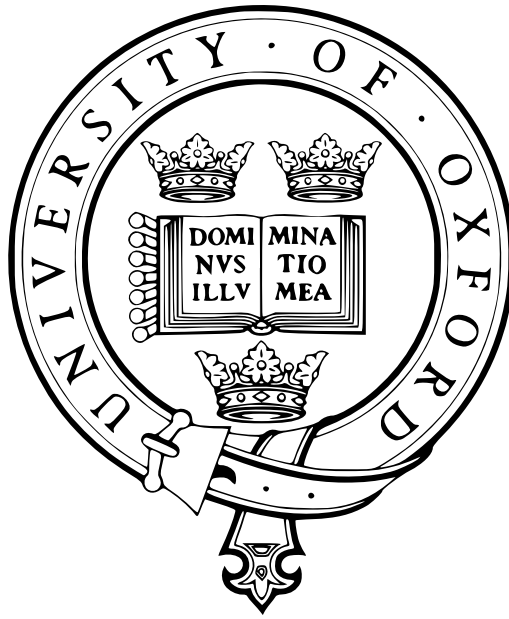




Developing A Combined Microfluidic and Hydrogel Platform for Single-Cell Assays

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Abstract

Microfluidic technologies can be utilised for the compartmentalisation of a bulk reaction into millions of water-in-oil droplets. Adapting bulk reaction methods to microfluidic environments enables sequences of multiple reactions to occur simultaneously on separate target molecules. Individual cells or strands of genetic material can be encapsulated within these droplets to compartmentalise biological assays for single cell resolution. Microfluidic bioassays can be used for the detection or sequencing of rare targets such as HIV provirus. This thesis develops robust experimental methods for several key steps in a proposed combined microfluidic and hydrogel platform for single-cell assays. While this work is shown with the context and motivation of characterising the HIV latent reservoir, the experimental systems developed could be used for a range of bioassays.

We develop a methodology to create hydrogel microspheres from water-in-oil droplets which are capable of trapping DNA at a single-cell resolution while allowing washing with reaction buffers. In essence these would act as micro-scale analogues to bulk size-based filtration techniques. We propose the encapsulation of cells and sol-phase hydrogel within water-in-oil droplets. Cell lysis and cross-linking of the hydrogel would trap the genomic DNA within hydrogel microspheres. Washing and re-encapsulation of the microspheres alongside the reagents required for polymerase chain reaction (PCR) would produce a new suspension. Successful PCR would produce a fluorescent droplet when the target DNA is present. Sorting these droplets allows for the sequencing of only genomic material containing target DNA.

We start by developing a reliable methodology to produce alginate and agarose microspheres with the physical and chemical properties required for the proposed assay. We investigate the viscoelastic properties of bulk alginate and agarose hydrogels and then predict how the composition of microspheres affects their pore size and trapping ability. We develop robust methodologies for cell lysis, the trapping of genomic DNA within hydrogel microspheres, and the re-encapsulation of these microspheres. We compare the efficiency of PCR within different

hydrogel networks, showing that alginate networks inhibit PCR, while agarose networks allow successful PCR. We discuss the work required to achieve successful PCR within alginate networks. Finally, we quantify the ability of the microspheres to trap particles, cells and DNA. We show how the composition of alginate hydrogel affects their trapping ability and investigate the diffusion of DNA through microspheres of various hydrogel compositions. We conclude by investigating the pore size of microspheres using fluorescent and confocal imaging techniques.

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Declaration

This thesis is submitted for the degree of Doctor of Philosophy in Physical and Theoretical Chemistry at the University of Oxford. No part of this thesis has been accepted or is currently being submitted for any degree, diploma, certificate or other qualification in this University or elsewhere. This thesis is wholly my own work, except where indicated.

