortened and replaced with a 300 μm wide outlet which was used to insert a capillary (Figure 5.6a). Bare fused silica capillaries with an external polyimide coating, identical to those used on CE instruments, were used. A 75 μm I.D. and 375 μm O.D. capillary of 6 cm total length was used for all experiments. Prior to insertion the capillary was prepared by burning a detection window, 3 to 4 cm long, from the point of insertion. Following which the capillary was conditioned. The conditioning steps were similar to

those used on the Peregrine Instrument (see Chapter 4). The capillary was rinsed with 1M HCl followed by 10% PVP for five minutes each. The sieving matrix was then loaded prior to insertion. The outlet end of the capillary was placed in a micro-centrifuge tube filled with sieving matrix (Figure 5.6a). The inlet reservoir and open section of channel was filled with x0.1 TBE prior to capillary insertion.

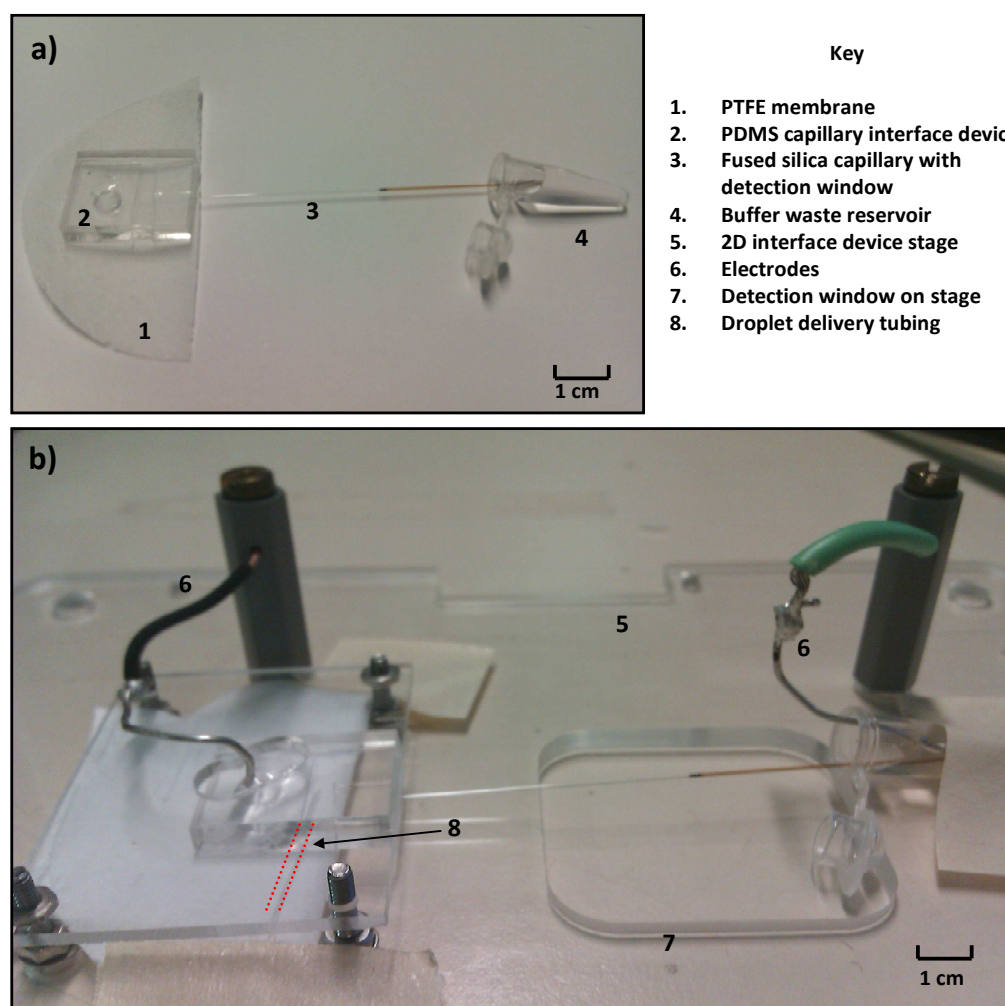


Figure 5.6: Modified droplet microchip electrophoresis device. The PDMS channel is replaced with a bare fused silica capillary. The main component of this device and the stage used for both PDMS and capillary device are labelled and listed. (a) The PDMS device used to connect the droplet delivery tubing to the capillary. The PDMS device is filled with x0.1 TBE buffer and mounted on the PTFE film. A detection window is burnt in to the capillary from the point of insertion prior to conditioning and loading the capillary with separation buffer. The outlet end of the capillary is placed in a micro-centrifuge tube which operates as the buffer waste reservoir. (b) The dual electrode microchip stage designed for the interface separations. The droplet delivery tubing is mounted on to the PTFE membrane (highlighted in red). The PDMS capillary device is then placed on top of the droplet delivery device such that the mouth of the tubing lies under the separation channel. This is held in place using the acrylic top plate, which has a circular opening to allow access to the inlet reservoir. The electrodes are inserted into the reservoirs at either end and a conductivity check is conducted. If successful the device is ready for use.

Both channel and capillary experiments were carried out on a purpose built stage (Figure 5.6b). The stage was made from acrylic with a 4 x 4 cm detection window and two electrode posts. The separation device, droplet delivery tubing and PTFE membrane were held in place using an acrylic top plate with inlet reservoir access. Figure 5.6b shows the device and stage set up while Figure 5.7 traces the overall experimental route from device fabrication to assembly, sample injection and detection.

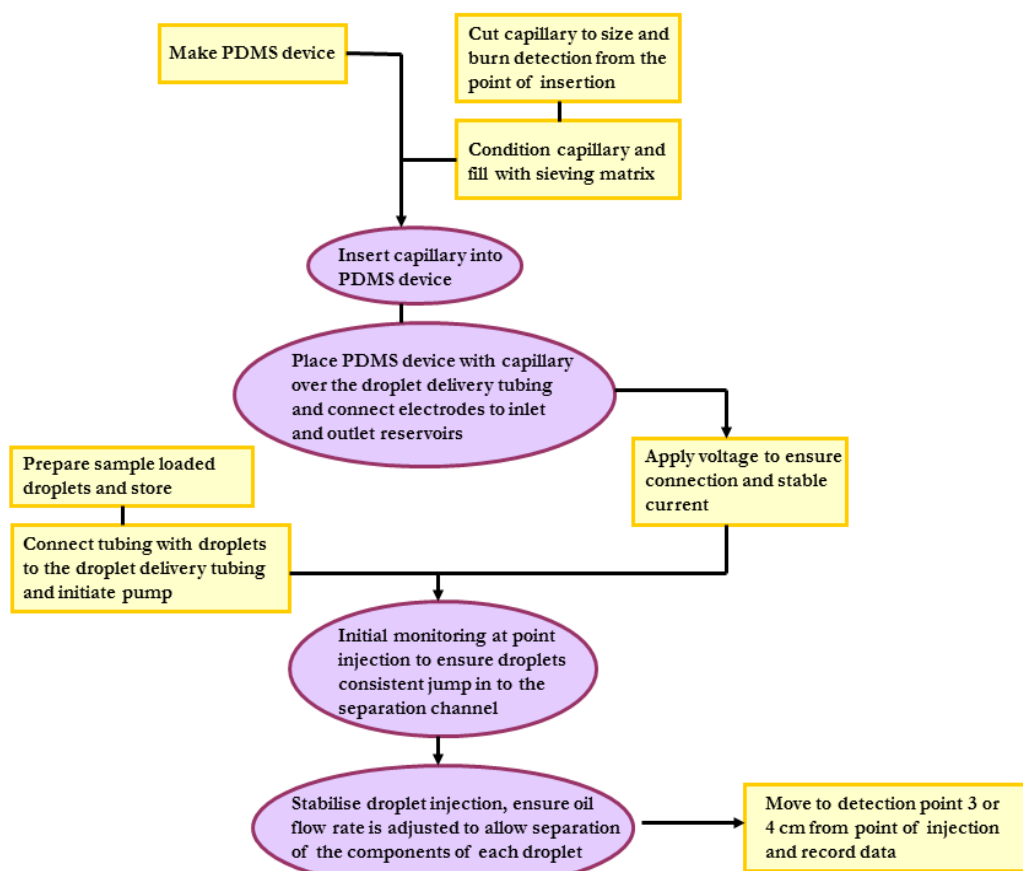


Figure 5.7: Overall experimental workflow for droplet to CE interface device. The PDMS interface device and capillary are prepared, loaded with separation matrix and then connected. Following which the entire device is placed above the droplet delivery tubing and a voltage is applied across the length of the separation channel. A stable voltage and current read-out is an indicator of good connectivity and absence of bubbles in the separation channel. Droplets, prepared earlier and stored in tubing, are connected to the droplet delivery tubing and pumped towards the interface device. Droplet injection is monitored at the interface to ensure that injection into the separation channel occurs efficiently at the set oil flow rate. For separation of complex mixtures with wide molecular weight dynamic range, droplet injection should be controlled to allow separation between components of a single droplet before the next is injected. This will prevent co-elution of bands from neighbouring droplets. Finally, detection is carried out several centimeters from the point of injection.

The change in design from channel to capillary for molecular weight assessment separations occurred due to problems faced when loading the sieving matrix. Typically, loading was initiated from the outlet reservoir using a syringe. The high viscosity matrix led to delamination of the sealing layer around the outlet reservoir. A thicker PDMS base would have prevented this; however a thickness greater than 500 μm would have adversely affected droplet ‘jumping’ from delivery tubing to the separation channel. Furthermore, the gel could only be filled up to just before the end of the channel. Gel in the open section of the channel led to sample ‘trapping’ and buffer leakage outside the channel. Therefore the channel was first loaded with x0.1 TBE and then filled with sieving matrix. If the separation matrix was loaded first, the open channel and channel leading to the inlet reservoir could not be filled. Additionally, monitoring loading with the TBE in the separation channel made it difficult to identify how far down the channel the matrix had reached. Consequently, the separation channel was either filled half-way or the matrix would reach the open section. A red food dye was employed to monitor the retreat of TBE from the channel and loading of the gel. This worked, however the dye had to be used at high concentrations to allow monitoring by eye. Prior to separation the dye was diluted out of the channel. Higher concentrations were harder to dilute, leading to non-standard current profiles. The initial PDMS channel device on the whole was simple to assemble, but awkward when applied to mass-based separations.

5.5 Injection Format

The traditional format of injection and separation on a microchip electrophoresis device is discussed in Chapter 4. Briefly, the channels are arranged in a cross format as depicted in Figure 5.8a. The shorter channel is used for sample loading while the longer channel is the separation channel. Injection is typically a two step process; initially the sample is moved under an applied field to the sample waste reservoir, where a discrete plug is maintained by applying ‘pinching’ voltages via the separation channel (Figure 5.8bi). Then the sample plug formed at the cross section of the channels is injected into the separation channel (Figure 5.8bii). The excess sample is simultaneously extracted into the sample and sample waste reservoirs by applying pull-back voltages. This form of injection requires four electrodes and can only analyse a single sample at a time. In comparison, the injection technique developed using the droplet interface device

requires only two electrodes, can inject multiple samples without cross-contamination or sample wastage, overcoming some of the problems associated with the traditional format.

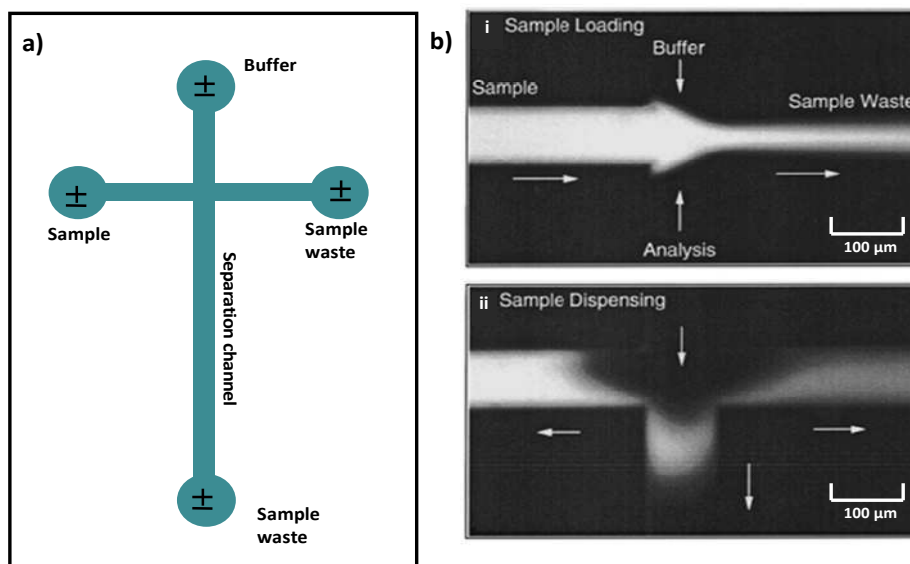


Figure 5.8: The traditional cross piece injector format microchip electrophoresis device. (a) A schematic of the cross piece device while (b) shows the typical pinching injection used to deliver a discrete sample plug in to the separation channel. See Chapter 4 for more details.

Once the device is assembled, droplets are pumped to the mouth of the droplet delivery tubing. The delivery tubing is cut at a 30° angle allowing the droplet carrier phase, FC-40 oil, to flow down and into the PTFE membrane placed below it and leaving the aqueous droplet at the mouth of the tubing. Schematics of ‘top down’ views are detailed in Figures 5.3 and 5.9. With such geometry, droplets exiting the tubing will adopt a spherical shape, due to surface tension (Figure 5.3c). The environment around the droplet is almost completely hydrophobic except for the buffer loaded separation channel suspended above it. Flow of oil from the channel lifts the droplet, connecting it to the aqueous phase contained in the separation channel. No surfactant is added in the oil phase (FC-40), therefore, when the spherical droplet makes contact with the aqueous buffer in the separation channel, droplet merging will occur in less than a millisecond [41]. During the sample injection process, the electrical field for separation is maintained; therefore separation of the analyte inside the injected droplet occurs immediately after merging. There are two major factors controlling the jumping of droplets from the segmented flow to the separation channel. The first is the distance between the mouth of the droplet delivery tubing and the separation channel, which in turn is controlled by the thickness of the PDMS layer. The

second is the size of the droplets. As the outer diameter of the delivery tubing remains constant ($370\text{ }\mu\text{m}$), changes in the foresaid factors will either inhibit or promote droplet jumping.

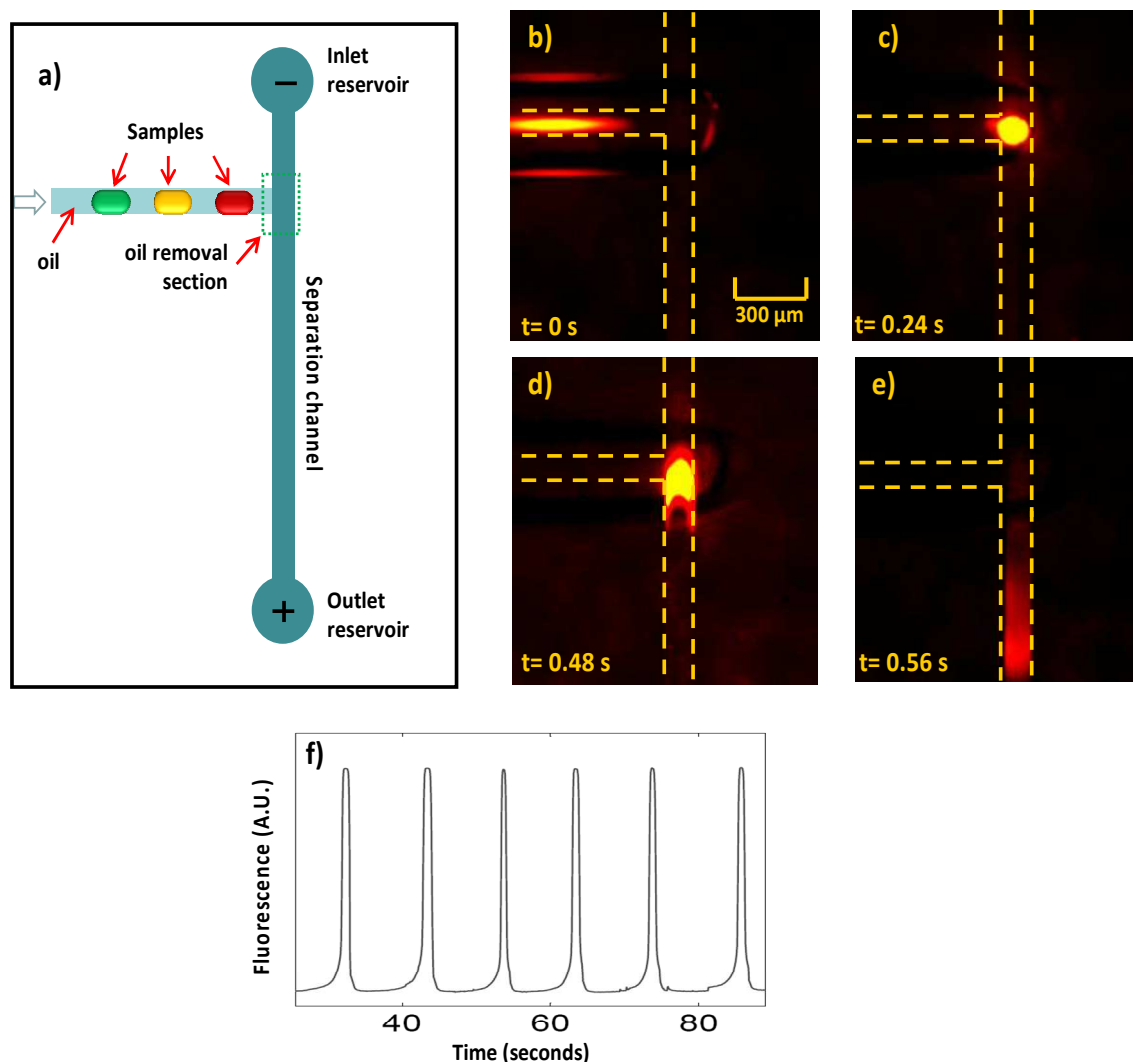


Figure 5.9: Sample injection using droplet CE interface device. (a) A top down schematic of the interface device. (b), (c), (d) and (e) show the delivery, jumping and movement of a FITC loaded aqueous droplet into a PDMS separation channel. The droplet arrives at the intersection, the carrier phase is absorbed into the PTFE film while droplet remains suspended beneath the channel. Further oil flow lift the droplet connecting it to the liquid in the separation. The charged analyte and surrounding water is dragged into the channel under the applied field. In the absence of the field the droplet jumps is pushed towards the inlet reservoir due to the pressure difference between the inlet and outlet reservoir. (f) The plot recording droplet jumping over time.

The injection volume in a cross or double –T injector can be varied by changing the height and width of the channel and the distance between the tees. These variations require different

masters, devices and operational protocols. Using the interface device developed herein the injection volume can be controlled by the size of droplet and the distance between the droplet delivery tubing and the separation channel. This allows exquisite control over injection volume, although there will be a lower and upper limit to the size of the droplets that can be injected whole.

Initial studies using microdroplets containing 500 μM FITC in x0.1 TBE encapsulated by FC-40 oil were used for calibration. The droplets were pre-generated on the automated robotic instrument and stored in PTFE tubing. Two kinds of tubing were used, with an inner diameter of either 50 μm and 200 μm . Droplets in the 50 μm tubing had an average volume of 80 picolitres, while the droplets in 200 μm tubing had an average volume of 1.2 nL, both with a droplet volume RSD of 4%. The droplets were injected into the separation channel with an approximate time interval of 10 seconds. Figure 5.9b to e show the time sequence of an 80 picolitre droplet being injected into a separation channel with a cross section of 50 x 50 μm^2 . After droplet merging, dye molecules are driven down the separation channel. A detailed inspection of the tubing head found no trace of the fluorescent dye employed. Such inspection was performed for more than 100 droplets, with the same results. Significantly, this demonstrates that such an injection format can efficiently avoid cross-contamination.

An important feature of the current design is that it permits consecutive injection of droplets into the separation channel, without accumulation of buffer at the intersection. Buffer accumulation will dilute the sample, increasing the width of the injection plug and thus broaden the injected bands. To prevent this from occurring, the chip was designed to account for surface tension at different points along the channel. At the intersection, the channel is confined with three solid hydrophilic walls while the bottom is open to the air. Therefore, the liquid in the channel adopts a curvature at the open side which may be either concave or convex, depending on whether the liquid is above or below the open surface. The radius of curvature is inversely proportional to the local pressure at the liquid surface (P_1) due to surface tension and may be described by:

$$R_1 \propto \frac{\gamma}{P_1} \quad (5.1)$$

Here γ is the surface tension and R_i the axial radius of the curvature along the channel direction. The local surface pressure has a tendency of minimizing the total surface area. The surplus liquid can flow to or be provided by the inlet reservoir as it is closer to the intersection than the outlet reservoir. Furthermore, in the presence of a sieving matrix in the separation channel, the $\times 0.1$ TBE in the inlet reservoir and intersection will flow more easily than the gel. As the width of the reservoir ($W_2 = 4\text{mm}$) is much larger than the width of the channel ($W_1 = 25$ or $50\text{ }\mu\text{m}$), the radius of curvature is much greater in the channel and consequently the surface tension in the reservoir is almost negligible compared to the surface tension at the intersection. Therefore, in Figure 5.8b to e and video (No.5.1), post droplet injection, there is a pulse of liquid flow towards the inlet reservoir. This flow ceases immediately after injection and the analyte is then driven to the separation channel under the applied field.

5.6 Current Controls

Compared to the closed channels used in traditional MCE, the short section of open channel at the interface is a unique feature of the droplet-CE platform. The open section was essential for droplet loading, however if this injection format hindered the efficiency of the CE separation the interface would have required a redesign.

To ensure that a stable current was maintained in the presence of the gap, the droplet interface was fabricated to contain different gaps from 12 to 0 mm (an entirely sealed channel). Using an applied field strength of 100 V/cm, the electric current was recorded for each gap (Figure 5.10a). Results showed that the size of the gap was inversely proportional to the fluctuation in current. However, the fluctuation does not disappear even when there is no gap. The reason behind this remains unclear. The RSD of current values determined from each reading were plotted as a function of gap size (Figure 5.10b). For a gap above 6 mm in length a sharp increase in current fluctuation was observed, while below 3 mm the current fluctuation is similar to that of a closed channel. Consequently the 3 mm gap length was used for all experiments detailed here. Moreover, the 3 mm gap was more than sufficient to place the droplet delivery (O.D. about 300 μm) including its metal jacket. Further experiments indicated that the current remains stable during the droplet loading process.