Common Abbreviations

AA: Acetic acid

AIDS: Acquired immune deficiency syndrome

ART: Antiretroviral Therapy

BF: Bright-field

CDC: The Centers for Disease Control

CLEX: Competitive ligand exchange cross-linking

DI: De-ionised

DIC: Differential interference contrast

DNA: Deoxyribonucleic acid

EA: A fluorosurfactant

EDTA: Ethylenediaminetetraacetic acid

FACS: Fluorescence activated cell sorting

FADS: Fluorescence activated droplet sorting

FCS: Fetal calf serum

FFJ: Flow focusing junction

FITC: Fluorescein isothiocyanate

HAART: Highly active antiretroviral therapy

HFE: Hydrofluoroether oil

HIV: Human immunodeficiency virus

IPDA: Intact proviral DNA assay

NGS: Next generation sequencing

PC: Phase-contrast

PCR: Polymerase chain reaction

PDMS: Polydimethylsiloxane

PFO: 1H,1H,2H,2H-Perfluoro-1-octanol

PGMEA: Propylene glycol methyl ether acetate

PLWH: People living with HIV

PrEP: Pre-exposure prophylaxis

PTFE: Polytetrafluoroethylene

PVA: Poly(vinyl) alcohol

QVOA: Quantitative viral outgrowth assay

RNA: Ribonucleic acid

SDS: Sodium dodecyl sulfate

SE: Single emulsion

UNAIDS: Joint United Nations Programme on HIV/AIDS

W/O: Water-in-oil

Contents

Abstract	iii
Acknowledgements	v
Declaration	vii
Common Abbreviations	ix
1 Introduction	1
1.1 Microfluidics and Emulsions	3
1.1.1 The History of Microfluidic Technologies	3
1.1.2 Emulsions	4
1.1.3 Droplet Production	6
1.1.4 Droplet Manipulation	10
1.1.5 Encapsulation of Cells	11
1.2 Hydrogels	13
1.2.1 Hydrogel Structure and Properties	13
1.2.2 Choosing a Hydrogel	14
1.3 HIV and the Latent Reservoir	15
1.3.1 The Global HIV Pandemic	15
1.3.2 The Structure and Pathogenesis of HIV	17
1.3.3 The Latent Reservoir	19
1.3.4 Quantifying the HIV Reservoir	21
1.4 Sequencing Technologies	22
1.4.1 DNA and RNA Analysis	22
1.4.2 HIV Sequencing	23

1.4.3	Single-Cell Sequencing Technologies	24
1.5	Thesis Outline	26
2	Experimental Techniques	29
2.1	Reagent Information, Preparation, and Storage	29
2.2	Microfluidic Techniques	31
2.2.1	Soft Lithography	31
2.2.2	Microfluidic Chip Production	33
2.2.3	Droplet Production	34
2.2.4	Droplet Breaking	36
2.2.5	Droplet Heating	36
2.2.6	Imaging	36
2.3	Biological Procedures	38
2.3.1	Cell Culture	38
2.3.2	Cell Staining	39
2.3.3	DNA Staining	39
3	Hydrogel Properties and Microsphere Formation	41
3.1	Hydrogel Microsphere Formation	41
3.1.1	Agarose Microsphere Formation	42
3.1.2	Alginate Microsphere Formation	44
3.1.3	Variables in Alginate Microsphere Formation	49
3.1.4	Comparison of Agarose and Alginate Microsphere Production	51
3.2	Viscoelastic Behaviour of Agarose and Alginate Hydrogels	52
3.2.1	Introduction	52
3.2.2	Theory	53
3.2.3	Methods	55
3.2.4	Results and Discussion	57
3.3	Properties of Hydrogel Microspheres	63
3.3.1	Time Stability	64
3.3.2	Thermal Stability	64
3.3.3	Microsphere Sedimentation and Washing	65
3.3.4	The Swelling of Hydrogel Microspheres	67