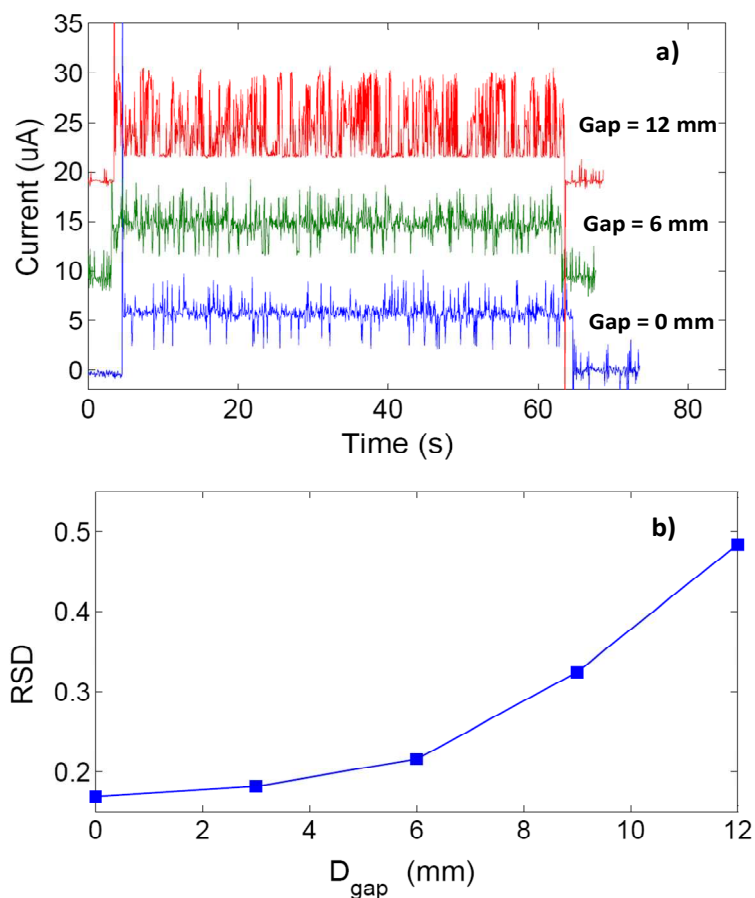


Figure 5.10: Current trace for channels with gaps between 0 mm and 12 mm. (a) The current trace obtained when there is a gap of 6 mm and 12 mm and for no gap (0 mm). (b) The RSD value for current versus the gap size. The variation observed with an open section up to 3 mm in length is similar to that observed in a completely sealed channel.

One of the main challenges with any form of electrophoresis is to keep the system free of air bubbles. Small bubbles may cause variations in current and disrupt band migration through the channel. While larger bubbles can disconnect sections of the channel leading to current disruption. Bubbles can also generate false peaks in electrophoretic profiles. During droplet generation, formation of air bubbles can occur due to insufficient sealing at one or more inlets. When these air bubbles were delivered to the continuous flow CE channel using the interface set-up, air bubbles did not enter the channel. Figure 5.11a details the course of an air bubble at the droplet-CE interface. Once the oil surrounding the air bubble was absorbed into the membrane the air dissipates into the surrounding atmosphere. The current and voltage were monitored during this process (Figure 5.11b) and only small variations were observed. These fluctuations were similar to those observed in the absence of air bubbles, indicating that air bubbles did not affect the electrophoretic process.

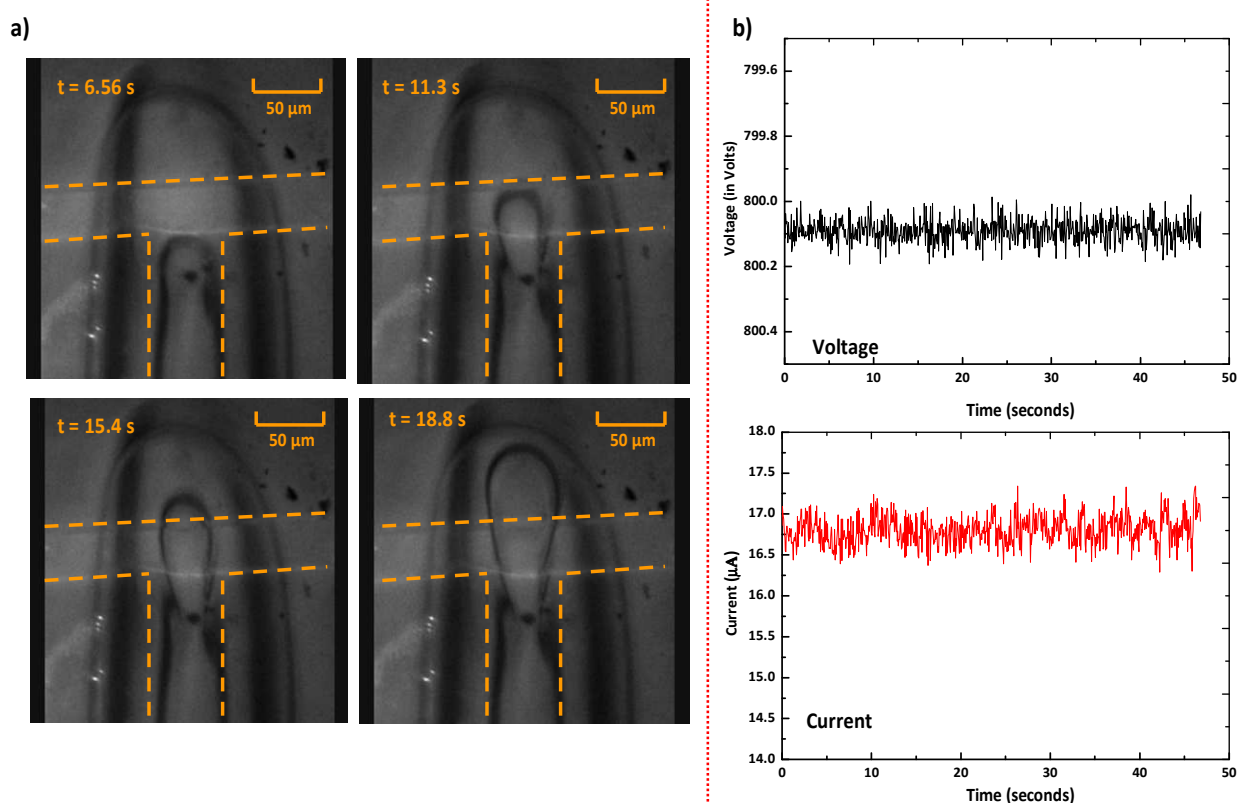


Figure 5.11: Effect of air bubbles on the droplet CE interface. (a) Illustrates the passage of a air bubble across the droplet interface. The emerging air bubble does not enter the separation channel but dissipates into the surrounding atmosphere once the oil containing is absorbed in to the PTFE membrane. (b) The voltage and current plots taken during the expulsion of the air bubble; both voltage and current values remain stable.

5.7 Results and Discussion

5.7.1 Effect of Droplet Size on the Injection

A variation in droplet size not only affects the signal obtained, but is a critical part of the jumping mechanism associated with the interface. As discussed previously the transfer of analyte to the separation channel depends on several factors, including droplet size. If the droplets are smaller than required they will accumulate at the mouth of the droplet delivery tubing and jump when a threshold volume is reached. Consequently, cross-contamination between the droplets and dilution of the analyte held within each droplet will occur. Figure 5.12a, b, c and d illustrate the effect of larger droplets. Here a large FITC droplet is injected into a PDMS separation channel using the droplet CE interface. Figure 5.12b to d illustrates the extended injection initiated by the large droplet. The droplet fractionates and injects three separate plugs into the

separation channel. Therefore, using 200 μm I.D. droplet delivery tubing, and a flow rate of 50 $\mu\text{m}/\text{second}$, a droplet with a length of less than 500 μm can jump into the separation channel whole. Above this length, the droplet tends to break during jumping, leaving a sister droplet in the tubing, due to Plateau–Rayleigh instability. The effect is similar to a multiple injection; however the period between each jump, 0.5 seconds in this case, is insufficient to allow the bands in each injection to resolve. The bands from the previous injection overlap with the initial injection. If the sample is relatively simple the resulting peaks may be de-convoluted, however in a complex mixture low intensity bands from one injection may co-elute with higher intensity bands and be lost.

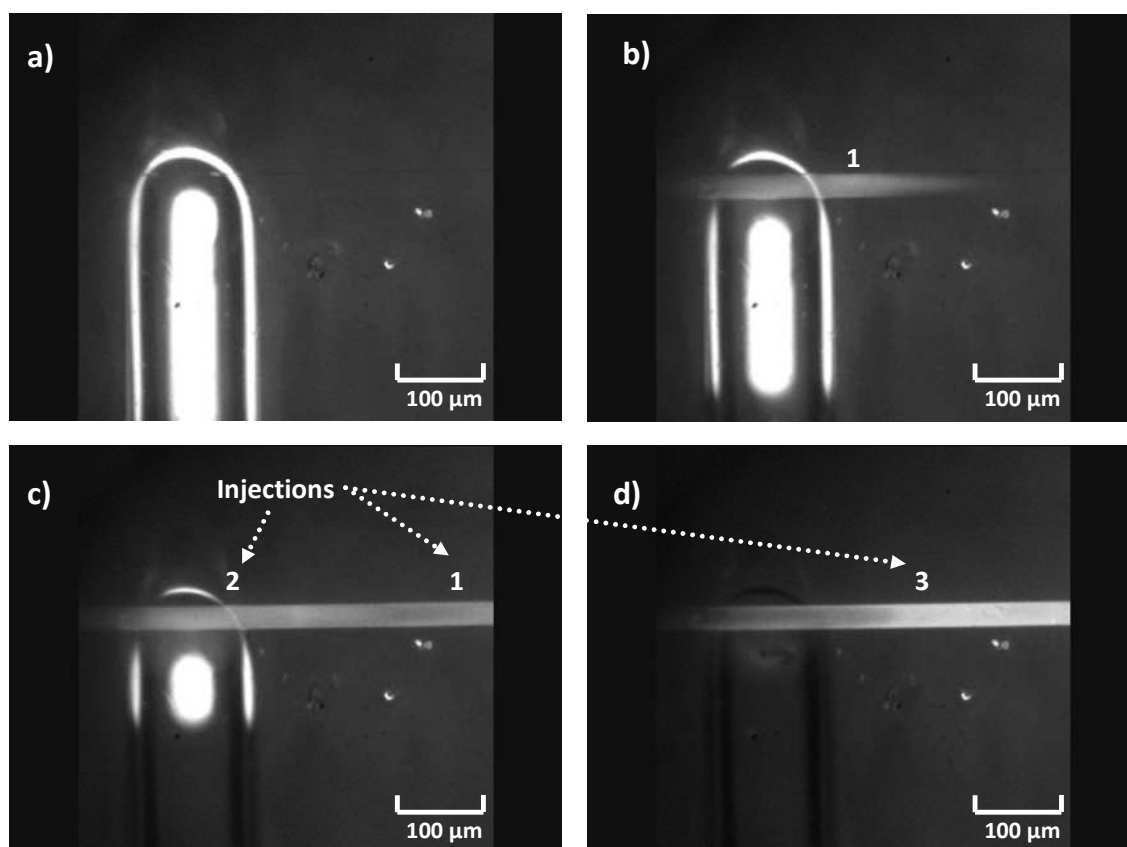


Figure 5.12: Images (a) to (d) illustrating the effect of large droplets on the injection. The droplets feed into the continuous flow electrophoretic channel and fractionate into several small plugs. These injections are often insufficiently spaced to allow all the components of each sample plug to resolve without overlap. Injections 1, 2 and 3 detailed in images (b), (c) and (d) respectively are separated by 0.5 seconds each.

5.7.2 Zone Electrophoresis using The Interface Device

Initial experiments testing the droplet interface were performed using a PDMS microchannel. Zone electrophoretic separations were performed using EOF to drive the separation. Separation channels were initially loaded with 0.1M NaOH and then replaced with a x0.1 TBE run buffer (pH 8.3) prior to separation. A conductivity check was performed to ensure electrical connection between the inlet and outlet reservoir. Following this, droplet flow into the separation channel was initiated. Droplet flow rates were set to allow a 20 to 40 second interval between injections. Injected droplets formed discrete sample plugs and migrated towards the detector. Fluorescein iso-thiocyanate and Eosin Y solutions at final concentrations of 100 μ M and 20 μ M respectively were prepared as a mixture in x 0.1 TBE. At a pH of 8.3 both dyes are negatively charged and migrate behind the electroosmotic flow. The PDMS separation channel supported the free solution separation of FITC and Eosin Y allowing resolution of the two components (Figure 5.13a). The time interval between droplet injection and separation was maintained during the analysis with a 40 second gap between each set. The difference in peak area reflects the difference in droplet size.

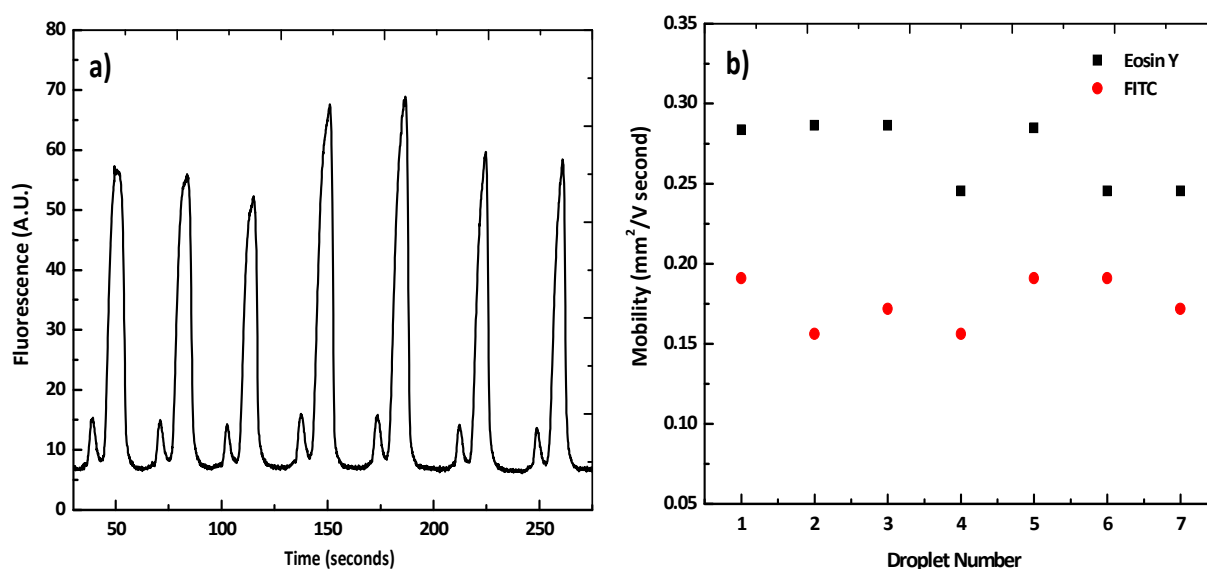


Figure 5.13: Free solution separation of fluorescent dyes, Eosin Y and FITC using a single channel droplet interface device. (a) The electrophoretic profile obtained for seven consecutive multiple injections. (b) Mobility versus injection number plot for the Eosin Y and FITC. Separation was performed in x0.1 TBE under an applied voltage of 266.66 V/cm.

A plot of mobility versus droplet number (Figure 5.13b) illustrates the variation in mobility values for Eosin Y and FITC over seven consecutive injections. The RSD values for Eosin and FITC were determined as 7.9% and 8.9% respectively. A similar experiment was performed using a dual channel device containing two 50 μm separation channels with a 50 μm gap between them. The concentrations of FITC and Eosin Y were 20 μM and 80 μM respectively. A droplet arriving at the interface splits and injects into both channels. Figure 5.14a shows the analyte bands migrating through the two channels. Separation profiles obtained for multiple injections of dye sample droplets into both channels is shown in Figure 5.14b. Mobility values for each peak were determined based on the velocity of each peak and were plotted against droplet number for each channel. The average mobility over five injections for peak 1 (Eosin Y) in channel 1 and 2 was 0.066 and 0.058 $\text{mm}^2/\text{V sec}$ respectively, while the average mobility determined for peak 2 (FITC) in channels 1 and 2 was 0.046 and 0.044 058 $\text{mm}^2/\text{V sec}$ respectively. Lower mobility values recorded for both peaks 1 and 2 in channel 2 indicates a reduced electroosmotic flow in this channel.

Zone electrophoretic separations on polymer microchips are notoriously difficult reproduce without altering the surface chemistry of the plastic microchannel. Additionally, controlling injection in the presence of EOF at the cross-piece is problematic. In the hands of the author, unless the device was well conditioned with 1 M NaOH prior to loading the buffer, turbulence was observed at the junction of the cross-piece. In the presence of EOF, carrying out a pinched injection would require optimising the injection till the strength of the flows from each arm were balanced, allowing the formation of a pinch. In addition, the pull back step led to increased variability and failed injections. The absence of a cross piece on the interface device allowed the droplets to move into the separation channel with the EOF moving in a single direction. The bare fused silica capillary employed in the capillary iteration of the interface device presents the experienced CE analyst with a long-tested and well-understood surface chemistry. Aside from a short portion of the channel which is made from PDMS, the analyte is exposed to the same conditions as in a standard CE system.

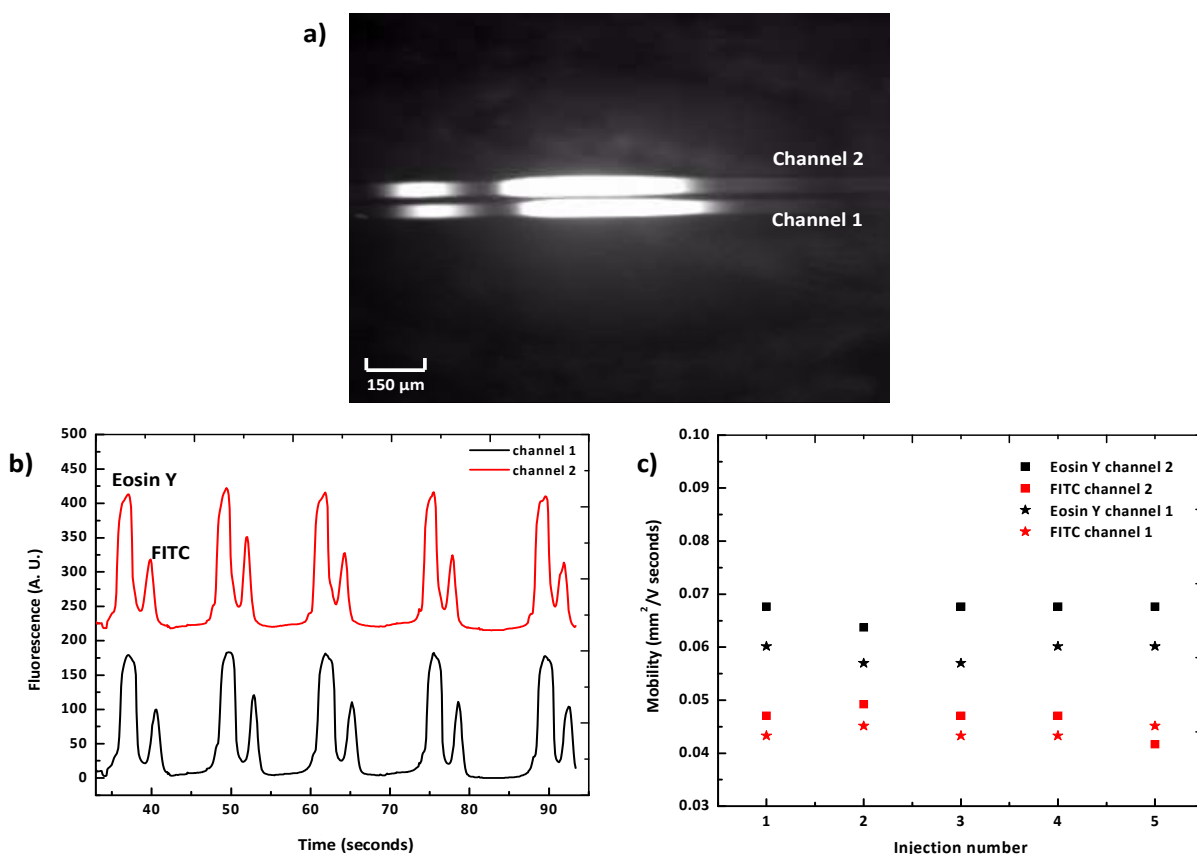


Figure 5.14: Free solution separation of fluorescent dyes, Eosin Y and FITC using a dual channel droplet interface device. (a) A fluorescent image of the separated bands on both channels. (b) An overlay of the electrophoretic profiles obtained from both channels. (c) Mobility versus injection number plot for the Eosin Y and FITC on both channels. Separation was performed in x0.1 TBE under an applied voltage of 436.36 V/cm.

5.7.3 Gel Electrophoresis Using the Interface Device

Although problems were encountered when loading sieving matrices into the PDMS channel of the interface device, several separations were attempted with successful outcomes. The separation of a mixture of FITC and Eosin Y using the gel system yielded several peaks (Figure 5.15). Similar separations performed on a commercial CE instrument (PEREGRINE, deltaDOT, London, UK) showed that each dye did indeed partition into several peaks (Figure 5.15d). In particular, at least six peaks were detected in the FITC electrophoretic profile. A closer view of the separation profile generated from the analysis performed on the interface device shows that seven peaks were consistently observed (Figure 5.15b). In addition, detection at two points along the capillary, at 1.5 cm (Figure 5.15) and 4 cm (Figure 5.15) from the injection point, showed an expected increase in resolution.

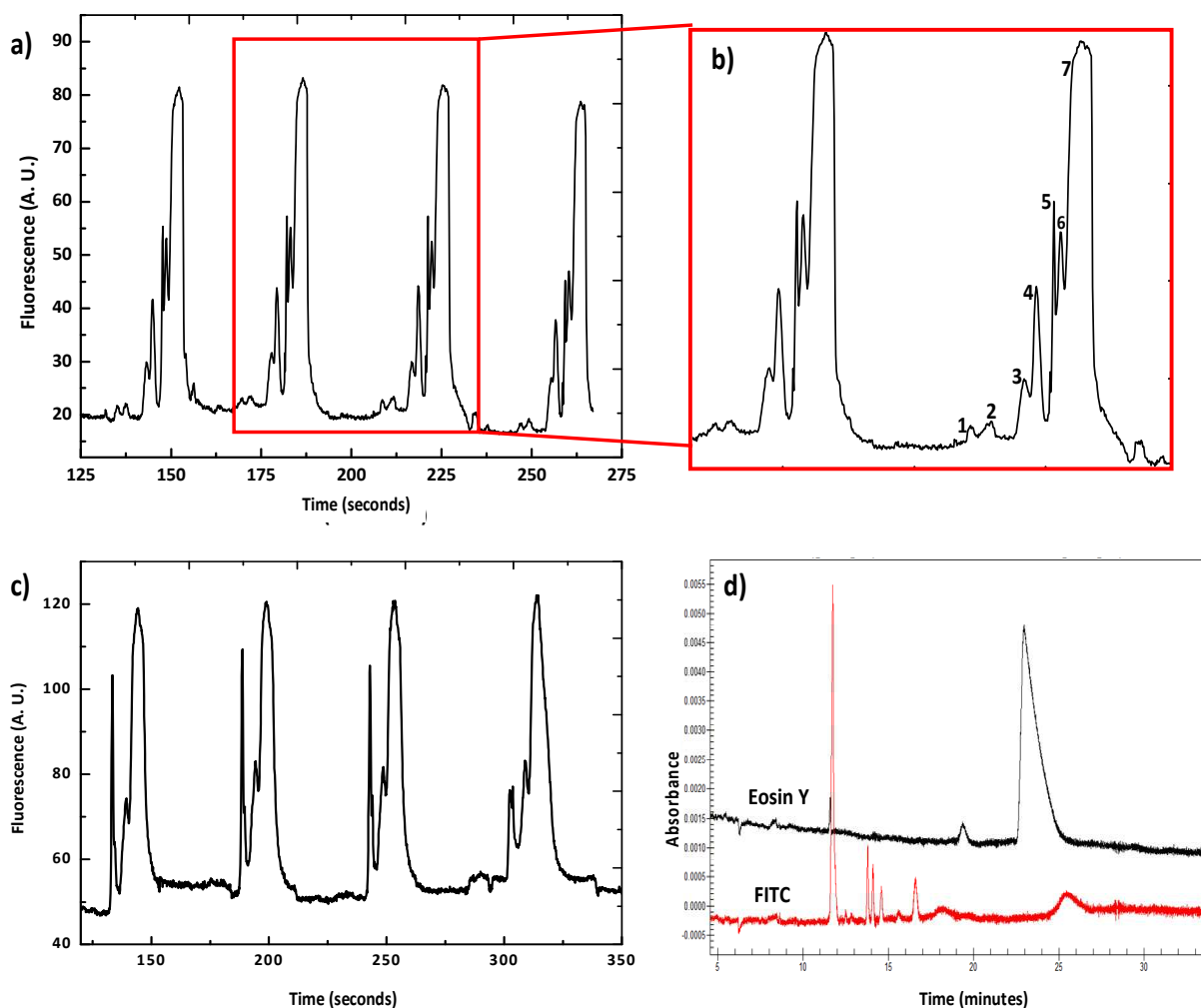


Figure 5.15: Gel separation of a mixture of FITC and Eosin Y on the droplet CE interface device, using a 2.5% PEO (5MDa) in x0.1 TBE sieving matrix. (a) and (c) show the multiple injection of the mixture detected at 4 cm and 1.5 cm from the injection point respectively. (b) A closer view of two consecutive injections, each set consists of 7 peaks. (d) The CE separation of each dye in 1.75% PEO (5MDa) in x1TBE sieving matrix using UV absorbance detection. Separations were carried out under a field strength of ~ 253.9 V/cm on the droplet CE interface device, and ~ 203 V/cm on the CE instrument.

Two dsDNA molecular weight standards were used to assess the performance of the droplet interface device with the capillary separation channel using the 2.5% PEO (5 MDa) in x0.1 TBE sieving matrix. The separation profiles for Marker VIII (Hoffman La Roche) obtained on the CE instrument, cross-piece microchannel device and droplet interface are illustrated in Figure 5.16c, b and a respectively. The agarose gel separation result provided by the supplier is also illustrated, along with component bands labelled by fragment size and DNA concentration for each band. Each fragment was assigned a peak number which was used to identify it on the separation profiles obtained from the other electrophoresis formats.

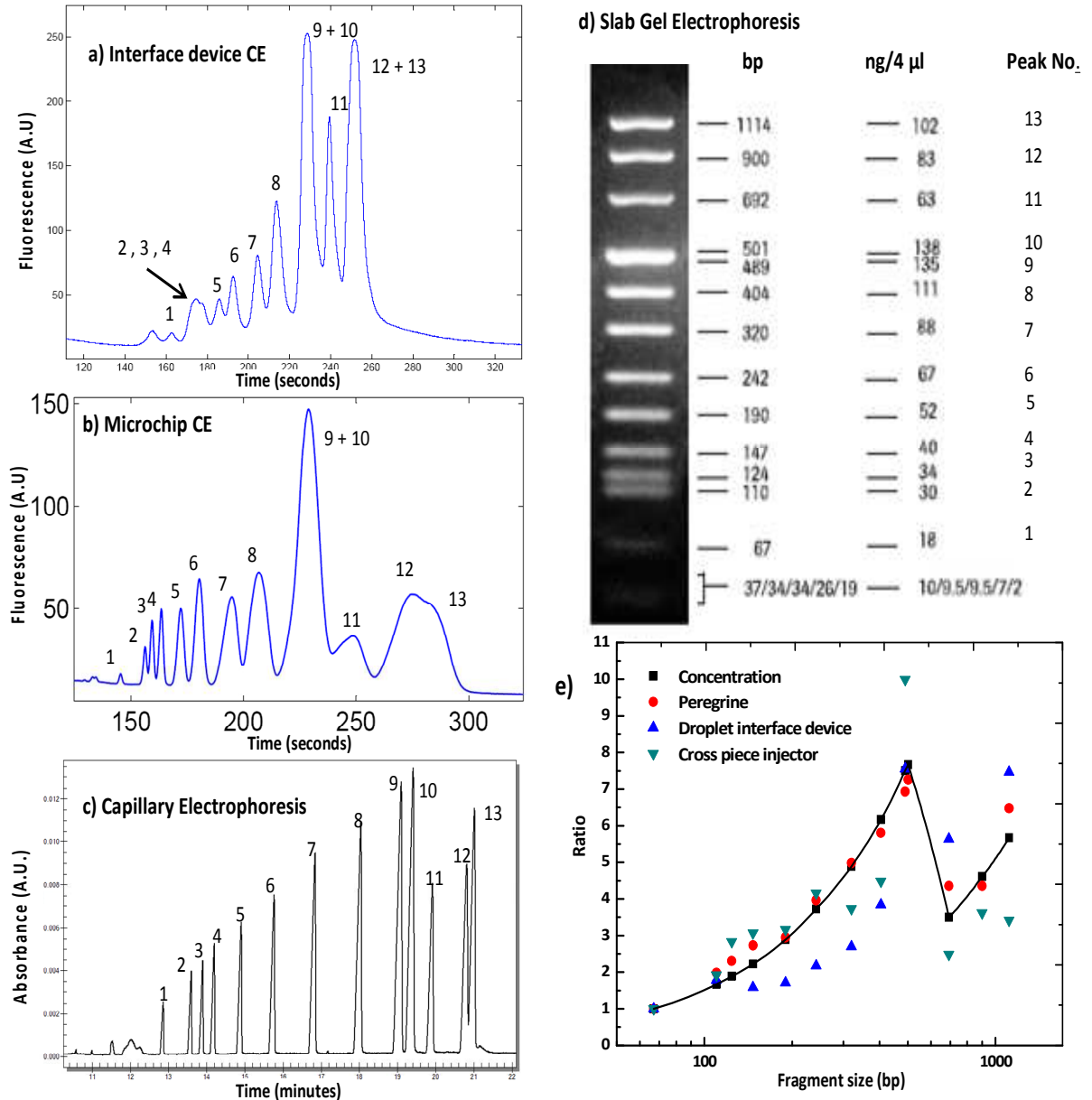


Figure 5.16: Comparison of different results obtained using standard techniques like capillary electrophoresis and Slab gel electrophoresis with those obtained on the microchip format. Separation of Roche marker VIII dsDNA restriction fragments (Hoffman La Roche, 0.034 μ g/ μ l) labelled with SYBR Green I (50 μ g/ml). Droplets of the labelled dsDNA step ladder were generated using the robot and injected in to the separation channel at a flow rate of 0.05 μ l per minute. (a) The electrophoretic profile obtained in a bare fused silica capillary of total length 5.5 cm using the droplet interface device. Total length of the separation channel from inlet to outlet reservoir was 6.3 cm. Detection was carried out $\sim$ 3.2 cm from the point of injection. (b) The separation of the same sample on a standard PDMS microchip. Total length of the separation channel on-chip was 6 cm and the effective length was 4.5 cm. (c) The capillary separation of the same unlabelled ladder in a capillary of total length 54cm and effective length 42 cm. Separations were carried out under a field strength of $\sim$ 253.9 V/cm on droplet CE interface device, $\sim$ 166.6 V/cm on microchip and $\sim$ 203 V/cm on the CE instrument. Both chip separations analysis were carried out in a 2.5% PEO in x0.1 TBE sieving matrix. The CE instrument separation was carried out in a 1.75% PEO (5MDa) in x1 TBE. Both capillaries were pre-coated with 10% PVP prior to loading with the sieving matrix. (d) The separation of the same ladder in 1.8% agarose as provided by the manufacturer (Roche). The band molecular weights and assigned peak numbers are also provided. (e) The ratio of peak height or fragment concentration to fragment size.

The DNA concentration (in ng/4 μ l) for each band was compared to the concentration of the smallest fragment (67 bp). These values were obtained from Promega Corporation and are listed in Figure 5.16d along side the fragment length and peak number. This ratio was plotted against fragment size (Figure 5.16e) and used as a control to monitor the fragment concentration observed in each separation format. Peak heights obtained from each separation profile were compared to the height of the first peak in each profile. Values were plotted as a ratio to allow comparison between the formats. Figure 5.16e compares the ratio of peak height or fragment concentration to fragment size. This plot permits comparison between the injection formats employed. Peak height ratios obtained from the separation performed on the PEREGRINE CE instrument follow the trend set by the fragment concentration closely. The increase noted for smaller fragments is in-line with the expected bias observed during electrokinetic injections. This bias is also observed for the cross-piece injector microdevice where higher amounts of smaller fragments were observed compared to the control. In addition, the larger fragments were under-represented with the lowest peak height ratios compared to the other injection formats.

The peak height ratios obtained for the droplet interface device follow the trend set by the concentration control. However, peaks heights observed were consistently lower than the control except for the larger fragments, which were higher. As peaks 9 and 10 and peaks 12 and 13 remain unresolved (under the separation conditions employed), it is likely that their combined signal caused the discrepancy in peak height. As indicated from this plot (Figure 5.16d) the injection geometry heavily influences the content of the sample plug. The greatest variation in sample content was observed using the cross-piece injector. An electrokinetic bias should not be observed using the droplet interface injection format as a discrete droplet is simply inserted into the separation channel. In comparison, using a continuous flow voltage-driven injection set-up analyte is drawn from the sample reservoir depleting it of highly charged or low molecular weight species. The droplet format also prevents sample wastage as the whole droplet is analysed. Long injection times (often 2-3 minutes on MCE devices with cross-piece injectors) leads to sample wastage with a large quantity of the sample moving to the waste reservoir. For large macromolecules such as proteins and DNA, sample volumes between 20 and 30 μ l are often required before an acceptable separation profile can be obtained. This total volume is similar to that used on most capillary systems today. By employing the droplet format a similar volume can be split up into thousands of nanolitre droplets allowing many separation profiles to

be extracted. Samples once prepared in the droplet format can be stored sealed and suspended in the oil carrier phase for 2 or 3 days, allowing re-use before another batch has to be prepared.

A log-log plot of mobility versus fragment size was generated for the separation of Marker VIII using the droplet interface separation device. This plot is detailed in Figure 5.17a, and shows the expected sigmoidal shape. As described in Chapter 4 each section of the curve corresponds to a specific sieving mechanism. The lower molecular weight fragments undergo Ogston sieving [42, 43], while the section of the curve showing an exponential decrease in mobility values corresponds to the reptation mode of migration [44]. Here the fragment mobility is inversely proportional to its molecular weight. Above a specific size the dsDNA fragments align to the applied field reducing the difference in mobility values obtained between them [45, 46]. This migration mechanism is described by the biased reptation theory.

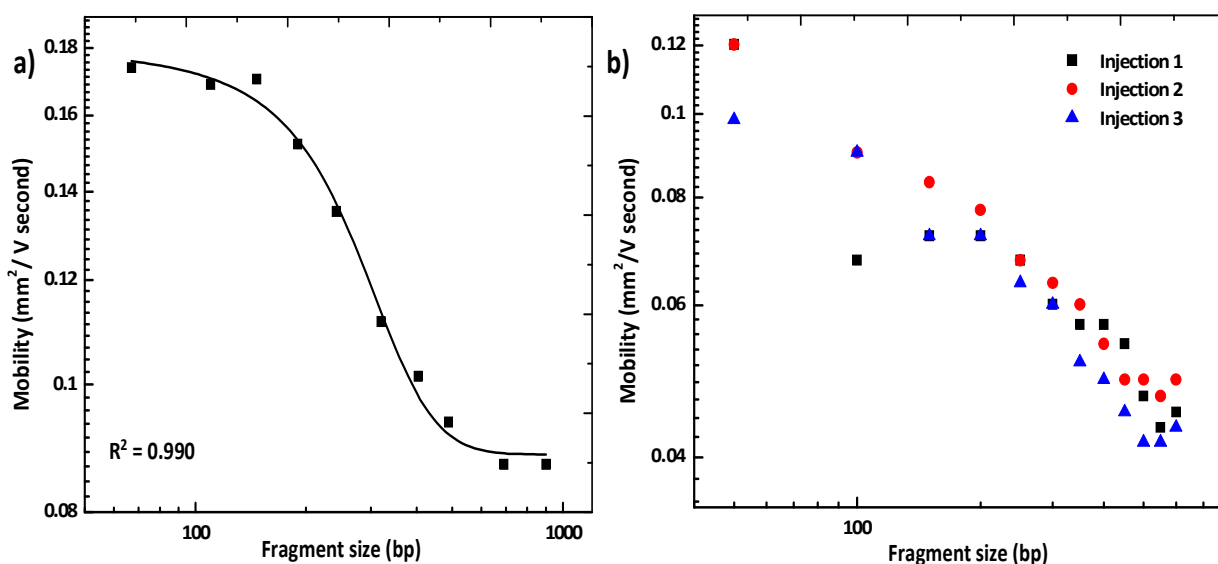


Figure 5.17: Log-log plots of mobility versus fragment size for droplet interface electrophoresis. (a) Results for Marker VIII (Roche). (b) The curve obtained for 3 consecutive injections of the 50 bp dsDNA step ladder. Both chip separations were carried out in a 2.5% PEO in x0.1 TBE sieving matrix and the capillaries employed were pre-coated with 10% PVP prior to loading with the sieving matrix. The Roche ladder and 50bp dsDNA step ladder were separated under a field strength of 253.9 V/cm and 246 V/cm respectively.

A similar plot was constructed for the 50bp step ladder separation. This dsDNA step ladder was used to demonstrate the multiple injection capability of the droplet interface. Fourteen out of sixteen fragments of this ladder were identified for all three injections. Detection was carried out 1cm from the point of injection. Increasing the effective length will allow baseline resolution of

all fragments. The sixteen fragments plus the 1,800 bp “backbone” fragment were separated in 55 seconds for each injection (Figure 5.18a). The log-log plot of mobility versus fragment size for each injection is detailed in Figure 5.17b.

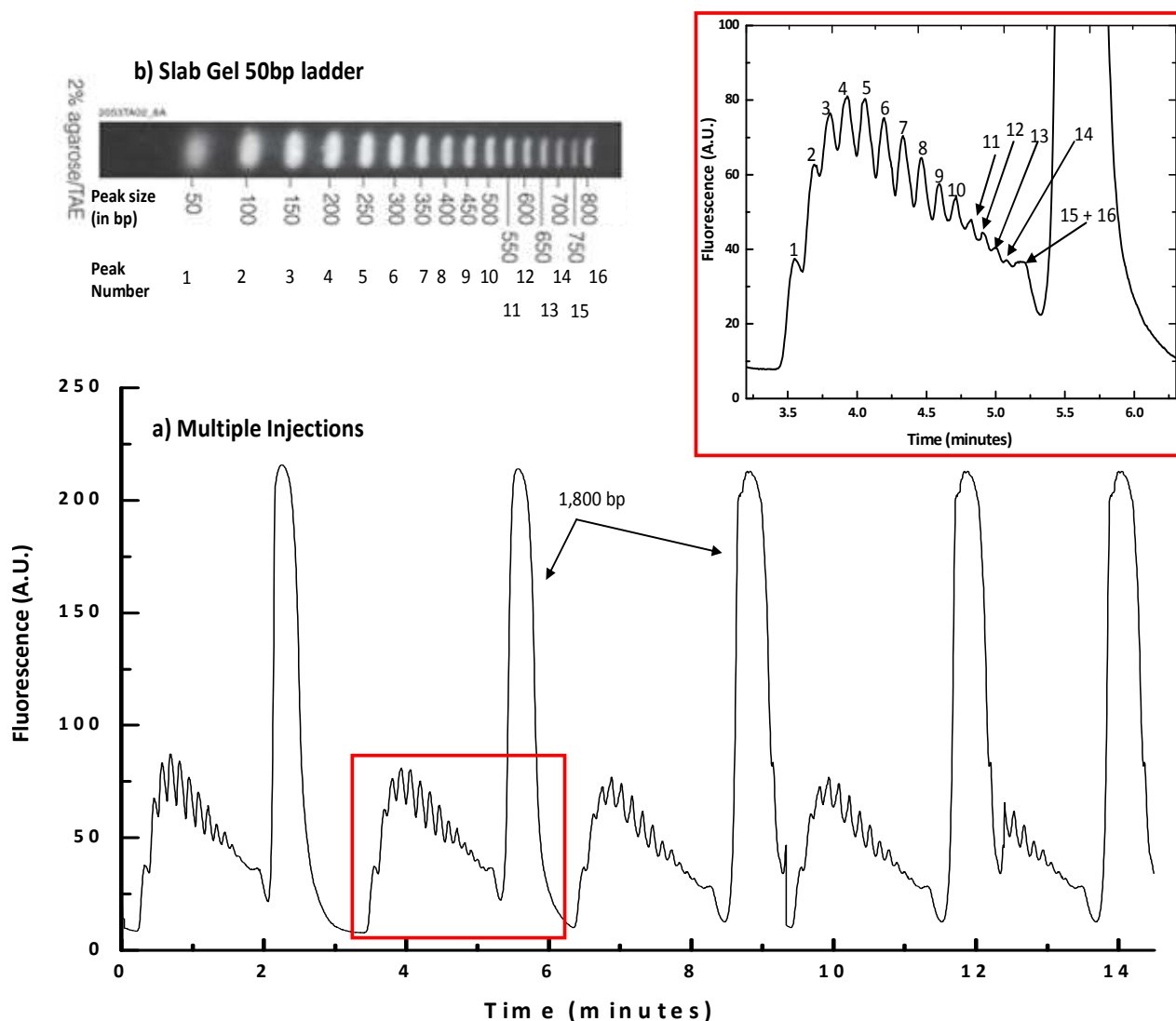


Figure 5.18: Multiple injection and separation of 50 bp dsDNA ladder (Promega, 0.034 $\mu\text{g}/\mu\text{l}$) labelled with SYBR Green I (50 $\mu\text{g}/\text{ml}$). Droplets of the labelled dsDNA step ladder were generated using the robot and injected in to the separation channel at a flow rate of 0.02 μl per minute. (a) The electrophoretic profile obtained for the multiple injection of the sample in to a bare fused silica capillary of total length 1 cm using the droplet interface device. Total length of the separation channel from inlet to outlet reservoir was 6.5 cm. Detection was carried out ~ 1 cm from the point of injection. The insert shows an expanded view of one separations with all the component peaks labelled. (b) The separation of the same ladder in 2% agarose as provided by the manufacturer (Promega). The band molecular weights and assigned peak numbers are also provided.

All three curves followed a similar trend described by the three possible sieving mechanisms. The mobility values between fragments varied between 2% and 10%. A larger variation was observed for the smaller fragments, due to stretching of matrix pores by the larger fragments of a prior injection. In other words, larger fragments distort the pores of a sieving matrix while they move through it; this effect is reversible until the polymer fragments forming the pore disentangle. Consequently, DNA in successive injections move through increasingly distorted pores. The multiple injection results presented here form part of a large group of sample droplets. The sieving matrix, which was not refreshed during the experiment, degrades with repetitive use. This is true of any multiple injection separation. Figure details the loss in resolution observed once the matrix degrades, shorter fragment lengths co-elute generating several large peaks. The maximum number of separations which will provide acceptable percentage reproducibility is yet to be established.

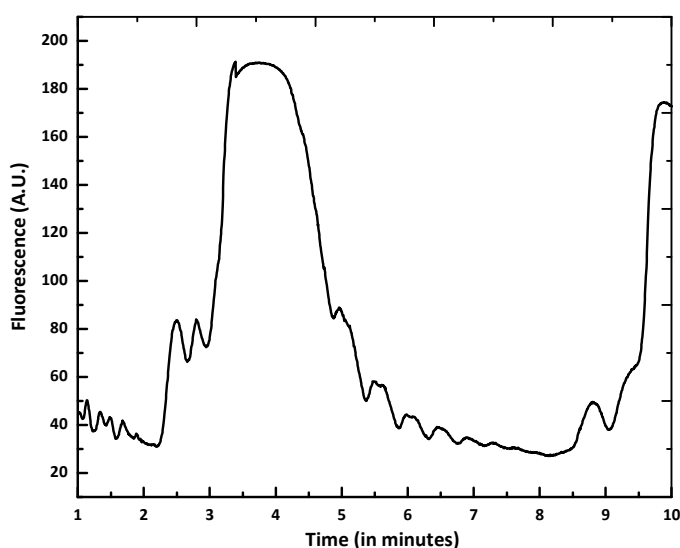


Figure 5.19: Co-elution of peaks due to matrix degradation. Multiple injections lead to stretching of matrix pores, consequently there is a loss in resolution between the shorter DNA fragments.

Importantly, the droplet interface allows injection and separation of consecutive droplets without interrupting the separation voltage. This is significant since typical multiple injection protocols on CE instruments involve three basic steps. First the separation voltage is discontinued, then the next sample is injected hydrodynamically or electrokinetically following which the separation voltage is re-applied. During the injection periods the bands already

separating within the capillary are assumed to be ‘parked’. Peak parking has been shown to lead to a loss in resolution between sample bands due to diffusion. In addition, the injection step will affect the parked peaks and their mobility values. Furthermore, injections must be timed to prevent the peaks being parked in the detection window. This will lead to photo-bleaching of fluorescently label samples and UV degradation of biomolecule samples in a transmission set up. The independence of the separation channel from the injection unit circumvents these issues. This makes the possibility of a droplet-interfaced multi-channel separation device with multiple injection capability real. Such a device, if realised, will provide extremely high throughput with minimal injection biasing.

5.7.4 Sample Quantification on Droplet Interface

Another advantage of the described droplet CE interface is the ability to perform high throughput injection of controlled volumes with no bias, permitting CE separations to be used as a tool in quantitative analysis. This function is illustrated using a set of droplets with varying concentrations of Fluorescein and Eosin dyes. Sample droplets were pre-generated with the fluorescein concentration decreasing from 100 to 40 μM while that of Eosin increasing from 20 to 80 μM , both in 20 μM steps. A 4% volume variation was observed between droplets. The Fluorescein and Eosin mix produces two peaks when separated using the 2.5% PEO in $\times 0.1$ TBE sieving matrix. The separation results obtained for one set of droplets is shown in Figure 5.20a, where red lines have been used to segregate bands from each droplet. Droplets were labelled from 1 to 4 and peak heights for Fluorescein and Eosin were determined for each droplet. Figure 5.20b shows a plot of peak amplitude versus droplet number. Linear fitting of the curves generated by each analyte showed R^2 values above 0.9, indicating a good fit in both cases. Consequently, the signal obtained from each droplet accurately reflects its concentration, permitting the generation of both calibration curves. Deviations from the fitted line are due to small variations in droplet size and manual error during sample preparation. Using these calibration curves a droplet with unknown concentration can be accurately quantified.