3.3.5	Summary	70
3.4	Chapter Summary	70
4	The HIV Latent Reservoir Assay	73
4.1	A Proposed New Assay	74
4.1.1	Lysis and PCR in Droplet Micro-reactors	74
4.1.2	A Hydrogel Solution	75
4.1.3	A Model Cell Line	76
4.2	Cell Lysis	76
4.2.1	Lysis Techniques	76
4.2.2	Testing Lysis Methods - Microsphere Survival	78
4.2.3	Testing Lysis Methods - Lysing Jurkat Cells	80
4.2.4	Lysing Cells in Hydrogel Microspheres	83
4.3	Re-encapsulation	86
4.3.1	Re-encapsulation of Microspheres	86
4.3.2	Re-encapsulation of Trapped Nuclear DNA	90
4.4	PCR	91
4.4.1	Theory	91
4.4.2	PCR Reagents and Methods	93
4.4.3	PCR in Agarose Hydrogel Matrices	94
4.4.4	PCR in Alginate Hydrogel Matrices	96
4.5	Chapter Summary	99
5	The Trapping Ability of Microspheres	101
5.1	Introduction	101
5.2	Trapping Particles and Cells	102
5.2.1	Trapping Colloidal Microparticles	102
5.2.2	Trapping Jurkat Cells	105
5.2.3	Trapping Nanoparticles	106
5.3	Trapping of DNA and RNA	108
5.3.1	Introduction	108
5.3.2	Methods	109
5.3.3	Results and Discussion	111

5.4	Determining Pore Size using Imaging Techniques	115
5.4.1	Introduction	115
5.4.2	Fluorescent Imaging	115
5.4.3	Confocal Imaging	117
5.5	Chapter Summary	121
6	Conclusions and Outlook	123
	Bibliography	126
	Appendix	155

Chapter 1

Introduction

Despite the existence of anti-retroviral therapy (ART) and pre-exposure prophylaxis (PrEP) to control symptoms and transmission of HIV and AIDS, the number of people living with HIV has still increased every year since the 1990s [1]. HIV (human immunodeficiency virus) evades typical treatments and detection methods by integrating proviral DNA into the genetic material of host T cells. These latently infected T cells can persist after treatment and then activate to produce new HIV virions. It is therefore of interest to sequence this integrated HIV provirus to understand how to detect and study this latent reservoir.

Cells can be lysed and sequenced at a single cell resolution, but only 300 per 10^6 resting CD4+ T cells contain provirus [2], and only 0.1 to 10 per 10^6 contain replication competent provirus [3]. If we sequenced every cell at a single cell resolution in order to obtain the sequence of just one HIV intact provirus, we could have to sequence up to 10^7 cells. This would be costly, labour intensive, and outside the scope of most currently available single cell sequencing techniques [4, 5]. Instead we propose a technique to sort the genetic material containing HIV from that which does not, maintaining a single cell resolution. Droplet microfluidics are used regularly in biological assays to compartmentalise reactions into millions of individual micro-droplet reaction vessels, allowing high throughput assays [6, 7]. Previous work done by the Frater group has attempted to encapsulate cells, lysis buffers and polymerase chain reaction (PCR) reagents into droplets [8]. This would allow for cell lysis to release nuclear DNA into the droplet, and then PCR could produce localised fluorescence for droplets containing HIV DNA. These could then be sorted based on fluorescence integrity, and we could sequence only the HIV positive population. Unfortunately this previous work, alongside others, show that PCR is inhibited by cell lysis reagents or cell lysates, and so this method does not work [9]. Other attempts involve instead

using microsphere hydrogel networks to trap the relatively large genomic DNA, while inhibitory material can be washed out, before these hydrogel microspheres can be re-encapsulated into new droplets for subsequent PCR. While the field has focussed on using agarose for this purpose [10], this thesis aimed to achieve a similar result using alginate hydrogels, and compares the trapping ability of the two methods. As such, this thesis mostly focused on alginate microspheres, pausing to compare these to agarose microspheres where relevant.

The aims of this thesis are threefold, and the research involved in investigating each of these is detailed in Chapters 3, 4 and 5. Chapter 3 aimed to produce alginate and agarose microspheres, and determine whether they had the necessary properties to be compatible with the proposed bioassay. Chapter 4 aimed to develop three key steps in this bioassay, using the microspheres to accomplish cells lysis, microsphere re-encapsulation and PCR. Chapter 5 then aimed to characterise the ability of microspheres of different compositions to trap cells, colloids and DNA.

The rest of Chapter 1 discusses the topics introduced briefly above in more detail. Section 1.1 describes the development of microfluidic technologies, in particular as a tool for developing immunological assays. Microfluidic techniques to create droplets are then investigated using the dimensionless numbers of fluid dynamics. Next the stability of emulsions is discussed, and various methods used to avoid the destabilisation pathways. This section concludes by comparing the ability of droplets to act as micro scale analogues macroscopic reaction vessels. Section 1.2 then discusses the structure and properties of hydrogels, as well as the biological applications of hydrogels with varying cross-linking methods. The use of hydrogels as molecular filters is shown to be a useful addition to the droplet microfluidics toolbox, and the hydrogels used in this thesis are chosen. Section 1.3 introduces the history of the HIV pandemic, the structure and pathogenesis of HIV and how the latent reservoir is a large barrier to a cure. This leads to a survey of methods to detect and quantify the latent reservoir, and the limitations of these. Section 1.4 explores DNA and RNA sequencing as applied to HIV research and the advantages of droplet single-cell technologies, explaining the current limitations in HIV single-cell DNA sequencing. Hydrogels microspheres are suggested as a potential solution to the limitations of the current assays. Finally, Section 1.5 outlines the structure of the rest of this thesis.

1.1 Microfluidics and Emulsions

This section discusses the development of microfluidic technologies from their beginnings in inkjet printing to the modern bioassays, and how certain technologies have enabled new developments in micro-assays. Section 1.1.2 then discusses the physical chemistry behind the creation and stability of droplet emulsions, the many pathways to destabilisation and how to avoid these. Section 1.1.3 discusses the microfluidic technologies used to produce droplets, and explores the variables that affect successful droplet production using Navier-Stokes flow and the Reynolds number. Finally, Section 1.1.4 compares droplet manipulation technologies to macroscopic manipulation of reaction vessels, showing the advantages of developing a size filtration system for droplet micro-reactors.

1.1.1 The History of Microfluidic Technologies

Scientific research in the latter half of the 20th century was dominated by the development of microtechnology. In the 1950s the earliest form of microfluidic technology was proposed; inkjet printing. Here ink micro-droplets consisting of a dye solute in a solvent are ejected from a nozzle onto a substrate, where they evaporate, leaving behind the dye. This technology has since evolved to produce conductive structures for LEDs, solar cells and other microcircuits, as well as magnetic nanoparticles, and for drug testing [11, 12]. In the 1960s the space race drove the miniaturisation of computers and circuits to reduce the mass and fuel required to send objects out of the reach of Earth's gravity. Across the 1970s the development of coating and etching patterns into silicon wafers culminated in 1979 with the fabrication of a miniaturised gas chromatographic air analyser [13]. Since the 80s, research into micro-electro-mechanical systems (MEMS) has moved away from development of techniques for micro-fabrication and towards applications of these devices [14], such as for displays [15], laser scanners [16], speakers [17], and mass spectrometers [18] among many others.

In the late 1990s, microfluidic devices were shown to be useful for bioassays. They provided a way to easily deliver samples to study the binding of antibodies/receptors in granulocytes [7] and immunoglobulins to surfaces [19], among others [20]. Microfluidic devices were beginning to be designed to produce micro-droplets [21, 22], with the long-term goal being able to run high-throughput reactions in each of these - each droplet can be considered a individual reaction vessel [23]. It was proposed that microfluidic devices could be used for DNA sequencing in a high-throughput and high-speed manner, but the difficulty and expense of producing devices at

the time limited this [24, 6].