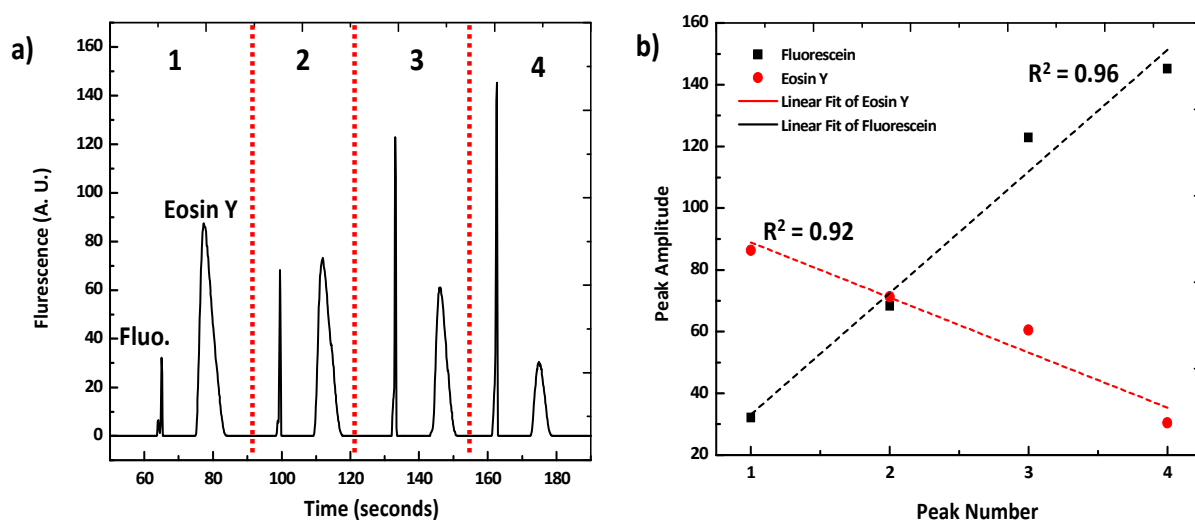


Figure 5.20: (a) Separation profile of four consecutive droplet injections containing sample with different ratios of Fluorescein and Eosin Y. Samples were prepared as detailed in the text. Change in sample concentration is reflected in the signal obtained. (b) The plot of peak amplitude versus droplet number. Separation was performed in 2.5 % PEO (5MDa) in x0.1 TBE sieving matrix with a total channel length of 6.5 cm and effective length of 0.5 cm. The inserted capillary was conditioned with 1M HCl and 10% PVP prior to loading the sieving matrix.

The droplet interface provides simple solutions to some of the fundamental problems of sample injection on capillary and microchip electrophoresis systems. Pre-defined sample volumes can be injected in their entirety, accurately reflecting the content of the sample. An electrokinetic bias, typically observed in continuous flow injection geometries is not seen in this segmented-flow assembly. Data comparing three injection strategies showed that the traditional cross-piece injector cannot be applied to quantitative analyses. In addition, on the droplet interface device, differing conductivities of sample and background electrolyte do not affect the moles of sample injected. Their differences affect the shape of the peaks obtained and can aid in on-column pre-concentration of the sample. Typically, field amplified sample stacking cannot easily be achieved employing a cross-piece. However, on the interface device only the capillary is filled with sieving matrix, while the inlet reservoir and interface area is loaded with x0.1 TBE. Adjusting the conductivities of each buffer will help focus the analyte bands at the mouth of the gel-filled capillary, pre-concentrating the sample. Furthermore, the entire sample contained in the droplet is analysed and thus sample wastage is minimal. The one to two minute injections carried out on the cross-piece injector to equilibrate sample at the intersection leads to high concentrations of sample collecting in the sample waste vial. This sample can be re-injected by reversing the

injection conditions; however it will primarily contain the highly charged, low molecular weight species of the original sample.

5.8 Conclusions

A droplet-interfaced CE platform for chemical separations is demonstrated. Local patterning of a hydrophobic but oleophilic film was proved shown to be effective in passive oil removal. This platform is much simpler in operation than the conventional cross channel MCE and provides the desired capabilities of small sample handling, high-throughput and quantitative analysis without cross-contamination between sample droplets. Both CZE and CGE separation modes were successfully demonstrated, proving the potential of such platform to be used in a wide range of applications such as small molecule separations, proteomics, genomics, metabolomics and other chemical and biological assays. Such a platform detaches the droplet generation and handling assembly from the separation channel, consequently most of the sample handling and preparation modules developed for droplet microfluidics can be integrated. For instance, individual cells may be encapsulated in droplets and chemically lysed. These sample droplets may be delivered to multi-dimensional separation analyses using the interface. Furthermore, post-separation interfacing with matrix assisted laser desorption ionisation mass spectrometry (MALDI-MS) has also been investigated and is described in the next chapter. For multidimensional separation, transfer between dimensions will be limited by the speed of the separation, rather than droplet injection. In addition, the interface can be set up to accommodate a multi-channel separation device permitting the development of an automated high-throughput electrophoretic system. Such a system has the potential to handle hundreds of samples with minimal user intervention.

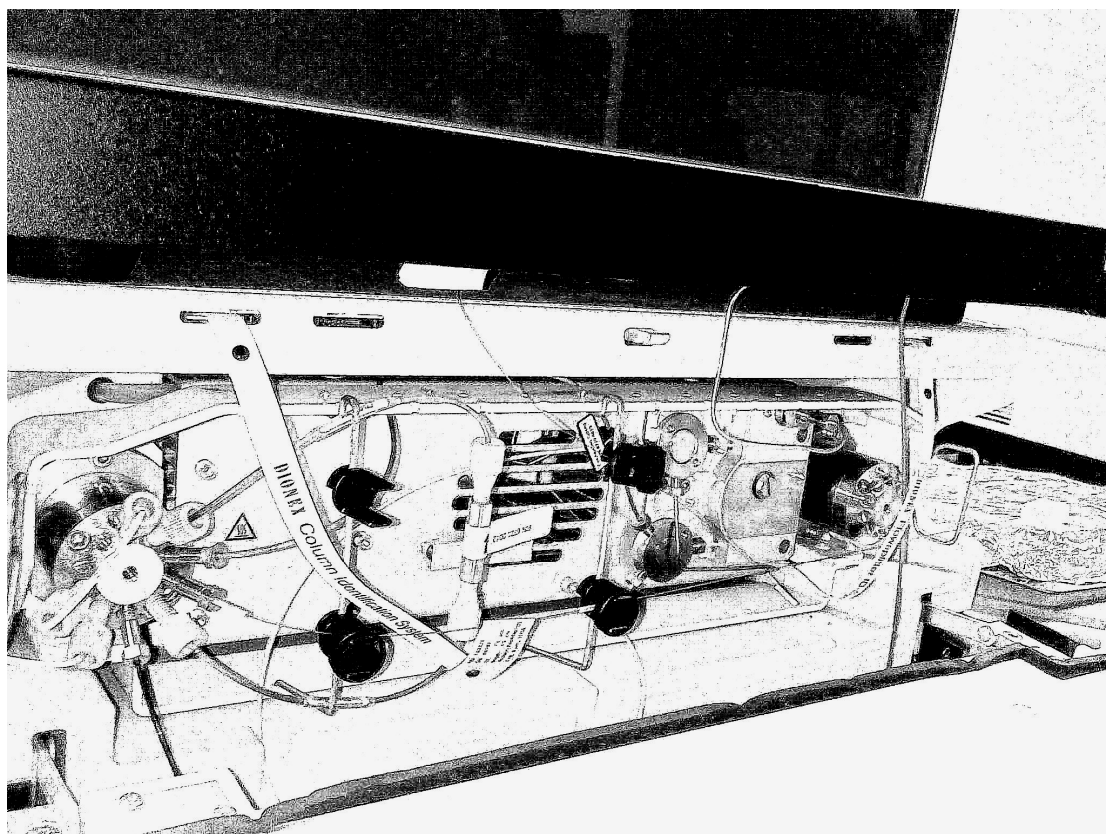
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Chapter VI: Nano LC MALDI mass spectrometry droplet interface



Synopsis

The work presented in the following pages described a novel droplet interface between Nano-liquid chromatography and Matrix assisted laser desorption/ionisation mass spectrometry (MALDI). The interface fractionates the eluate from the Nano-LC into droplets and transports them to a deposition probe. The probe absorbs the oil phase and collects the aqueous phase at the mouth of the spotter prior to deposition. Fractionation of the eluate as it exits the LC column retains the resolution obtained during the separation and simplifies the downstream MS analysis. The interface was placed approximately 10cm from the end of the LC column while the LC column is connected to the commercial spotter using 97cm long fused silica capillary. At the point of droplet formation the LC eluate is mixed with MALDI matrix. A droplet may contain several bands depending on the resolution and conversely a series of droplets may contain fractions of a single band. Typically, an average volume of 250 nL was spotted every 15 seconds, therefore between 15 to 20 droplets are collected at the probe tip before they are spotted on to the MALDI target. Peptide mixtures resulting from the Trypsin digestion of Cytochrome c and Bovine serum Albumin (BSA) were used to test the manual droplet spotter and compare it to the automated commercial spotter. Employing the commercial spotter the Cytochrome c sample showed 7 peaks out of 12 while the manual droplet spotter showed 11. A similar trend was observed for the more complex BSA peptide digest, with the commercial spotter showing only 12 out of a possible 22 peaks while the droplet spotter showed 19. MS data also indicates that the peaks were spotted in the same order in which they were eluted from the column using the droplet spotter. However, this was not observed for the samples spotted using the commercial spotter. In both cases the droplet interface device performed better, seeing over 86% of all peptides present compared to 56% observed by the automated spotter.

Although the use of droplet helps to maintain the separation obtained by LC, the oil carrier phase adversely affects the matrix-analyte crystallisation and desorption/ionisation process. As demonstrated here, the presence of oil, leads to the formation of large matrix crystals which are distributed non-uniformly across the surface of the sample well. Signal acquisition under these conditions is erratic due to the inhomogeneous surface and the overlying oil layer which may prevent desorption or ionisation from occurring. This chapter also investigates the potential of cross-contamination between droplets at different points on the interface device and the effect of MALDI matrix solvents on the PDMS devices and droplet formation.

6.1 Introduction

Proteomic studies have traditionally been conducted using 2-dimensional (2D) gel separations [1-6]. Here a complex protein mixture, often derived from a cellular lysate, is separated on the basis of iso-electric points (pI) of the proteins in the first dimension followed by a mass-based separation in the second dimension. This technique can separate up to 11,000 proteins from a lysate [7], but it is restricted to analysis of the most highly abundant proteins in the sample and proteins with extremes of mass or pI tend to be underrepresented. Following separation, protein spots are picked and digested with Trypsin. The resulting peptides from each protein are then analysed using mass spectrometry. This sequence of analysis, though commonly used, is tedious, time consuming, irreproducible, requires large volumes of buffers and an experienced operator. An alternative approach which does not suffer from these problems is to employ a single dimension mass separation followed by liquid chromatography (LC) coupled to on-line to mass spectrometry [8]. Following this trend, a recently developed 'shotgun' technique employs a multi-dimensional liquid chromatography [9, 10] separation of a mixture of proteins that have been digested with Trypsin prior to peptide analysis on a mass spectrometer. This process does away with the need to separate, isolate and clean samples, reducing the complexity of traditional process. This minimises manual error and improves reproducibility.

Mass spectrometry (MS) is a powerful technique capable of obtaining data from a wide range of samples [11-17]. However, MS methods are unable to provide results when applied to complex sample mixtures. To reduce sample complexity, mass spectrometers are often coupled to one or more separation techniques either in an on-line or off-line format. Liquid chromatography [18-22] and capillary electrophoresis [23-25] in their various forms are some of the most commonly used and efficient separation techniques available and thus are among the primary techniques that have been hyphenated to the mass spectrometer. Due to their inherent nature, liquid chromatography and electro-spray ionisation (ESI) mass spectrometry are the most frequently coupled techniques. The compatibility of the mobile phase (acetonitrile) and the nanolitre to microlitre per minute flow rates of micro-column LC separation with nano-flow ESI sources has facilitated on-line coupling. However, as an ionisation technique, matrix-assisted laser-desorption ionisation (MALDI) is less responsive to the presence of additives like trifluoroacetic acid (TFA), sodium dodecylsulphate (SDS) and salts that are common in the separation media of LC and CE. MALDI is also known to produce simpler spectra than ESI as the majority of ions formed have single charges. Multiply

charged ions are usually formed due to sodium ion contamination and this effect can be ameliorated by washing crystallised MALDI spots in water prior to analysis [26]. In addition, biomolecule separations using nano-LC can take several hours depending on the gradient applied. For an off-line MALDI analysis, the eluted samples can be spotted onto the MALDI plate and analysed in minutes after the completion of the separation. Furthermore, these spotted samples can be stored over a period of days and analysed using different spectrometric techniques. Conversely, running an on-line ESI MS analysis under these conditions would require the mass spectra to be acquired over the duration of the LC run. In complex samples by the time MS/MS analysis on a selected peak is conducted co-eluting peaks may be missed and the LC separation will have to be re-run.

Integration of LC and MALDI MS using both off-line and on-line interfaces has been developed by many groups [27, 28]. Typically, LC effluent is channelled to a tee-junction where it is mixed with the appropriate MALDI matrix and then spotted on to the target using contact or non-contact deposition. Contact deposition of LC effluent on to a MALDI target can be carried out in two basic ways. The effluent can be collected and deposited in the form of discrete droplets or in a continuous ‘streak’ across the MALDI target [29, 30]. The latter is harder to perform and can lead to a loss of resolution due to axial diffusion of the sample prior to matrix crystallisation. The most common form of deposition is contact deposition of a discrete droplet. A drawback of this technique is potential scratching of the MALDI target plate or fracture of the deposition probe. A number of commercially available LC to MALDI spotting systems based on contact deposition have been developed, for example the Probot (Dionex, Amsterdam). Several methods of non-contact deposition have also been developed by different groups including electrospray deposition [31, 32], impulse driven deposition [33], heated drop deposition [19], pulse electric field driven deposition [34]. On-line MALDI has also been achieved using spray [35, 36], continuous flow [37, 38] or mechanical interfaces, of which only continuous flow and mechanical interfaces have been applied to microfluidic devices.

Conventional methods of fraction collection involve the transfer of eluting bands into sample vials or microtitre plates, with a minimum fraction volume of several microlitres. Depending on the instrumental set up the fraction is often diluted and requires further sample processing to allow MS analysis. Niu *et al.* [39] and Edgar *et al.* [40] have recently demonstrated fraction collection and compartmentalisation into droplets from microfluidic separation systems. These approaches retain

separation efficiency during transfer between analytical techniques and allow the collection and transfer of nanolitre volumes without dilution. However, both groups have not investigated the interfacing of these systems to mass spectrometry. Furthermore, in 2010, Li and co-workers [41] described a commercial set up for LC effluent fraction collection and integration to offline ESI-MS. The authors report the deleterious effect of oil on the electro-spray ionisation process and the MS signal obtained. They report a passive method for oil removal where the oil collects at the emitter tip migrates away from it and is then siphoned away in Teflon tubing. A set of 4 small molecule metabolites and a Trypsin digest of Corticotropin releasing factor were used in demonstrating the potential of this approach.

The efficiency of oil removal is a critical parameter when employing droplets to connect analytical techniques. In 2006, Hatakeyama *et al.* [42] demonstrated the use of droplets as micro-reactors followed by MALDI-MS analysis via contact deposition. The authors did not discuss oil removal or the effect of oil on the MS results. Other studies [43, 44] have described different techniques to transfer droplets between segmented and aqueous streams, all of which involve dilution of the fractionated peaks. Recently, Zhu and Fang [45] have reported the extraction of aqueous droplets using a hydrophilic ‘tongue’. The sample is siphoned into a hydrophilic carrier channel from the hydrophobic segmented flow channel from where it is delivered to ESI-MS using a monolithic ESI emitter. The microdevice was used to monitor peptide alkylation and although this was achieved, the extraction format described diluted the sample post extraction. Neither groups explored integration of their devices to an LC separation.

The LC-MALDI interface designed and discussed herein employs a hydrophobic and oleophilic PTFE membrane to actively absorb the oil at the tip of the deposition probe. The device consists of two parts; a droplet generation device which fractionates the LC effluent into droplets and a deposition probe which is used to spot the sample on to the MALDI-MS target. The device can be positioned several centimetres from the outlet of the LC separation column ensuring that the resolution gained by the separation is preserved once the analytes are fractionated into droplets. The device mixes the sample with matrix prior to droplet formation, allowing the droplets to be delivered to the target in a MALDI-ready format. Initial experiments involved calibration of the droplet generation and deposition system using native proteins. Cross contamination between deposited droplets was also investigated. The effect of the oil on the MALDI MS signal and matrix

crystallisation process was examined since oil has been shown to effect signal obtained when using electrospray ionisation.

6.1.2 MALDI Mass Spectrometry

Matrix-assisted laser desorption was introduced as an ionisation method for large biomolecules in 1988 by Hillenkamp and Karas [46]. Since then it has become a common tool for the analysis of proteins and peptides [47, 48]. In this technique the protein sample is mixed in solvent containing small organic molecules (the matrix). Compared to other laser desorption and ionisation techniques employed in mass spectrometry, the laser energy is absorbed by the matrix instead of the sample analyte. This allows efficient and controllable energy transfer to the analyte from the laser via the matrix. Furthermore, biological sample integrity is maintained during the transfer of energy, preventing thermal decomposition due to overheating. The matrix, which is present in large molar excess, is also used to isolate analyte molecules from one another preventing cluster formation. Association between analyte molecules may lead to the formation of complexes which are too large to desorb and analyse [49, 50].

Matrix selection and optimisation of sample preparation are important steps in the analysis of biopolymers using MALDI-MS. There are several methods used for sample preparation which are almost all based on a modified version of the traditional dried drop technique. Here, the aqueous sample solution is mixed in a 1:1 ratio with the appropriate matrix and applied to the MALDI target. Typically small volumes in the range of 0.25 μl to 2 μl are applied to the probe and allowed to dry at room temperature. UV-MALDI matrices are made up in volatile organic solvents such as ethanol and acetonitrile since this increase the rate at which the spotted sample dries. Faster drying normally leads to the generation of a homogenous thin layer of a 'solid solution' of analyte-doped matrix crystals. The presence of carrier phase above the matrix-analyte crystals would adversely affect the sublimation and matrix plume formation process, inhibiting any ionisation mechanism. The matrix-analyte crystals are then exposed to short, intense pulses of laser energy. It is this process that leads to ionisation of the analyte; however the underlying mechanisms leading to ionisation are not well understood. There are several theories explaining primary and secondary ion formation. In essence, photo-excitation of the matrix by laser energy causes localised sublimation and expansion of the matrix. The co-crystallised analyte molecules move into the matrix plume and ionisation may occur at anytime during this process. The simplest and most

widely accepted ion formation pathway involves the transfer of protons from the ionised matrix molecules to the analyte in the matrix plume [51, 52].

The initial studies were carried out using nicotinic acid as a matrix [46], however over the years a large number of matrices have been identified with improved ionisation efficiencies [53-55]. The requirements of a MALDI matrix are strong absorbance at the laser wavelength employed, vacuum stability, low molecular weight to allow sublimation and chemical inertness [47-49, 51, 52]. Different classes of analyte require different matrices. In the current work protein and peptide desorption and ionisation were achieved using 'Trans-3,5-dimethoxy-4-hydroxy cinnamic acid, commonly known as Sinapinic or Sinapic acid and 4-hydroxy α -cyanocinnamic acid (HCCA) respectively.

6.2 Experimental

6.2.1 Interface Device and Deposition Probe

The nano liquid chromatography to MALDI mass spectrometry interface was designed in two parts; a schematic of each part is illustrated in Figure 6.1. The first was a two inlet T-junction droplet generation microdevice used to mix the LC effluent with MALDI matrix and then fractionate the mixture into droplets. The second part was a deposition device which delivers and deposits the droplets on to the MALDI target and extracts oil from the segmented flow. The interface device as shown in Figure 6.1 was fabricated in PDMS using an SU-8 master as described in Chapter 2. The channels accommodating the liquid chromatography capillary (30 μm I.D. and 100 μm O.D.) and Teflon tubing (200 μm I.D. and 320 μm O.D.) were 300 μm wide and 150 μm high. The capillary and tubing were inserted into the microdevice after plasma bonding and sealed using wax or PDMS. The PDMS seal was created by depositing uncured PDMS around the tubing and heating to 150°C until the PDMS cures. Narrower channel dimensions were used for droplet generation and mixing. The 300 μm wide LC inlet channel narrows to 100 μm which is the size of all other channels in the device, while the height of all the channels remained 150 μm .

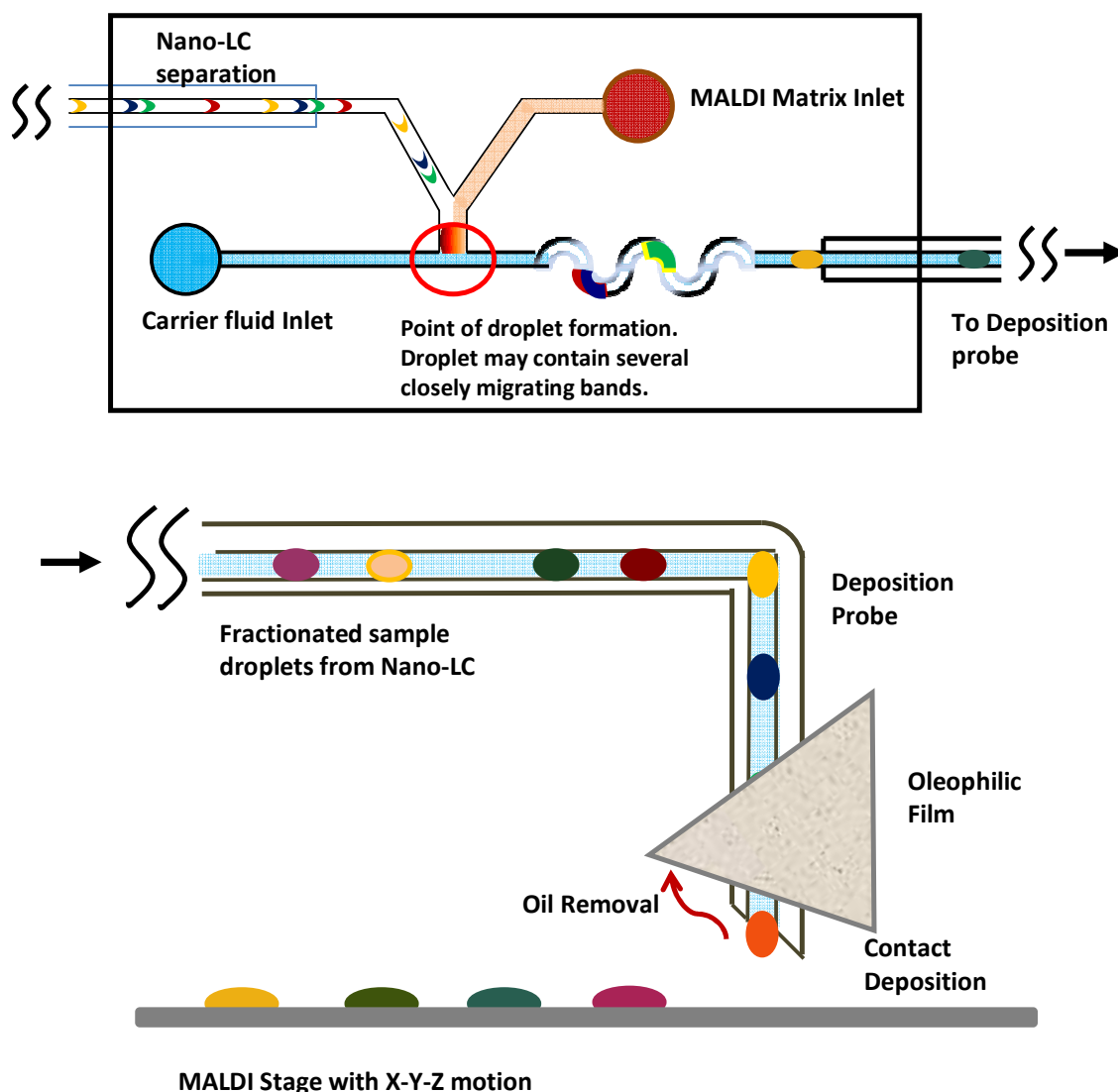


Figure 6.1: Schematic of the Nano-LC MALDI-MS droplet interface device. The top panel details the interface device which was fabricated in PDMS. Water-in-oil droplets were generated from oil and aqueous solutions using the T-junction microchip having two aqueous inlets, one oil inlet and an outlet. The LC effluent was transferred in to the device via a silica capillary which was inserted into the side of the PDMS device using a specially designed channel. The second aqueous inlet was used to deliver MALDI matrix. FC-40 oil was used as the carrier phase. The LC effluent and MALDI matrix meet at the T-junction and are then pumped into the oil stream where they are broken into droplets due to the shear force of the oil. A single droplet may contain several bands or a large band (high concentration sample) may be split into several droplets. The S-bends in the device are used to induce rapid mixing between the LC-effluent and the MALDI matrix. The lower panel illustrates the components forming the deposition probe. These include the 200 μm internal diameter Teflon tubing and the oleophilic PTFE membrane. Droplets generated in the interface device are transported to the MALDI target using the Teflon tubing. The tubing is wrapped with the PTFE membrane which extracts the oil leaving the aqueous droplet suspended at the tip of the tubing. This droplet is then spotted on to the MALDI target via contact deposition.

The two other inlets on the micro-device were for the MALDI matrix and FC-40 oil introduction. The inlets were created by punching holes using a 1mm biopsy punch (Premier Healthcare and Hygiene, UK) through to the channels which were connected to syringes (containing stock solutions of each reagent) using Teflon tubing (Cole Parmer, London, UK). Liquids were driven through the device at specified volumetric flow rates using syringe pumps (PHD Programmable 2000, Harvard Apparatus, UK). To assess and calibrate the deposition probe, initial experiments were carried out without a nano-LC separation using native proteins. The LC capillary was replaced with a standard capillary of similar internal and external diameters. For calibration experiments using proteins the LC capillary inlet was also attached to a syringe loaded with stock protein solution and the flow rates set depending on the size of the droplets required. For all experiments the flow rates were set to 0.5 $\mu\text{l}/\text{minute}$.

The outlet end of the Teflon tubing was cut at an angle ($\sim 30^\circ$) and inserted into the oleophilic membrane such that the tubing protruded 1 to 2 mm from the edge of the membrane. Prior to insertion the tubing end was coated with Duxback (PPG Industries, Pittsburgh, PA). This arrangement of tubing and film ensures that oil is absorbed as it exits the tubing whilst the aqueous droplets collect at the tip of the tubing. The set-up was secured using a staple clip, adhesive tape or an acrylic holder mounted on a stage with XYZ motion. All three securing modes worked efficiently as long as the tubing was maintained at the appropriate distance from the film. The Teflon tubing, along with the oleophilic membrane and described mooring form the deposition probe. Deposition on to the MALDI plate was carried out by contacting the tip of the probe to the surface of the plate either for a specific amount of time (e.g. 25 or 15 seconds) or until the aqueous droplet fell under the effects of gravity. As the deposition probe was constructed from a flexible polymer the MALDI target and deposition probe remained undamaged during the spotting process. Due to the location of the LC system used to accomplish this work, the staple or tape technique which requires less space was found to be simpler to implement. However, for all protein calibration experiments when the LC instrument was not required, the acrylic holder with the XYZ stage proved useful.

6.2.2 Sample Preparation

The interface device and deposition probe were tested prior to attachment to the nano-LC. These experiments were performed using protein stock solutions of Bovine Serum Albumin (BSA),

Cytochrome C, and Lysozyme (Hen Egg white) each prepared in a 0.1% trifluoroacetic acid (TFA) solution (Sigma Aldrich, UK). The concentration and molecular weight of each protein used is detailed in Table 6.1. Each protein solution was mixed using a vortex, then filtered and degassed prior to use. The MALDI matrix used for this work was sinapinic acid (Sigma Aldrich, UK). The matrix was prepared at a concentration of 12.5 mg/ml in 45% acetonitrile (ACN), 45% ethanol and 10% of an aqueous solution of 0.1% TFA. The TFA and matrix solutions were freshly prepared fresh each day.

Protein	Molecular Weight (in kDa)	Stock Concentration (mg/ml)
BSA	66	7.0
Lysozyme	14.3	18.0
Cytochrome C	12.4	5.5

Table 6.1: Proteins used in this work, along with their molecular weight and stock concentrations. Each protein was prepared in a 0.1% TFA stock solution. The samples were diluted as detailed in the text prior to use. The molecular weights listed here are those provided by the manufacturer.

6.2.3 Mass Spectrometry Instrumentation

Mass spectrometry was carried out on two different instruments. Mass analysis of proteins was carried out on a Micromass® MALDI micro MXTM mass spectrometer (Waters, Manchester, UK). Positively charged ions in the mass range of 5000-150000 Daltons were analysed in the linear mode. A hundred single-shot spectra were gathered manually in groups of 10 from random spots within each sample well on the MALDI plate. The spectra gathered were summed and processed using the smoothing and base line correction functions provided in the Mass Lynx software.

Mass analysis of positively charged peptide ions was carried out on the MALDI 4800 (Applied Biosystems, California, USA). Positively charged ions in the mass range of 500-5000 Daltons were analysed automatically in the reflector mode. A thousand single-shot spectra were gathered in groups of 25 from random spots within each sample well on the MALDI plate. A peptide calibration ladder (Figure 6.2) was analysed prior to each 'real' sample set to ensure mass accuracy. The peaks are labelled with the expected mass value which varied by 0.1Da from the experimental values obtained. The peptide ladder consisted of des-Arg1-Bradykinin (904.47 Da), Angiotensin I

(1296.685 Da), Glu1-Fibrinopeptide B (1570.677 Da), Adrenocorticotrophic hormone 1-17 (ACTH, 2093.084 Da) and ACTH 18-39 (2465.197 Da) and was obtained from Dionex Corporation.

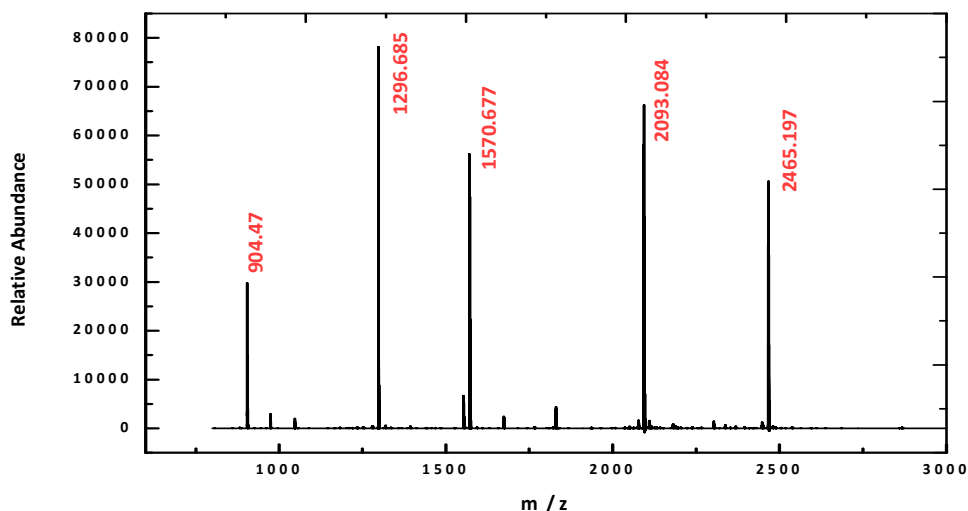


Figure 6.2: Peptide calibration ladder analysed prior to MALDI mass spectrometry analysis. The sample contained 5 peptides listed in the text above and was prepared in 0.1% TFA. Each peak is labelled with the expected mass value.

6.2.4 Nano-LC Instrument and Automated MALDI Spotter

The LC instrument used to test the microfluidic interface was the UltiMate™ 3000 system (Dionex Corporation, Amsterdam) consisting of a degasser, an autosampler (WPS 3000), a thermostatted flow manager module (FLM 3000), a UV flow cell (UVD 3000) and Micro-pumps (LPG 3000). A reproducible flow rate was maintained through the separation column by using a chromatographic splitter; the split ratio was adjusted to approximately 100:1. The mobile phases used for the reverse phase separation were Buffer A: 0.1% trifluoroacetic acid and 2% acetonitrile v/v and Buffer B: 0.1% TFA and 90% acetonitrile v/v. Two commercially available peptide digests were used to test the microfluidic interface and compare it to the Probot automated LC-MALDI sample spotter (Dionex Corporation, Amsterdam). Lyophilised Trypsin digests of Cytochrome C (Dionex Corporation, Amsterdam) and Bovine Serum Albumin (New England BioLabs, USA) were reconstituted in 200 µl of 0.1% TFA.

The peptide digest samples were loaded on to a PepMap™ 300 µm x 5 mm C₁₈ reverse phase trapping column. After 4 minutes the sample was eluted into the PepMap™ 75 µm x 150 mm C₁₈ analytical separation column in back flush mode at a flow rate of 0.4 µl/minute. The separation

was performed across a gradient of 0-60% Buffer B in 36 minutes. The peptides eluted off the analytical column were passed through the UV flow cell to ascertain resolution between peaks and then run into the Probot spotter. Detection was carried out at 220 nm. The length of capillary (30 μm ID and OD) joining the analytical column to the UV flow cell was approximately 62 cm and the length of capillary connecting the flow cell to the spotter was 72 cm. Once required resolution was obtained the sample was run directly into the spotter without routing it through the UV flow cell. The length of capillary connecting the separation column to the spotter was 97 cm.

6.3 Results and Discussion

6.3.1 Protein Calibration Experiments

6.3.1.1 Droplet Generation

Droplets are generated within microfluidic channels using two immiscible fluids; typically oil and an aqueous solution [56-58]. Aqueous solution is extruded into the oil phase at the T-junction of the device and is broken into droplets due to the shear force of the oil. The aqueous droplets are enveloped by the oil and separated from the surface of the device by a thin layer of oil. Consequently, the droplets formed are discrete environments where biomolecules are shielded from the surface of the device preventing sample loss due to hydrophobic interaction between the sample and channel surface. The interface used to generate water-in-oil droplets functioned in a similar manner. The aqueous phase was a mixture of protein solution and MALDI matrix while the carrier phase was FC-40 oil. MALDI matrix, Sinapinic acid was prepared in a mixture of organic solvents, acetonitrile and ethanol, which are polar solvents completely miscible with water. The protein and matrix solutions meet at the junction of the two inlets and bud off into the oil carrier stream. Once generated, the droplets are channelled through several S-bends, which aid in the mixing of the two aqueous components and then pass into tubing leading to the deposition probe.

The droplet generation process was monitored for several hours to determine the effect of the matrix solvent on the device and droplet formation. During initial experiments the matrix solution

appeared to have a greater affinity for the hydrophobic PDMS surface than the oil carrier phase FC-40. This resulted in the matrix behaving like the carrier phase and splitting the oil into droplets (Figure 6.3). This was observed even when the matrix was diluted in a 1:1 ratio with aqueous protein stock solution, and when an HCCA matrix was used instead of sinapinic acid. Once the droplets transferred into the Teflon tubing this effect was not observed and the matrix and protein solutions formed droplets with concave edges carried by the oil phase.

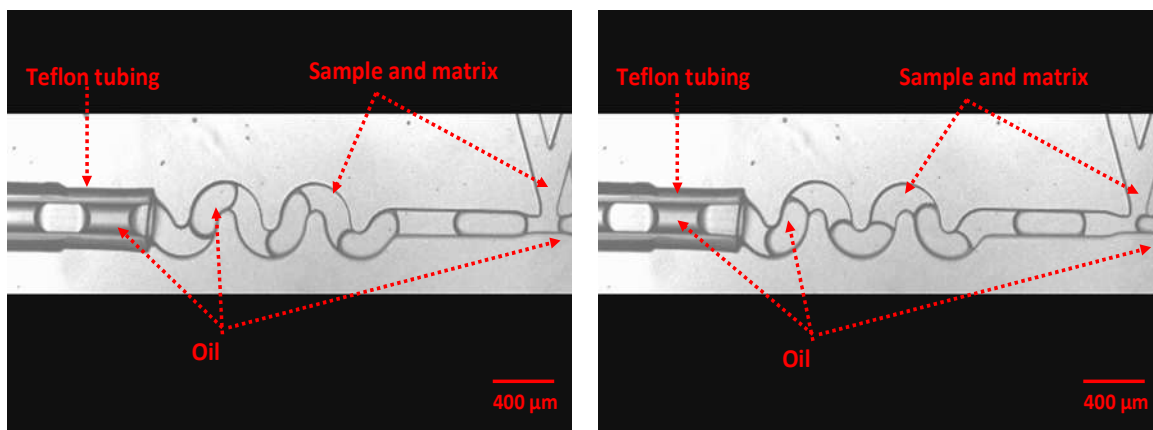


Figure 6.3: Droplet malformation in PDMS microdevice. The left and right panels are separated in time with the right showing the progress of droplet formation 2 seconds after the left. The mix of MALDI matrix and protein solution showed better wettability compared to the oil phase, generating oil in water droplets at the T-junction of the microdevice. The oil droplets migrate towards the outlet driven by the flow of sample and matrix. On entering the Teflon tubing inserted at the outlet the condition reverses to ‘normal’ with the formation of water in oil droplets. Within the Teflon tubing the oil phase showed greater wettability compared to the mixture of matrix and sample leading to the reversal.

Direct contact of matrix and protein solution on the PDMS surface was exacerbated due to this effect leading to increased sample loss due to adhesion to the PDMS surface. To minimise this effect the PDMS surface was treated with the commercial hydrophobic surface coating reagent Duxback® (PPG Industries, Pittsburgh, PA) after plasma bonding by pumping the solution through the entire device. It is ideally suited to treating glass, however a number of authors have successfully used it in treating PDMS surfaces to aid droplet generation. Droplet generation videos and contact angle experiments also indicate some level of change in the surface chemistry of the PDMS channels. Normally, PDMS presents a hydrophobic surface, however due to adhesion or adsorption of charged ions the surface may become hydrophilic. Duxback is usually applied to ensure a homogenous hydrophobic surface which will prevent any beading of aqueous droplets to the channel walls. The coating was carried out before sealing the inlet and outlet tubing, for two minutes. Following this air was blown through the device to remove any excess liquid, dry the

coating to the surface, and ensure that all the channels remained unblocked. Duxback treatment alters the surface characteristics of PDMS and allows the formation of water-in-oil droplets (Figure 6.4). However, the shape of the droplets formed was unusual. Typically, water-in-oil droplets show concave edges, the droplets produced after treatment with Duxback had flat edges (the leading edge appears concave due to motion of the droplet). This result indicated that the Duxback treatment increased the affinity of the oil carrier phase for the surface compared to the MALDI matrix and sample solution mixture. However, the relative affinities had not been altered sufficiently to generate ‘model’ water-in-oil droplets.

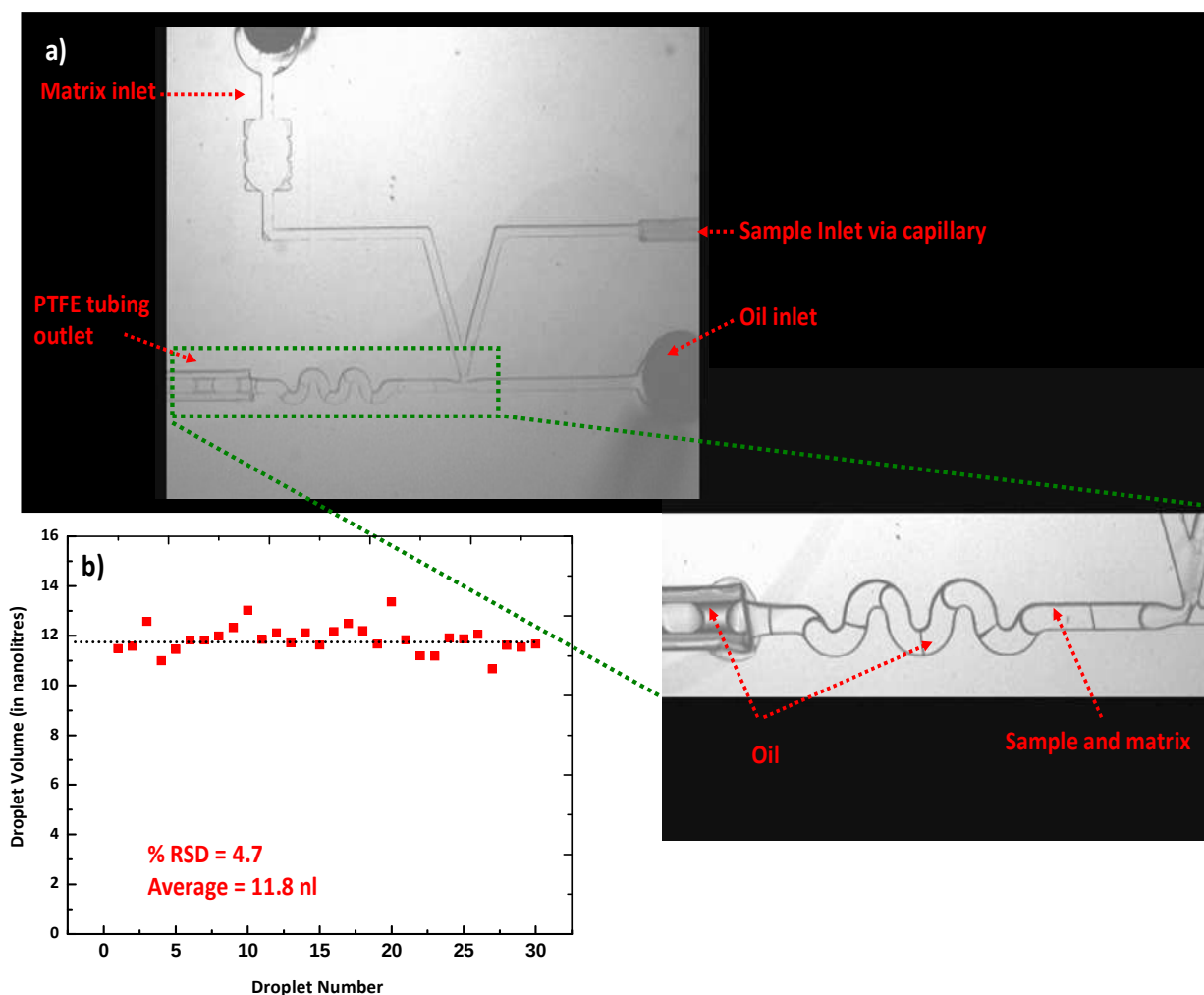


Figure 6.4: Droplet formation in the PDMS interface post Duxback treatment. (a) The effect of the Duxback hydrophobic surface coating on the formation of water-in-oil droplets. The matrix -analyte solution shows decreased surface wettability from contact angle measurements taken post surface treatment. (b) A plot of the variation in droplet volume obtained post treatment with Duxback. An average volume of 11.8 nl with a 4.7% RSD was obtained over N=30 droplets. Oil, MALDI matrix and protein sample were loaded under similar flow rates of 0.5 $\mu\text{l}/\text{minute}$.

Droplets produced under these conditions had an average volume of 11.8 nl with a relative standard deviation of 4.7% (Figure 6.4b). These values were determined using the known width of the Teflon tubing (200 μm) to determine the length from which the approximate volume was calculated using the equation for volume of a cylinder ($\pi r^2 h$), where r is the radius of the cylinder or half width of the droplet and h is the height of the cylinder or length of the droplet. The lengths of the droplets were determined using Image J analysis software. Employing a Teflon coating to circumvent this problem is currently under investigation. There are several practical problems associated with coating the internal surface of PDMS channels with Teflon. Commercially available coatings have curing temperatures above 200 °C. In addition Teflon applied to a PDMS device is absorbed into the body of the device, due to its porous nature. Therefore, post curing, most devices have blocked channels and irregularly coated surfaces.

Surface wettability and hydrophobic loss and recovery of silicone rubbers coating is generally characterised using contact angle measurements [59]. The contact angle can be defined as the angle formed by a liquid at the three phase boundary where a liquid, gas and solid intersect; it is a quantitative measure of the wetting of a solid by a liquid [60, 61]. Low values of the contact angle indicate that the liquid wets the surface well, whilst high values of the contact angle indicate poor wetting [61]. Consequently, measurements were taken to analyse surface wettability of PDMS prior to and post Duxback treatment. Contact angle measurements were taken of a sessile droplet using a Krüss goniometer (Krüss GMBH, Germany). The basic elements of a goniometer include a light source, sample stage, lens and camera. Contact angles were measured using drop shape analysis software provided with the instrument. Typically, a value is obtained by functional fitting of the shape of the drop followed by determination of the slope of the tangent to the drop at the solid-liquid-vapour interface.

Cured PDMS layers approximately 2 mm thick were cleaned by sonicating in water for 5 minutes followed by drying under nitrogen. Duxback was applied to the PDMS by wiping the surface with a reagent soaked fibre-free tissue. For each reading, 5 μl droplets of water, 0.1% TFA, ethanol, acetonitrile (ACN), FC-40 oil, sinapinic acid and HCCA each at 12.5 mg/ml prepared as MALDI matrices were pipetted on to the surfaces. Additionally, both matrices diluted 1:1 in 0.1 % TFA, to mimic the aqueous sample prepared on-chip, were also prepared and pipetted on the the PDMS surface. The contact angle value obtained under each condition is listed in Table 6.2.

Sample	LEFT/ RIGHT	PDMS	Average	PDMS with	Average	Surface Wettability
		Contact Angle (degrees)		Duxback Contact Angle (degrees)		
Water	L	104.9	104.85	107.5	106.5	↓
	R	104.8		105.5		
FC40	L	34	38.05	20.7	19.85	↑
	R	42.1		19		
Ethanol	L	35.5	35.6	44.5	45.75	↓
	R	35.7		47		
Acetonitrile	L	51.1	51.9	45.6	44.85	↑
	R	52.7		44.1		
HCCA matrix	L	46.2	46.4	51	50.2	↓
	R	46.6		49.4		
Sinapinic acid	L	47.4	47.6	52.9	53.5	↓
	R	47.8		54.1		
TFA 0.1% in water	L	104.4	104.65	109.3	109.25	↓
	R	104.9		109.2		
HCCA with TFA (1:1)	L	76.4	76.65	80.3	80.8	↓
	R	76.9		81.3		
Sinapinic with TFA (1:1)	L	70.7	71.2	83.7	84.4	↓
	R	71.7		85.1		

Table 6.2: Contact angle measurements performed on PDMS and Duxback treated PDMS. PDMS layers were cleaned and dried prior to testing. The Duxback coating was applied using a fibre free tissue. Measurements were taken for both aqueous and oil phases on each surface. All components of the aqueous phases were tested individually and as prepared when used for droplet formation. Both left and right contact angles are listed as significant differences were observed between them for certain reagents. Larger differences between left and right values were observed on the Duxback treated PDMS due to an increase in surface roughness. Average contact angle values and the change in surface wettability post treatment are also listed.

The average contact angles obtained over three readings (Left and Right) for each condition are listed in Table 6.2. The contact angle values for the non-coated surface of PDMS indicates that the oil should wet the surface preferentially compared to the matrix solutions in TFA. However as this does not occur an alternative explanation must be sought. One possible explanation is that the TFA solution and Matrix in ACN and ethanol do not thoroughly mix at the T-junction. The contact angles values of the individual components of the matrix solution, specifically ethanol, show that they have similar or greater surface wettability compared to FC-40 oil. After the application of Duxback to the surface of PDMS the contact angle for all tested reagents increases except for FC-40 oil and acetonitrile. However the change in surface wettability prompted by the coating acts to return the droplet formation process back to the expected water-in-oil format. The flat-edge shape of the droplets obtained after the coating could be due to the small increase in surface wettability observed for acetonitrile. Contact angles from both the left and right hand side of the droplet were recorded. The difference in values observed can be attributed to surface roughness, swelling of the PDMS, surface heterogeneity and impurities from the applied solution adsorbing on the surface. A greater difference between left and right contact angles was observed on the Duxback treated PDMS surfaces due to increased surface roughness caused by an uneven surface coating. Less variation may have been observed if the Duxback had been spin coated on to the surface of the PDMS and then dried. However this would not accurately reflect the nature of the coating within the microchannel.

6.3.1.2 Contamination

A major concern and potential vulnerability of the deposition probe was sample cross - contamination at the tip of the probe and on the surface of the oleophilic film. Both these possibilities were investigated to ascertain if they occurred. Cross contamination between droplets at the tip of the deposition probe was examined. Fluorescein iso-thiocyanate (FITC) loaded droplets were generated and fed into the probe, and the tip was placed on an oleophilic membrane. Images of the tip were taken prior to, during expulsion and post removal of the droplet as shown in Figure 6.5. The droplet was removed by spotting the droplet on to the MALDI target ensuring the tip remained untouched. As can be seen in Figure 6.5c, post spotting no FITC can be observed on the tip of the probe. The brightness and contrast of the image were increased to 90% (Figure 6.5d) and the tubing is observed to exhibit a low-level fluorescence signal. This indicates that once spotted an aqueous droplet leaves almost no trace on the

deposition probe preventing cross contamination between spotted droplets and those still contained in the probe.

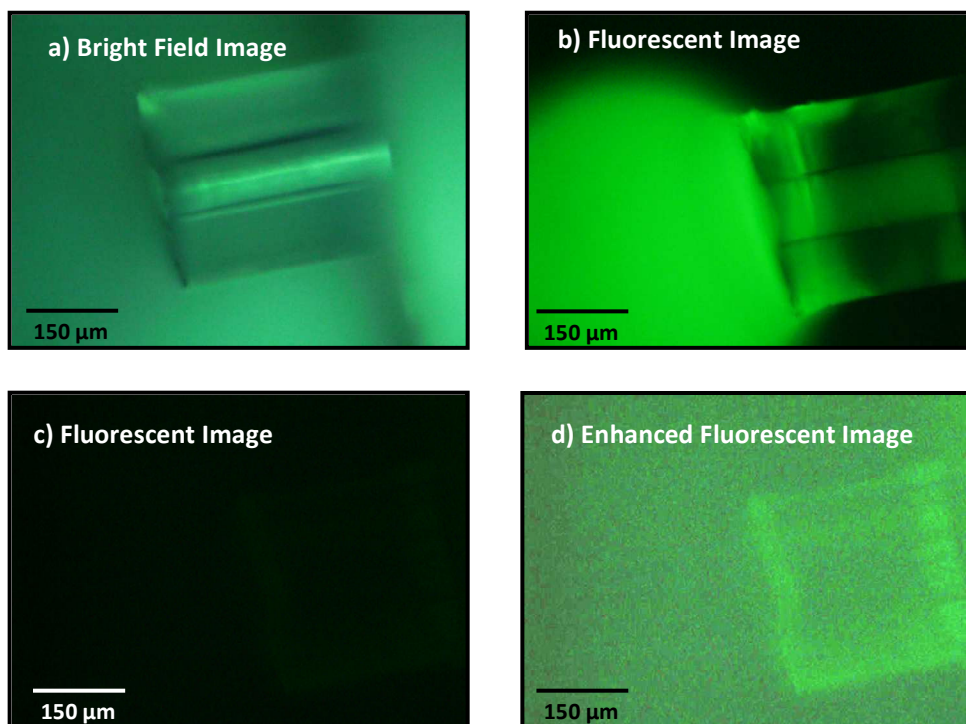


Figure 6.5: Assessment of cross-contamination at the tip of the deposition probe during contact deposition. (a) A bright field image of the flat cut PTFE tubing in the absence of droplets. (b), (c) and (d) are fluorescent images of the tubing during and post expulsion of a 180 $\mu\text{g}/\text{ml}$ FITC droplet. The brightness and contrast of Panel C was increased to 90% to produce the image in Panel D. Once spotted the tubing does not retain significant aqueous volume of the droplet.

Contamination of the oleophilic membrane by the protein sample and cross-contamination between droplets on the surface of the membrane was also studied. A dilution series of BSA in water was prepared and used to generate a calibration curve of absorbance versus concentration using the Nano Drop 2000 (Thermo Scientific, Massachusetts, USA) (Figure 6.6). The calibration curve showed good linearity over the range tested with an R^2 value of 0.9991. 5 μl samples from two points along the curve, (232.5 and 465 $\mu\text{g}/\text{ml}$ BSA), were spotted on to the oleophilic film and left for 1 minute, following which it was re-collected and an absorption reading taken. The concentration of the spotted sample was determined using the calibration curve. A 5-10% drop in protein concentration was observed post spotting on the membrane (Table 6.3). BSA is a highly adhesive protein used in many microfluidic applications to reduce adhesion of reaction components to the walls of the microfluidic device by competitively adhering to the wall. One of

several reasons for its adherence to the surface of PDMS devices is the hydrophobic interaction [62, 63]. A similar interaction could be the reason for the drop in protein concentration observed on the 'dry' film.

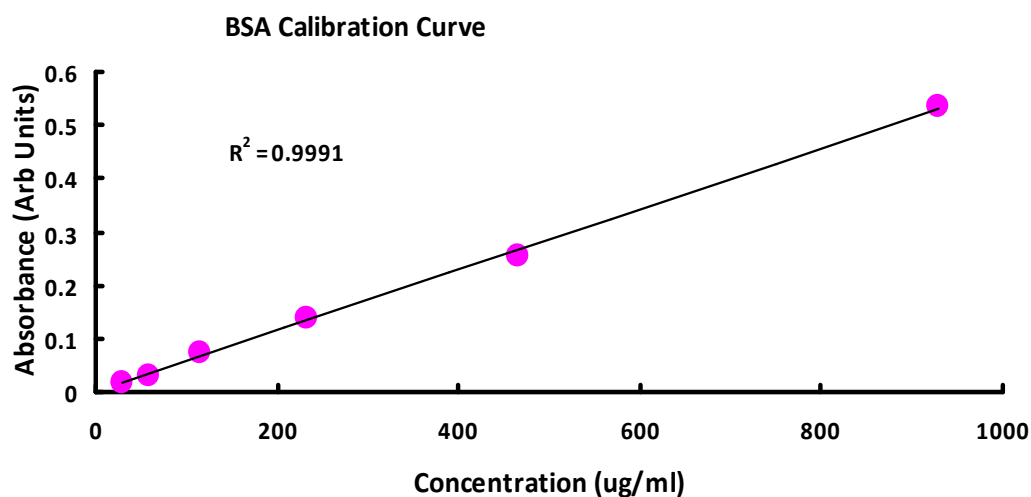


Figure 6.6: Protein adhesion to the 'dry' PTFE membrane. The calibration curve generated to quantify changes in BSA concentration after it is applied to the membrane. An R^2 value of 0.999 was obtained, indicating excellent linearity.

BSA Sample Concentration ($\mu\text{g/ml}$)	UV absorbance at 214 nm	Concentration as determined from Calibration curve	
232.5	0.129	214	
	0.126	209	
	0.138	229	
	0.127	210.67	
	0.13	215.67	Mean
		7.24	% RSD
465	0.267	444	
	0.247	410.67	
	0.255	424.00	
	0.235	390.67	
	0.251	417.33	Mean
		10.25	% RSD

Table 6.3: UV absorbance values and concentrations derived from the calibration of BSA after application to the membrane. A 5 to 10 % decrease in protein concentration was observed.

The same experiment was repeated with the membrane wetted with the FC-40 oil carrier phase. The BSA was applied to the wetted film at a concentration of 465 $\mu\text{g}/\text{ml}$ and showed an increase in UV absorbance post removal as shown in Table 6.4. However, using UV absorbance values to establish concentration in the presence of a possible contaminant absorbing in the same region is inaccurate. Absorbance readings for FC-40 oil samples were taken and are listed in Table 6.4. A ratio of absorbance values at two wavelengths is often used to assess contamination. However, as FC-40 shows significant absorbance values at both wavelengths recorded, a ratio cannot be used to determine contamination. The increase in absorbance values obtained is most likely due to a combination of effects; low level evaporation, minute amounts of oil transferred during the re-collection process and absorption of oil into the protein droplet during its residence time on the film. Therefore, compared to the ‘dry’ film oil wetted PTFE membrane shows less adsorption of protein.

Sample	220 nm	280 nm	Ratio
BSA Concentration 465$\mu\text{g}/\text{ml}$			
1	0.25	0.022	11.364
2	0.248	0.021	11.810
3	0.241	0.021	11.476
FC-40 with surfactant			
1	1.171	0.327	3.581
2	0.757	0.348	2.175
3	1.138	0.309	3.683
BSA post retrieval from Membrane			
1	0.281	0.021	13.381
2	0.258	0.023	11.217
3	0.287	0.024	11.958

Table 6.4: UV absorbance values at 220nm and 280nm for BSA (465 $\mu\text{g}/\text{ml}$) prior to and post retrieval from the PTFE membrane. Absorbance values for FC-40 are also listed.

In use the membrane is wrapped around the tip of the probe. Before droplet generation it is standard procedure to flow oil through the entire micro-device to ensure all surfaces are wetted by the oil. During this process oil is flowed down the PTFE tubing to the tip of the probe wetting the membrane that is wrapped around the tip of the probe. As the membrane used in the deposition probe is first wetted with the carrier fluid, the matrix-analyte aqueous solution does not have the opportunity to come in contact with the 'dry' film. The use of contact deposition on to the MALDI sample support and the distance between the tip of the probe and membrane ensures that both modes of cross contamination discussed above remain at a minimal level.

6.3.1.4 Droplet Deposition

All control protein samples were spotted using the traditional dried droplet preparation technique. Here 2 μ l of Sinapinic acid matrix was mixed with 2 μ l of protein stock solution on the surface of a layer of Parafilm (VWR, UK), from this 1 μ l of the mix was spotted on each sample well and left to air dry in a clean, dust free area.

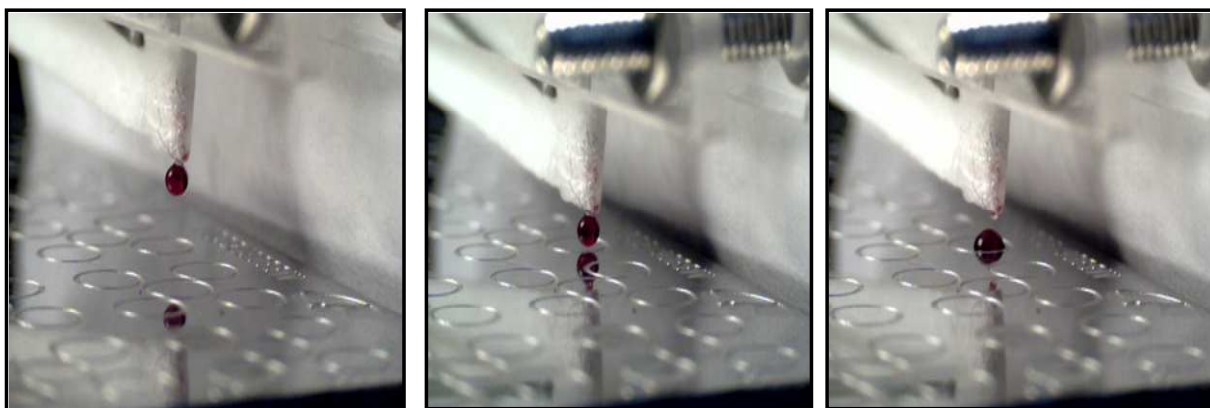


Figure 6.7: Droplet spotting on to a MALDI target. The droplet generation interface device with deposition probe was attached to an X, Y, Z stage using the acrylic holder. Matrix-analyte-dye droplets were generated and spotted on to the MALDI target. The stage was lowered till the analyte droplet made contact with the MALDI target and was then raised. The droplet detaches from the probe tip and is spotted on to the target. BSA at a stock concentration of 7mg/ml BSA prepared in 0.1% TFA spiked with red food dye was mixed in a 1:1 ratio with 12.5 mg/ml Sinapinic acid and fractioned into droplets prior to spotting.

Droplets generated on the interface were spotted on to the MALDI sample support using the deposition probe (Figure 6.7). The emerging aqueous droplets collected at the tip of the probe

while the carrier fluid (FC-40 oil) was absorbed by the oleophilic film wrapped around it. Figure 6.7 illustrates the deposition procedure with the probe mounted on an X, Y, Z motion stage. The probe is lowered manually until the droplet makes contact with the MALDI target and the matrix-analyte droplet adheres to the hydrophilic metal surface, detaching itself from the PTFE tubing when it is lifted away from the target. Once spotted on to the plate the samples air dry, mimicking the dried droplet format of matrix crystallisation. The volume of liquid spotted depended on the inlet flow rate and rate of deposition. For the protein calibration experiments the flow rates at the matrix and protein sample inlets were 0.5 $\mu\text{L}/\text{minute}$ with a spot deposited every 15 seconds. The droplet volume spotted was 250 nL based on the combined inlet flow rates. With an average droplet volume of 12 nL, generally, 21 droplets were collated and spotted per MALDI sample well. As the spots are applied using contact deposition, lower volumes or smaller numbers or droplets can be analysed per deposition by decreasing the deposition time. When connected to the nano-LC, the droplet fractionated effluent was also manually spotted on the MALDI support every 15 seconds over 40 minutes, leading to the generation of 160 spots. The matrix and carrier phase flow rates were set to 0.5 $\mu\text{L}/\text{minute}$ each while the effluent from the LC had a flow rate of 0.4 $\mu\text{L}/\text{minute}$. The spotted drop volumes were therefore 225 nL. MALDI mass spectrometers employed here can obtain signal from drop volumes down to 150 nL with peptide concentrations as low as 300 fM. The limit of detection varies considerably with the ionisation potential of the peptide. Accordingly, an average of 13 droplets are required to obtain a signal, however for samples with high peptide concentrations or ionisation potentials a smaller number of droplets is sufficient to generate a signal. A calibration mix and controls were spotted using pipettes after completing the LC separations.

6.3.2 Effect of Oil on MALDI MS Spectra

In order to use droplets to fractionate and deliver MALDI-ready LC effluent the aqueous phase should be extracted from the carrier phase before it is spotted onto the MALDI target. This is done using the oleophilic film fitted to the deposition probe. The presence of the oil over the matrix-analyte solution is similar to the slow crystallisation procedures demonstrated by several authors. Factors such as the matrix solvent composition, the matrix-sample preparation procedure and the analyte to matrix ratios affect the quality of the MALDI MS spectra obtained for biopolymers. The effect of oil on matrix crystallisation and MALDI MS spectra is demonstrated in Figures 6.8, 6.9 and 6.10.

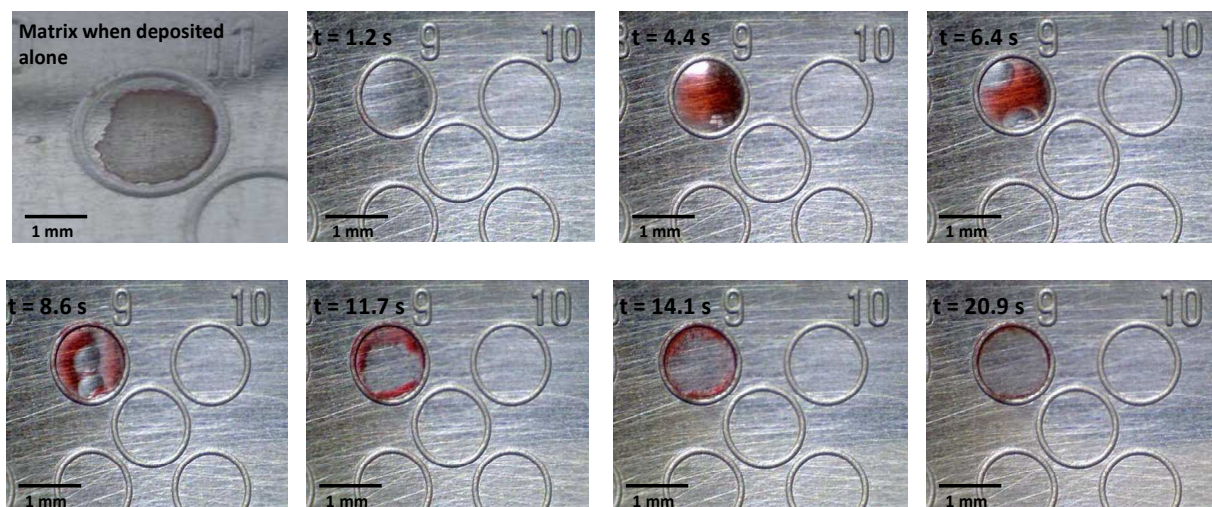


Figure 6.8: Time slices taken from a video recording the effect of FC-40 oil on the crystallisation of HCCA. The first panel illustrates the crystal surface formed using 1 μ l of the matrix prepared in the dried drop format. The first and second time annotated panels show the loading of 0.5 μ l FC-40 oil and 0.5 μ l dye loaded matrix on to the MALDI target. Time slices $t = 6.4$ seconds and $t = 8.6$ seconds show the formation of bubbles within the oil. These bubble swirl around the surface of the sample well, redistributing the dye loaded matrix. Finally the bubbles erupt from the surface of the oil and evaporate, leaving the matrix crystallised at the edges of the sample well. The oil either returns to the depression created in the centre of the well, or is pushed out of the well when the bubbles evaporate.

The HCCA matrix prepared at a 12.5 mg/ml concentration in 45% acetonitrile, 45 % ethanol and 0.1% TFA in deionised water was mixed at a 1:1 ratio with Bovine Serum Albumin at 7 mg/ml in 0.1% TFA spiked with red food colouring dye. Two wells on the MALDI target plate were loaded with 1 μ l of the mix. A further 1 μ l of FC-40 oil was added on top of the matrix-analyte droplet on one well and both samples were dried at room temperature. Figure 6.8 illustrates the effect of oil on the matrix crystallisation compared to the standard dried droplet format shown in the first panel. At 4.4 seconds the sample loaded matrix appears to sink beneath the oil following which the matrix solvent, acetonitrile and ethanol, endeavour to evaporate through the oil forming bubbles. Bubble formation moves the matrix and analyte around the sample well and finally to its edge. The oil gets pushed from the sample well during evaporation and returns to some areas when the solvent has evaporated. This leaves the sample and matrix distributed around the edges of the well, partially or wholly covered with oil. The effect is volume dependent, and increasing ratios of oil to matrix-analyte solution exacerbate the observed edge effect. In the presence of oil most of the solvent evaporates in under 30 seconds so the sample is completely dried in approximately 120

seconds compared to less than 60 seconds for the dried droplet format. Similar behaviour was also noted for Sinapinic acid, the other matrix used in this study.

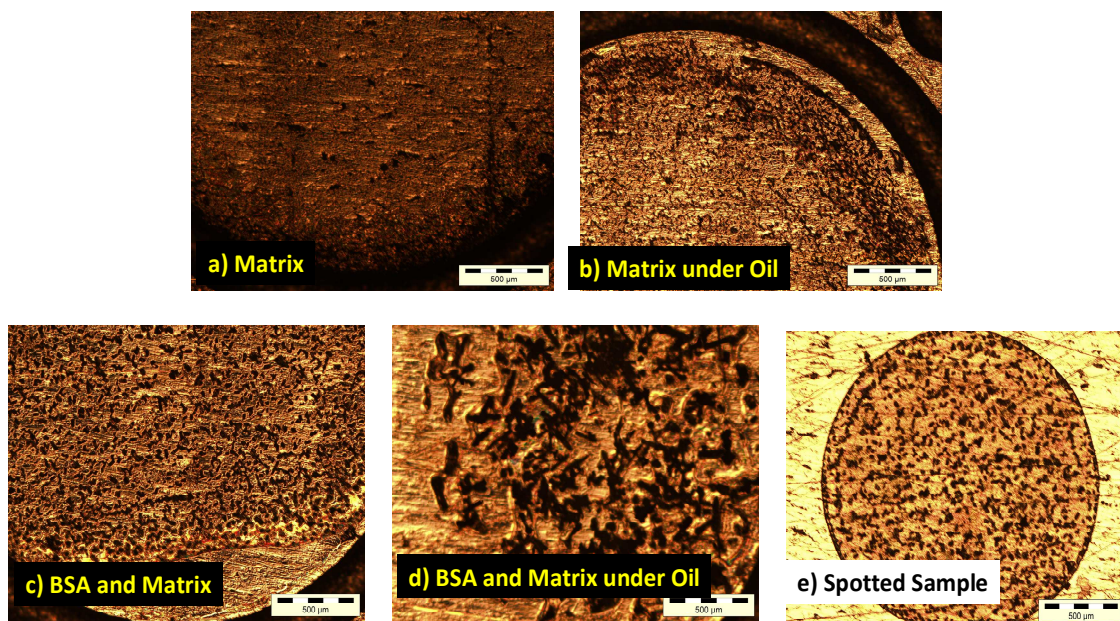


Figure 6.9: Sinapinic acid matrix crystallisation when prepared using the dried drop technique and in the presence of oil. The former is depicted in (a) and (c) while the latter is shown in (b) and (d) for crystals formed from the matrix alone and the matrix mixed with BSA respectively. (e) Image of the crystals formed when a sample is spotted using the deposition probe.

Matrix surfaces produced using the two aforementioned matrix-analyte sample preparation techniques were imaged at x5 magnification. Figures 6.9 and 6.10 show the matrix (HCCA or Sinapinic acid), the matrix with BSA, matrix under FC-40 oil and matrix and BSA under FC-40 oil. Figure 6.9 also shows an image of matrix and BSA spotted using the deposition device at x5 magnification. To generate the spot in the image droplets were collected for 15 seconds prior to spotting. Images for both matrices show large crystals when the matrix or the matrix-analyte solution is crystallised under oil compared to those obtained when prepared under the traditional dried drop method. Under visual comparison, the spotted sample in Figure 6.9e showed crystals of similar size to those prepared using the traditional dried drop method. Crystallisation of the HCCA matrix under FC-40 oil also showed the ‘edging’ effect described previously with the matrix distributed in patches around the edge of the deposition zone leaving large areas of the sample well empty (Figure 6.10). The presence of oil leads to the formation of an inhomogeneous surface with large matrix crystals distributed randomly over the surface of the sample and protein

'hot spots' making signal acquisition complicated. The edging effect may lead to little or no signal if acquired using the automated mode which is pre-set to examine a specific target area within the sample well.

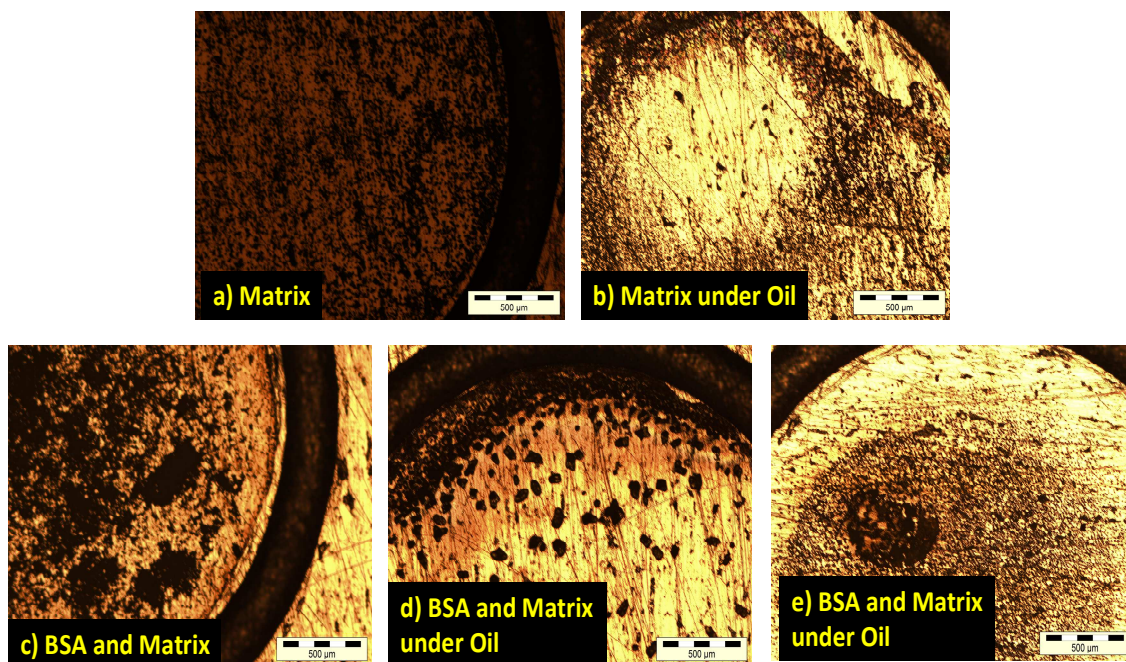


Figure 6.10: HCCA matrix crystallisation when prepared using the dried drop technique and in the presence of oil. The former is depicted in (a) and (c) while the latter is shown in (b), (d) and (e) for crystals formed from the matrix alone and the matrix mixed with BSA respectively.

The mass spectra extracted from samples deposited in the presence of FC-40 oil were non-reproducible (Figure 6.11a). Depending on the matrix distribution on the MALDI plate, manually acquired signals showed protein peaks in the regions where matrix crystals were observed. However, under automatic acquisition conditions, extremely low-level signal or no signal at all was observed for 50% of the spotted samples. As described previously, the presence of oil leads to large matrix crystals spread inhomogeneously over the surface of the sample well. Accordingly, little or no correlation between signal intensity and sample concentration was observed. Cohen and Chait [50] have compared the effect of dried droplet and rapid and slow crystallisation methods on the quality of MALDI mass spectra using two different HCCA matrix solvent systems. In both cases they found that the slower crystallisation procedures lead to a mass discrimination effect. Only the higher molecular weight peptides were observed in the MALDI spectra, while the dried drop and rapid techniques showed most or all the peptide peaks expected. It is important to note that their slow crystallisation period is defined in terms of hours.

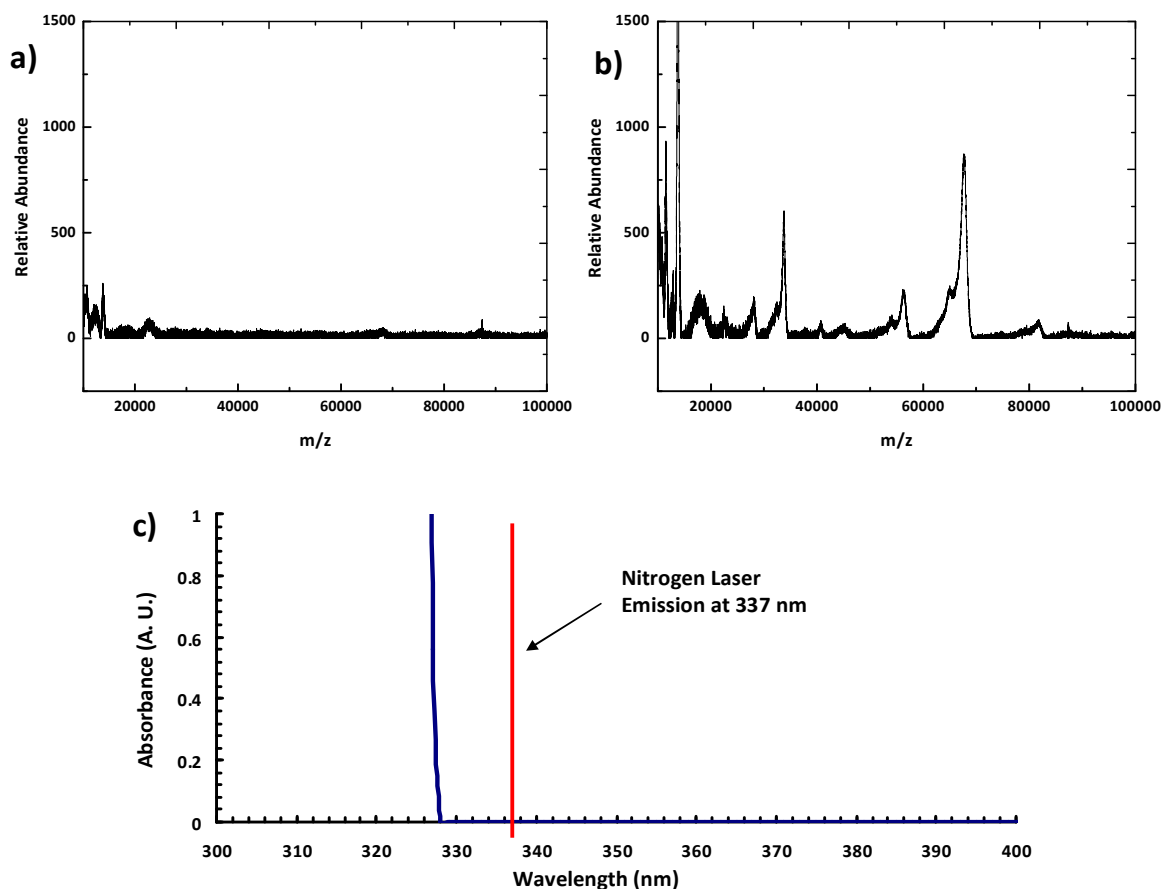


Figure 6.11: Effect of oil on MALDI MS spectra. (a) and (b) detail MALDI mass spectra for matrix-BSA samples spotted under FC-40 oil. (a) shows no peaks while a BSA peak is observed in (b). (c) UV spectra of FC-40 in the wavelength range of 300 to 400 nm. The highlighted area reflects spectral region in which the MALDI MS laser emits.

The Nitrogen laser employed within the Waters MALDI MS emits UV light at a wavelength of 337.1 nm. This is absorbed by the matrix-analyte crystals which sublime and protein ionisation occurs in the gas vapour phase above the matrix surface. These wavelengths are typically not absorbed by the protein analyte thus preventing thermal degradation. The absorption spectrum of FC-40 oil was measured between 300 to 400 nm using an air blank (Figure 6.11b). FC-40 oil does not absorb UV between 328 and 400 nm, therefore its presence over the sample post deposition and crystallisation should not reduce the amount of laser energy reaching the matrix. However, vaporisation of the photo-excited matrix with attached analyte may not occur due to the presence of an oil layer above the matrix-analyte crystals. This will trap gas phase ions preventing them from accelerating towards the detector. In addition, cooling of the matrix prior to sublimation due to the transfer of thermal energy to the oil layer may also impede desorption and ionisation. As

mass spectrometers operate under vacuum, and tests have shown that FC-40 oil evaporates when placed in a vacuum for 10 to 15 minutes, mass spectra obtained over a period of time from the same sample may vary depending on the amount of oil present.

6.4 Protein MALDI MS

Figure 6.12 shows the mass spectrometry results for protein samples spotted on to the MALDI plate using the traditional dried drop technique (Panels a1, a2 and a3) and those prepared using the droplet deposition probe (Panels b1, b2 and b3).

Samples spotted using the deposition probe all exhibit peaks in the appropriate mass-to-charge range for each protein tested and compared well with the control samples spotted using the traditional technique. The y-axis scales were set to similar values for each protein set to enable comparison between signal intensities. The proteins were prepared as detailed earlier (section 6.2.2) and diluted 1:1 in sinapinic acid, reducing their concentration to half the original value. Higher signal intensity values were observed for the high molecular weight protein (BSA) using the deposition probe while values for the lower molecular weight proteins remained comparable. MALDI mass spectrometry is not generally used to quantify proteins as the signal intensities obtained depend on several factors including sample preparation, matrix, laser energy and the presence of contaminants. Nonetheless, similar signal intensities obtained between the spotting techniques indicate that the interface and deposition probe are effective in generating droplets and then separating the oil stream from the aqueous droplets. As discussed previously, the presence of oil in the deposited droplet will affect both the matrix-analyte crystallisation and the analyte ionisation process.

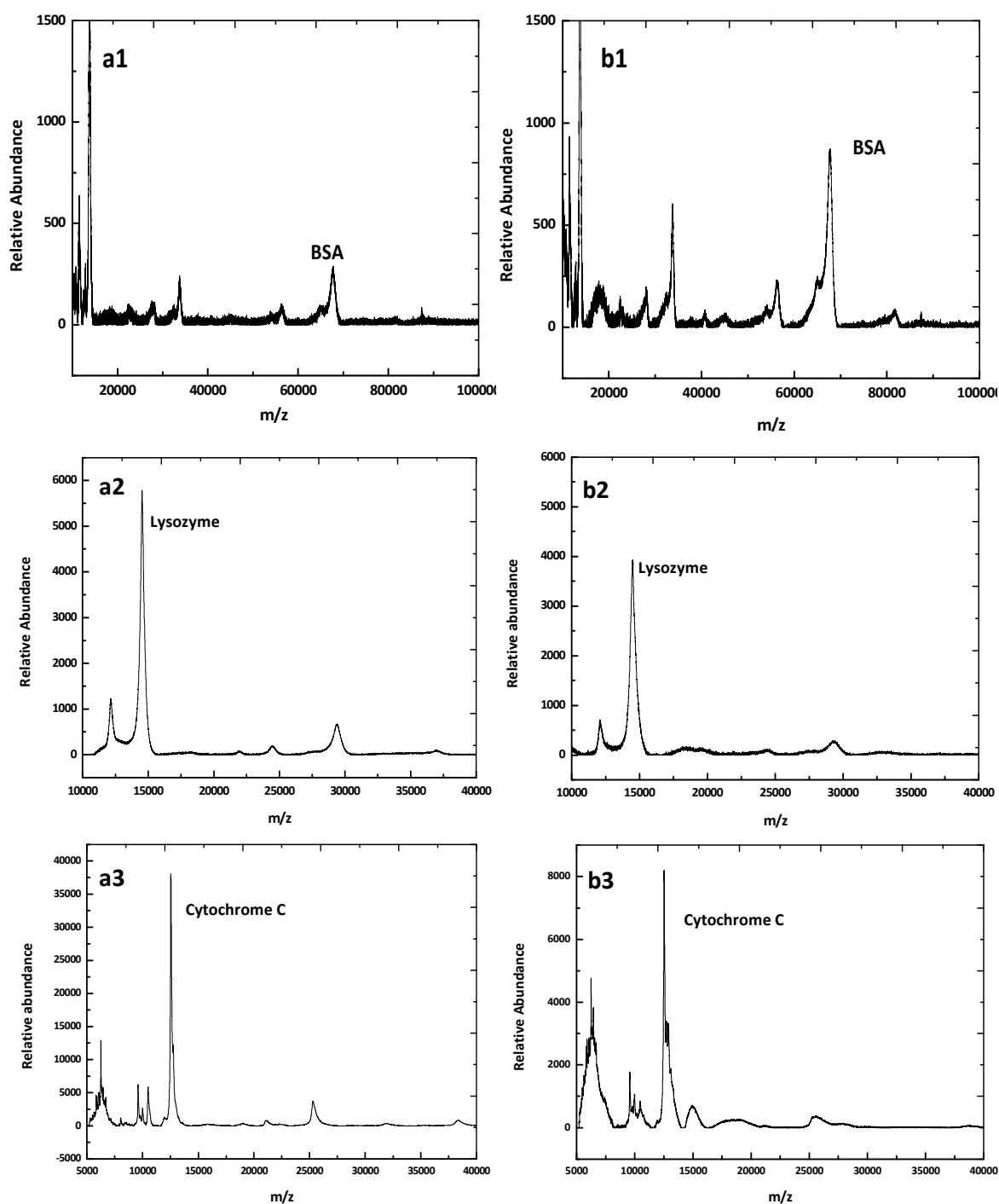


Figure 6.12: MALDI MS spectra for BSA, Cytochrome c and Lysozyme. (a1-3) The spectra obtained when spotted using the traditional dried drop technique. (b1-3) Spectra obtained when using the PDMS interface device and deposition probe. The proteins were prepared at the stock concentrations listed in Table 6.1, and diluted 1:1 in Sinapinic acid matrix prepared at a concentration of 12.5 mg/ml in 45% acetonitrile, 45% ethanol and ten percent 0.1% TFA.

6.5 Nano LC and MALDI MS

The Trypsin digest of Cytochrome C was analysed without a nano-LC separation to demonstrate the advantages of a separation step prior to MALDI mass spectrometry. The analysis of complex mixtures is often complicated by the phenomenon of ion suppression or competitive ionisation. Peptides with higher affinity for charge than others present in the mixture successfully compete for protons generated during the ionisation process and are preferentially detected. The signal corresponding to the low affinity peptides may disappear from the spectrum under these conditions even though they yield an easily detectable signal when analysed separately. Mono-isotopic mass values provided by the sample supplier (Dionex Corporation, Amsterdam) were used to locate peaks in the mass spectra obtained. Only nine out of twelve peaks were assigned (Figure 6.13). The large number of unassigned peaks seen in the mass spectra may be associated with other isotopic forms of the peptides or fragments with varying mass to charge ratio values.

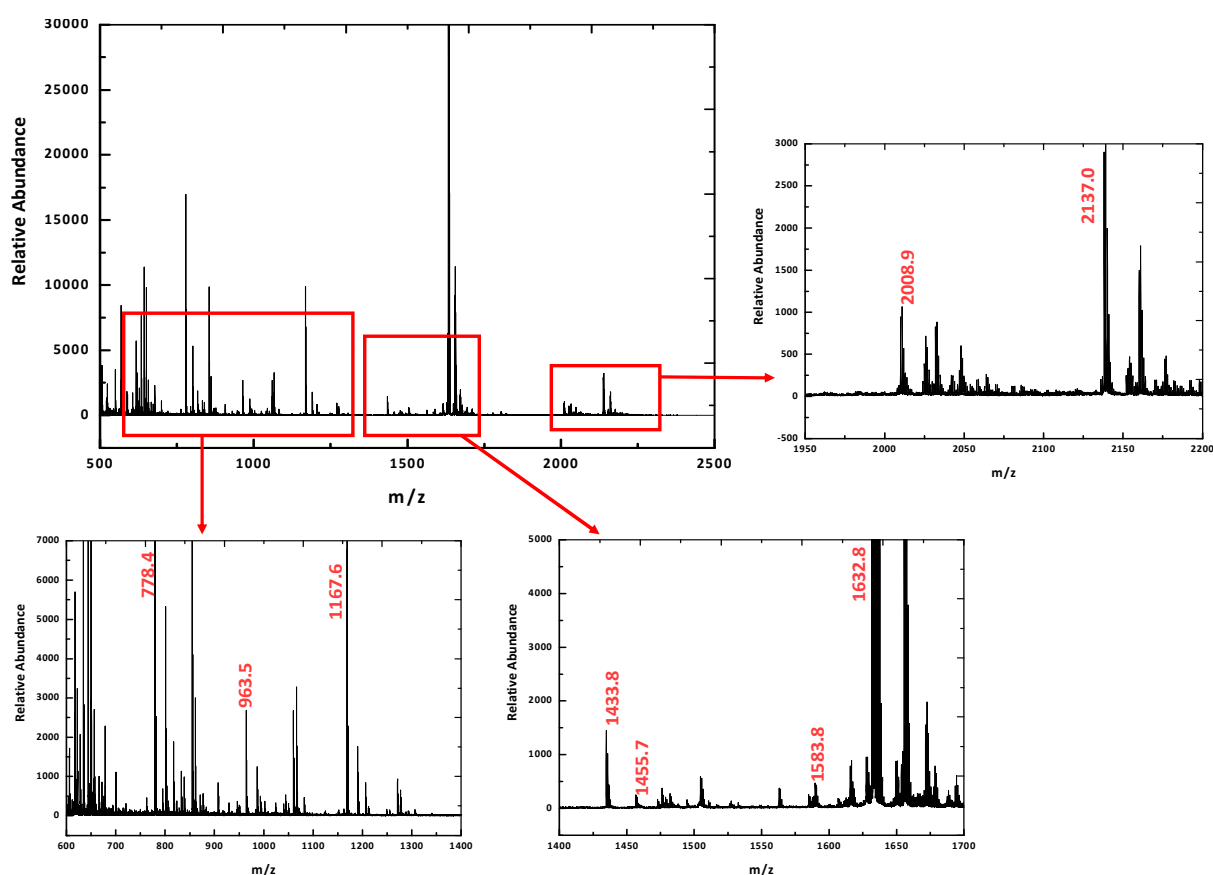


Figure 6.13: Cytochrome C spotted on to MALDI target without separation on Nano-LC. Sample spotted with HCCA matrix. The inset images highlight the peaks obtained in the low, middle and high m/z ratio range. The m/z ratio of Cytochrome C peptide fragments provided by the supplier have been identified in the images.

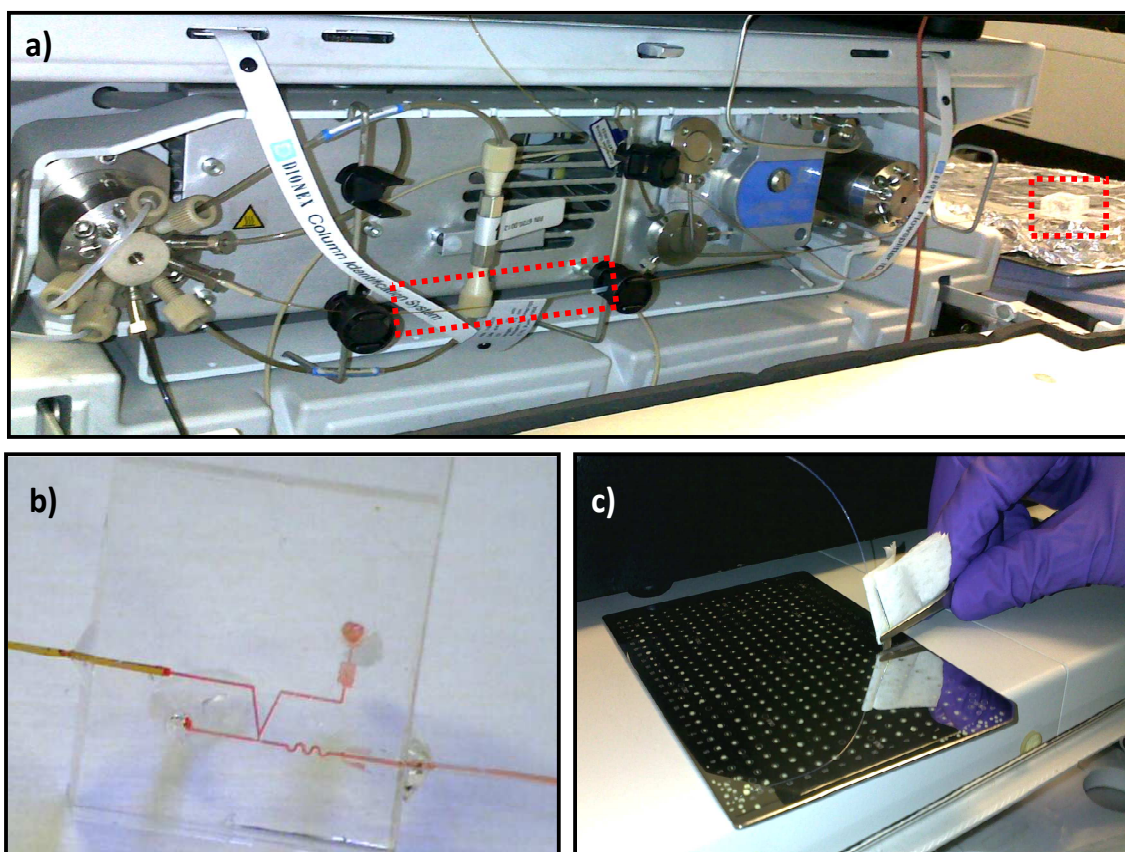


Figure 6.14: Attachment of the droplet generation interface device and the deposition probe to the Nano-Liquid Chromatography separation column. In (a) the chromatography column and PDMS interface device are identified, these components are connected via a 25 cm long fused silica capillary extending from the separation column. The capillary and deposition tubing are inserted into the interface device and sealed using PDMS as detailed (b). A solution of red food dye is used to visualise the channel design and flow through into the deposition tubing. (c) The manual spotting of droplets on to the MALDI target using the deposition probe.

Figure 6.14 illustrates the position of the PDMS interface device with respect to the LC separation column and the spotting procedure. The interface along with the deposition probe was attached to the liquid chromatography system are shown in Figure 6.14a. The 25 cm long fused silica capillary leading from the PepMap™ 75 μm x 150 mm C_{18} analytical separation column was inserted into the PDMS device (Figure 6.14b) and sealed with PDMS cured at 150°C for 5 minutes/hours. The interface device may be attached closer to the separation column; however the current set up of the LC instrument could not be modified to demonstrate this. The LC effluent travels a total of 26 cm before being mixed with the matrix and fractionated into droplets. This compared to the 97 cm travels before it is mixed with matrix using laminar flow and conventional spotting.

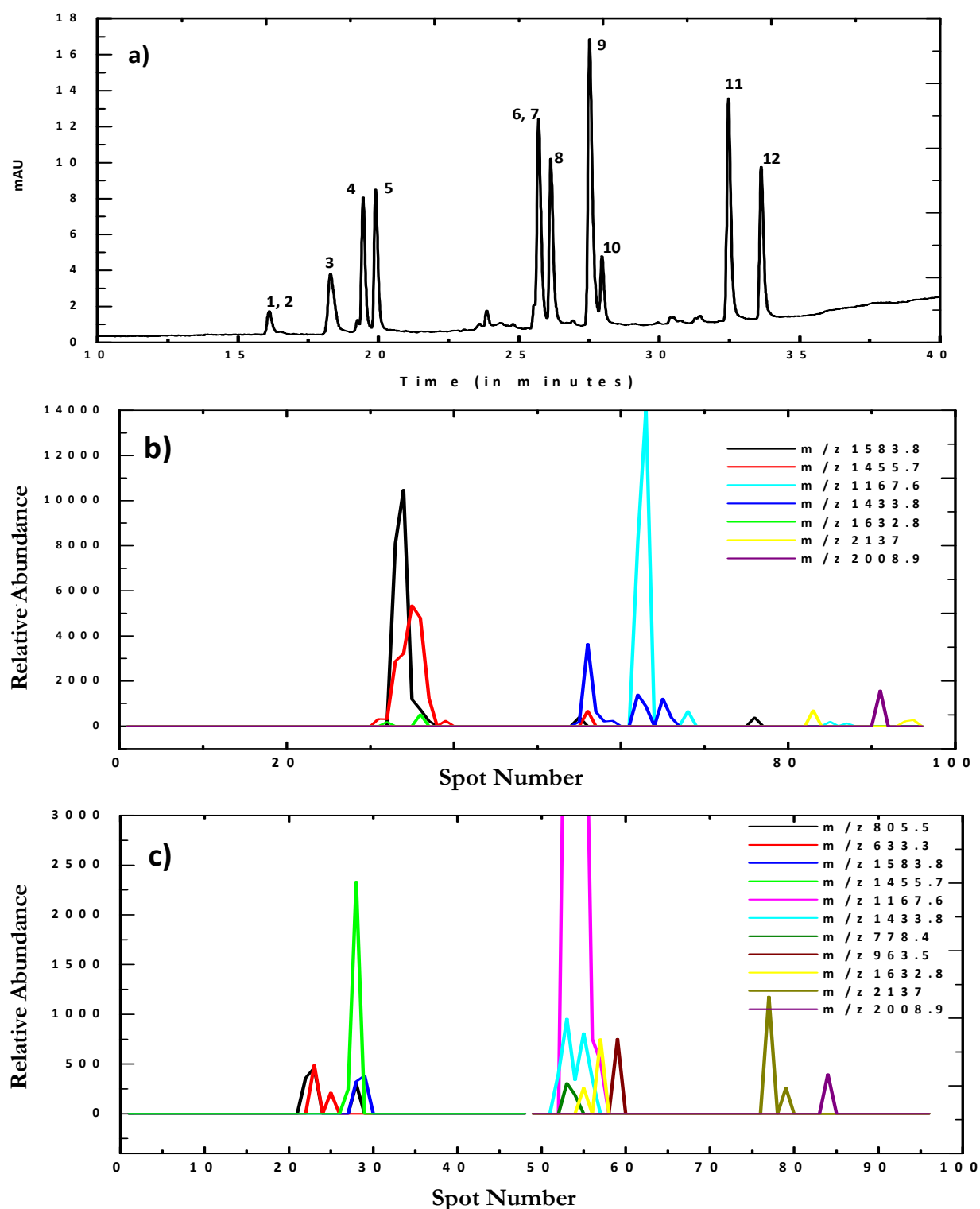


Figure 6.15: LC-MALDI MS separation of peptides generated from Trypsin digested Cytochrome c. (a) The LC separation profile of the protein digest. (b) and (c) detail the mass spectrometry results for the control spotted using a commercial spotter and the test spotted using the deposition probe respectively. The mass spectrometry results were plotted as relative abundance of the peptide versus the spot number or number of wells across which the LC effluent was spotted. The molecular weight of the numbered peaks is listed in Table 6.5.

The two inlet PDMS device mixed the sample eluting from the LC column with MALDI matrix (HCCA at 12.5 mg/ml) in a 1:1 ratio and then split the mixture into droplets (Figure 6.14b). The fractionated sample stored in droplets is transported down a 15 cm long deposition probe and spotted on to the MALDI target. Nano-LC eluate; spotted using a commercially available spotter, Probot (Dionex, Amsterdam), was mixed with the HCCA matrix prepared at 3.0 mg/ml in ACN and water in a 1:1 ratio and deposited using contact deposition. Matrix could not be used at saturation levels, as with the traditional dried drop technique of the deposition probe, due to precipitation and clogging of the deposition device. On the Probot, the sample target holder moves in the X, Y and Z planes to deposit the spots. The instrument allows spotting at a maximum frequency of 12 spots/ minute; however it is generally used at a rate of 2 spots/minute. To allow comparison between the manual deposition probe and automated commercial spotter, a spotting frequency of 4 spots/minute was employed for all experiments. Both methods of spotting generated approximately 90 spots for the Cytochrome c digest sample.

Two Trypsin digested proteins, Cytochrome c and BSA, were used to assess the performance of the deposition probe against a commercially available MALDI sample spotter. Separated peptide fragments were run initially through the UV flow cell of the LC instrument to produce a separation profile, after which the eluted peptides were channelled directly to the appropriate spotter. Routing the samples via the UV flow cell adds a further 32 cm of capillary that the separated peptides have to travel through and increases the total run time. Trypsin digested Cytochrome c produces twelve peptides when separated using Nano-liquid chromatography. The elution order and molecular weight information was provided by the manufacturer for all twelve peaks and are listed in Table 6.4. The LC separation profile obtained is shown in Figure 6.15a, where all peptides except peaks 1 and 2 and 6 and 7 were resolved.

A single peptide can be deposited across several sample spots. The software provided with the MALDI MS instrument scans the spectra obtained and plots the relative abundance versus spot number for each peptide. The distribution of peptide fragments of known mass can be assessed based on this function. The results for each Trypsin digest were collated and presented in Figures 6.15 and 6.16. MS results from the automated deposition of Cytochrome c Trypsin digest showed 7 out of 12 peaks which had mass values similar to those listed by the supplier within a mass tolerance of 0.1. The lower mass peptides were not observed. In comparison the manual deposition probe showed 11 out of 12 peaks within a mass tolerance of 0.1. The only peptide not

observed was the smallest fragment which had a mass of 633.5 Da (peak 3). The elution order is maintained in both spotting formats, although it is explicitly illustrated by the fragments spotted using the deposition probe. The LC separation profile of Cytochrome C can be divided in to 3 groups of peaks, i.e. 1-5, 6-10 and 11 and 12. Using the PDMS droplet interface device with deposition probe provided a MALDI MS result which preserved the resolution obtained during the chromatographic separation. The three distinct sections have been maintained at the peak level, peaks 1-5, 6-10 and peaks 11 and 12 are spotted in the same sequence in which they were eluted from the chromatographic column, with the exception of peak 3 which was not observed. There is a loss in resolution between the bands in each group; however resolution can be improved by increasing the frequency of spotting and reducing the distance between the chromatographic column and the droplet generation device. The results from the automated spotter also show three distinct regions; however they are not as highly preserved, as the presence of peak 10 in the first group demonstrates. Initial results (data not shown) using the automated spotter at a lower deposition frequency of 2 spots/second showed marginally better results than those demonstrated at the higher spotting frequency of 4 spots/minute.

Peak Number and Elution order	Mono-isotopic Mass	Probot automated spotter	Deposition probe manual spotter
1	805.5		X
2	677.4		X
3	633.4		
4	1583.8	X	X
5	1455.7	X	X
6	1167.6	X	X
7	1433.8	X	X
8	778.4		X
9	963.5		X
10	1632.8	X	X
11	2137.0	X	X
12	2008.9	X	X

Table 6.5: Columns 1 and 2 list the elution order and molecular weight of the peptide fragments from the Cytochrome c Trypsin digest. Columns 3 and 4 mark the peptide fragments that appear in the MALDI mass spectra for each spotter.

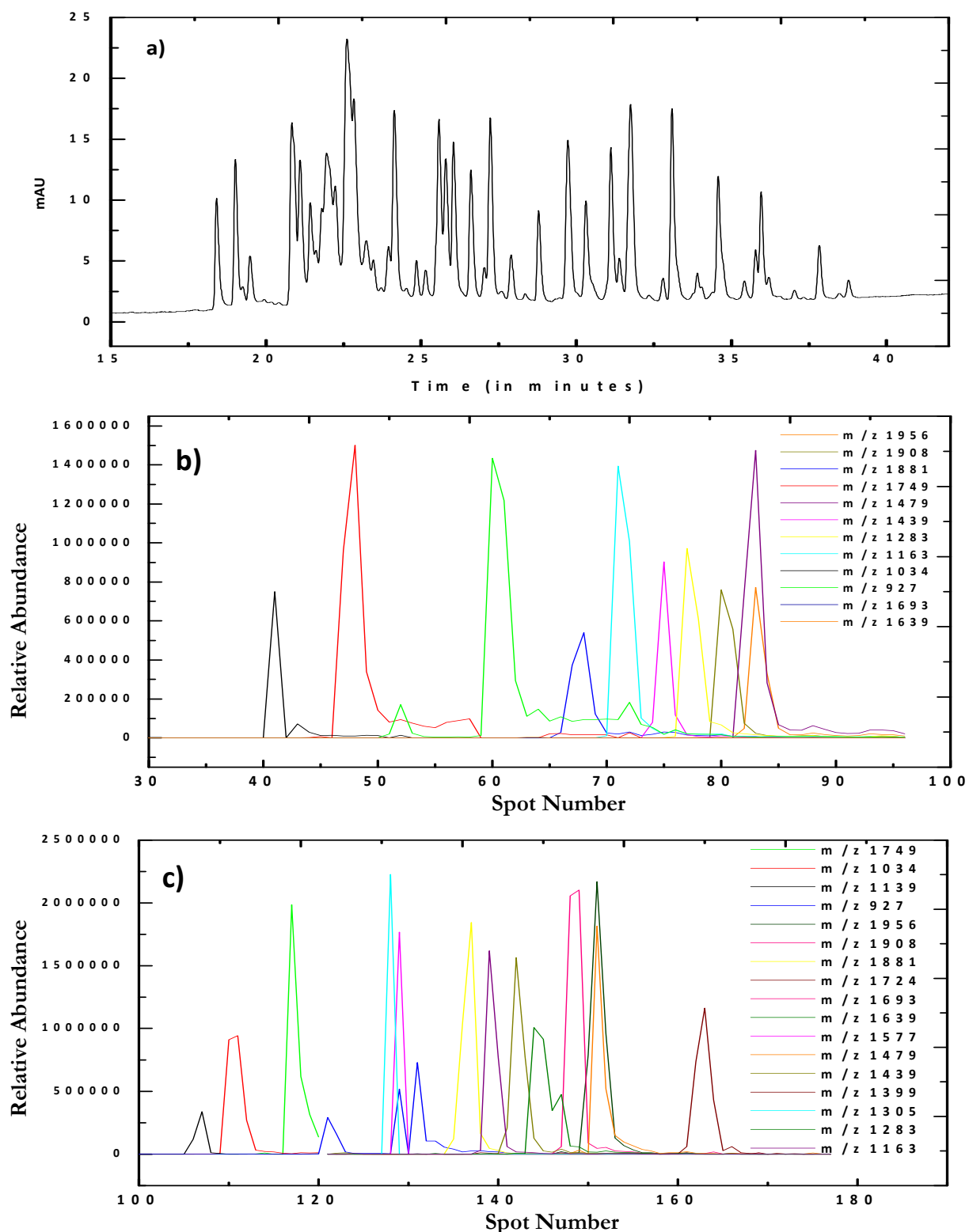


Figure 6.16: LC-MALDI MS separation of peptides generated from Trypsin digested Bovine Serum Albumin. (a) The nano-LC separation profile for the peptide digest. (b) and (c) detail the mass spectrometry results for the control spotted using a commercial spotter and the test spotted using the deposition probe respectively. The mass spectrometry results were plotted as relative abundance of the peptide versus the spot number or number of wells across which the LC effluent was spotted.

Mass	Probot automated spotter	Deposition probe manual spotter
544		
688		
927	X	X
1034	X	X
1107		X
1139		X
1163	X	X
1283	X	X
1305		X
1399		X
1439	X	X
1479	X	X
1567		X
1577		X
1639	X	X
1693	X	X
1724		X
1749	X	X
1881	X	X
1908	X	X
1956	X	X
2492		

Table 6.6: Columns 1 lists the molecular weight of the peptide fragments from the BSA Trypsin digest. Columns 2 and 3 mark the peptide fragments that appear in the MALDI mass spectra for each spotter.

The second sample, Trypsin digested BSA was employed to assess the deposition probe's performance with a more complex sample. The nano-LC separation profile (Figure 6.16a) shows numerous peaks which elute between 15 and 40 minutes. However, the suppliers provided the mass for only 22 out a possible 45 peaks. The molecular mass provided was derived from MALDI MS analysis using an HCCA matrix with no comment on the elution order. The results for the BSA sample followed the trend set by the Cytochrome c sample. The mass spectra data for the Probot micro-fraction spotter showed 12 fragments while the deposition probe showed 19 fragments both within a 0.1 mass tolerance of the mono-isotopic mass values listed by the supplier. Figure 6.16 compares the nano-LC separation with the mass spectra results plotted as relative abundance versus spot number for both deposition formats, whilst Table 6.6 lists the peaks found by each spotting format. Three peptide fragments at 544 Da, 688 Da and 2492 Da are not observed by either spotting techniques. This could be because the LC effluent was not spotted

for the entire duration of the run, losing peaks from the start or the end of the separation. Interference from the matrix could also obscure the signal from the smaller peptide fragments.

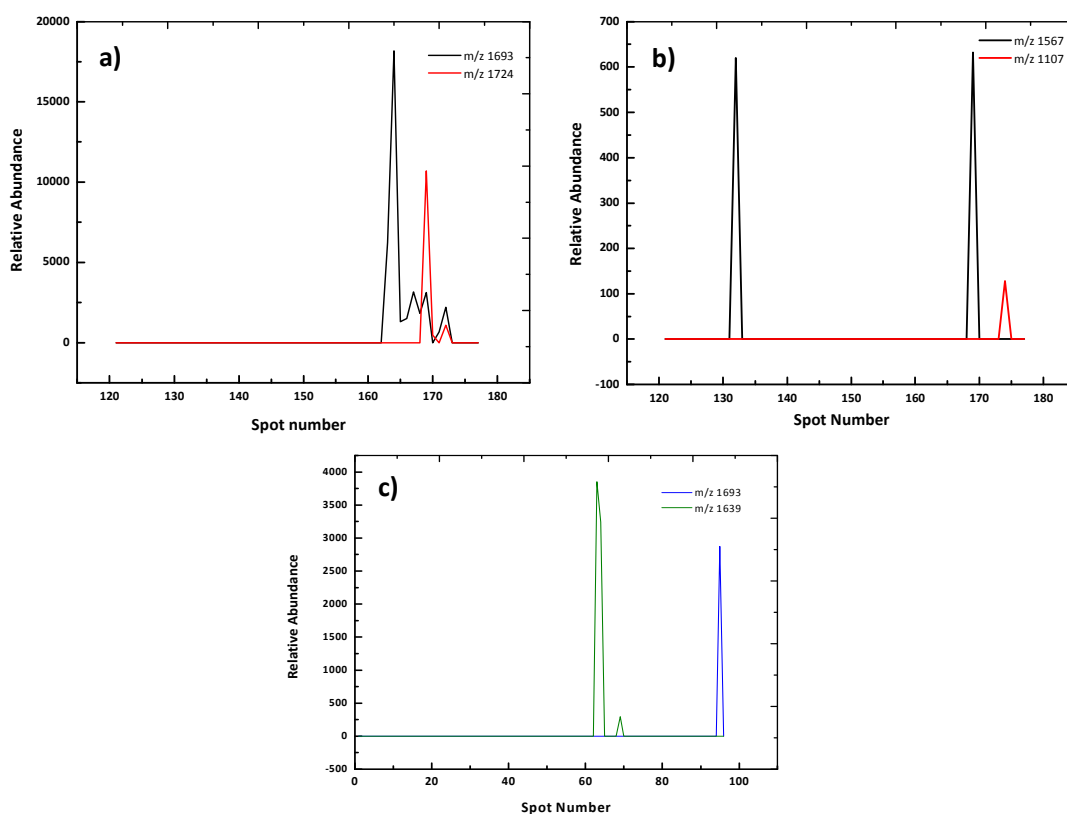


Figure 6.17: MALDI MS spectra for peptides generated from Trypsin digested Cytochrome c with relative abundance below 30,000. (a) and (b) detail the mass spectrometry results for the peptide sample spotted using the deposition probe with relative abundance below 30,000 and 700 respectively. (c) Details the mass spectrometry results for the peptide sample spotted using the commercial spotter with relative abundance below 4,000. The mass spectrometry results were plotted as relative abundance of the peptide versus the spot number.

Signal intensities of peptide fragments have been re-plotted and are presented in Figure 6.17 for those fragments not easily observed amongst the others whose signal intensities are several orders of magnitude higher. Seven peaks of known mass were not observed by the automated spotter. One reason for this could be ion suppression by co-eluting peaks. Nano-LC effluent is transported under pressure to the automated spotting instrument. Peak dispersion caused due to the parabolic flow profile in pressure driven systems would reduce resolution between bands, thus the longer the distance between the separation column and the spotting system the greater the loss of resolution. Also, sample adhesion to the surface of the capillary will increase cross contamination

between eluting bands. Consequently, the spotted sample may contain several peptides, as well as closely eluting fragments leading to competitive ionisation and loss of signal.

6.6 Conclusion

A novel droplet based interface between nano-liquid chromatography and matrix assisted laser desorption ionisation mass spectrometry has been successfully demonstrated. LC effluent was mixed with MALDI matrix and fractionated into droplets retaining the resolution obtained during the separation. The droplets were transported to a MALDI target via the deposition probe which also helps extract oil from the segmented flow. Compared to techniques currently described in the literature, this format of oil removal does not lead to dilution of the contents of the droplet and shows minimal cross contamination between droplets. Moreover, oil adversely affects the matrix-analyte crystallisation and desorption/ionisation process. In the presence of oil, large crystals are formed and deposited non-uniformly across the surface of the sample well. Signal acquisition under these conditions is erratic due to the inhomogeneous surface and the overlying oil layer which may prevent desorption or ionisation from occurring.

Mass spectrometry results using native proteins show that the interface provides results similar to those obtained if the sample was spotted using the traditional dried drop technique. Furthermore, the performance of the interface device and spotter was compared to a commercially available automated spotter using the Trypsin digest of two proteins. In both cases the droplet interface device performed better, seeing over 86% of all peptides present compared to $\sim 56\%$ observed by the automated spotter. In addition the elution order was also preserved demonstrating. The ability to reproducibly generate MALDI ready droplets and successfully extract them from an oil stream, if combined with automated spotting, would permit the deposition of single droplets and enable analysis of extremely complex peptide mixtures originating from several proteins.

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Chapter VII: Conclusion



7.1 Conclusion and Future work

The work in this thesis has been aimed at improving chip-based electrophoretic separations and developing microfluidic devices that can be integrated using droplets. The ultimate aim of the described work is to establish a modular lab-on-chip device where samples are transported between preparation, amplification and detection processes using encapsulated droplets. Accordingly, such devices may be interfaced at the discretion of the user, streamlining analysis and eliminating manual error. Integrated devices employing this approach adopt the advantages associated with both segmented and continuous flow microfluidics. Specifically, two novel interfaces have been described and demonstrated. The first is a droplet interfaced CE platform for chemical separations. A hydrophobic, oleophilic film was used to promote passive oil removal and consequently droplet segregation. The droplet-CE interface is simpler in operation than conventional formats of injection used in chip-based CE. It also provides the desired capabilities of low volume sample handling and high-throughput quantitative analysis without cross-contamination between sample droplets. Both CZE and CGE separation modes were successfully demonstrated on-chip, proving the potential of the platform in a wide range of applications such as small molecule separation, proteomics, genomics, metabolomics and other chemical and biological assays. Adaptation of this interface as an injection format for conventional CE instrumentation is currently in progress. The second is an interface between nano-liquid chromatography and matrix-assisted laser desorption ionisation mass spectrometry. LC eluate is mixed with MALDI matrix and fractionated into droplets retaining the resolution obtained during the separation. The droplets are transported to the MALDI target via a deposition probe which also helps to extract and remove the oil from the segmented flow. Compared to techniques currently described in literature, this interface does not contribute to sample dilution or cross contamination between droplets. The performance of the interface device was compared to a commercially available automated spotter using two complex peptide mixtures. In both cases the droplet interface device performed better, seeing 30% more peptides compared to those observed by the automated spotter.

The migration behaviour of DNA in capillary gel electrophoresis has been characterised at a fundamental level. The effect of polymer length, polymer concentration, polymer auto-oxidation, and buffer viscosity on the migration dynamics of DNA has been studied. This is of crucial importance when transferring separation protocols to chip-based formats where short separation

lengths and varying surface chemistry reduce the efficiency, reproducibility and resolution of the separation. Herein two PEO sieving matrices were developed for the analysis of dsDNA fragments. Both matrices were UV transparent, and permitted separation of a wide range of fragment sizes with sufficient resolution to enable mass calibration. Additionally, the matrices were stable over a wide temperature range. The low molecular weight PEO matrix exhibited a lower viscosity allowing efficient introduction into the capillary and microchannel. However the higher molecular weight and consequently higher viscosity matrix was more effective when protocols were transferred to a chip-based formats. To circumvent the practical problems associated with loading high viscosity matrices into chip-based separation channels a hybrid device was designed. Here the separation channel was replaced with a bare fused silica capillary while maintaining a cross-piece injection geometry on-chip. The hybrid format was also translated to the droplet interface device where the cross-piece was replaced with the droplet injector. The hybrid device unites well-understood silica surface chemistry employed in conventional CE and the superior injection capabilities of chip-based CE. This results in a device that can exploit the decades of protocol development in CE and the short analysis times of chip-based CE. Accordingly, future work will focus on optimising injection and separation parameters to achieve high resolution separations and short analysis times.

DNA amplification was successfully demonstrated using electroosmotic flow induced by diodes to achieve thermal cycling. The diode driven fluid pump embedded within a single channel device afforded the flexibility of performing any number of PCR cycles without changing the design or channel layout. The device format can be adapted to drive amplified product to a T-junction forming droplets. Consequently, using the droplet-CE interface sample can be amplified and then analysed.

The interfaces and hybrid devices described herein make a modular approach to microdevice integration attractive. For example, individual cells may be encapsulated in droplets, chemically lysed and prepared for DNA or protein analysis. The sample droplets can then delivered to multi-dimensional separation analyses using the segmented-to-continuous flow interface. Accordingly, DNA can be amplified in droplet format and then analysed via chip-based CGE. Additionally, high-throughput aptamer selection could be achieved by compartmentalising individual components of an aptamer library with the target protein or cell and then screening each droplet using chip-based CE. Traditional 2D protein analysis can also be accomplished using chip-based

formats for IEF and gel electrophoresis described in literature. Each separation could be connected via the droplet interface. After gel electrophoresis proteins of interest could be encapsulated into droplets and Tryptsin digested. Subsequently, mass spectrometric analysis could be performed using the droplet-MALDI-MS interface. Furthermore, the interface can be set up to accommodate a multi-channel separation device permitting the development of an automated high throughput electrophoretic system. Such a system has the potential to handle hundreds of samples with minimal user intervention. Additionally, similar devices could be used to integrate existing macroscale instrumentation reducing the error associated with manual transfer between analyses. The work described here has demonstrated several basic components of a modular lab-on-a-chip device facilitating its application to real world sample analysis.