At first microfluidic devices consisted of micro-channels etched on silicon wafers capped with glass [25], an approach that was expensive and difficult as the etching of silicon wafers was a complicated process. By investigating the development of plastic and glass chips [26], eventually devices made from a polydimethylsiloxane (PDMS) layer bonded to a glass slide became the industry standard [27]. By the early 2000s the production of microfluidic devices was quick, cheap, and easy, allowing the number of micro-assays in development to increase exponentially. The general proposal for droplet single-cell bioassays is the ability to conduct millions of identical reactions on individual cells or DNA strands at once by using each micro-droplet as its own micro-reactor. The modern world of droplet single cell assays, and the proposal of a new one to investigate the HIV latent reservoir is discussed further in Section 1.4.

1.1.2 Emulsions

An emulsion is a two-phase system of two normally immiscible liquids. It consists of a dispersed phase of droplets of one liquid in a continuous phase of another. This thesis uses water-in-oil emulsions (W/Os) in which aqueous droplets are dispersed in an oil phase. Most biological assays take place in an aqueous phase so this needs to be the content of our micro bioreactors. In order for these droplets to be used as bioreactors for individual assays, they are required to be stable over a timescale long enough for the assay to be complete with confidence there is no transfer of material between droplets. There are a few different pathways to the destabilisation of droplets which need to be avoided.

Emulsions are not thermodynamically stable. Over time droplet coalescence will occur, leading to eventual liquid-liquid phase separation. This is called de-emulsification or emulsion-breaking [28]. This is driven by the desire of a system to reduce the surface tension. Surface tension (γ),

$$\gamma = \frac{F}{l} = \frac{w}{\Delta A}, \quad (1.1)$$

originates from the energy cost of moving a molecule from the bulk to a surface due to reduced number of favourable interactions (decreased coordination number of like species), and therefore results in an energy cost (work, w) to increase the area of a surface, ΔA .

Droplet coalescence is driven by the reduction in overpressure inside a larger droplet resulting when two smaller droplets merge together. Laplace's law for spherical droplets gives this pressure

as

$$\Delta P = \frac{2\gamma}{R}. \quad (1.2)$$

A pressure (or energy per unit volume) needs to be supplied to counteract this overpressure in order to stabilise the emulsion with respect to phase separation. This overpressure is minimised by reducing surface tension or increasing the size of the droplet.

Emulsions can be stabilised for certain timescales with a monolayer of an adsorbed substance at the liquid-liquid interface [29, 30]. The molecules which make up these adsorbed monolayers are known as surface-active agents, or surfactants. Surfactants will form favourable interactions with both phases of the emulsion, reducing the surface tension. Typically they consist of a hydrophilic "head" and a hydrophobic "tail".

Droplet coalescence will still occur over time, usually described via a creaming pathway. Creaming of droplets occurs where the dispersed phase has a lower density than the continuous phase, so the droplets rise to the top of the continuous phase and bunch together. The droplets are then in contact with each other so the displacement of surfactant molecules allows the creation of a larger dispersed droplet by merging of the interfaces, reducing surface tension and Laplace pressure [31].

Droplets are also destabilised upon heating. Surface tension decreases at higher temperatures [32], which we would expect to result in an increased stabilisation of emulsions. Instead this destabilisation of water-in-oil droplets is proposed to be caused by water evaporation and droplet coalescence [33]. Droplet evaporation typically results in phase-separated aqueous and oil layers, and a loss of size monodispersity of droplets. Normally the entropy change of mixing of two liquids is positive, so on increasing the temperature, the Gibbs energy change of mixing, $\Delta G = \Delta H - T\Delta S$, becomes more favourable. However for oil and water mixtures the "hydrophobic effect" instead drives a negative entropy of mixing at low temperatures. This is due to water molecules surrounding a non-polar molecule becoming arranged so as to minimise loss of hydrogen bonds compared to bulk water [34]. This entropy change reduces upon heating as at high temperatures the average kinetic energy of a water molecule dominates over hydrogen bonding. Therefore heating drives phase-separation rather than phase-mixing in this system.

DLVO theory describes the coagulation and flocculation of emulsions, but as this typically involves solvated ions in the continuous phase it is more applicable to oil-in-water or water-in-oil-in-water suspensions. As the subject of this thesis is water-in-oil emulsions, this is not

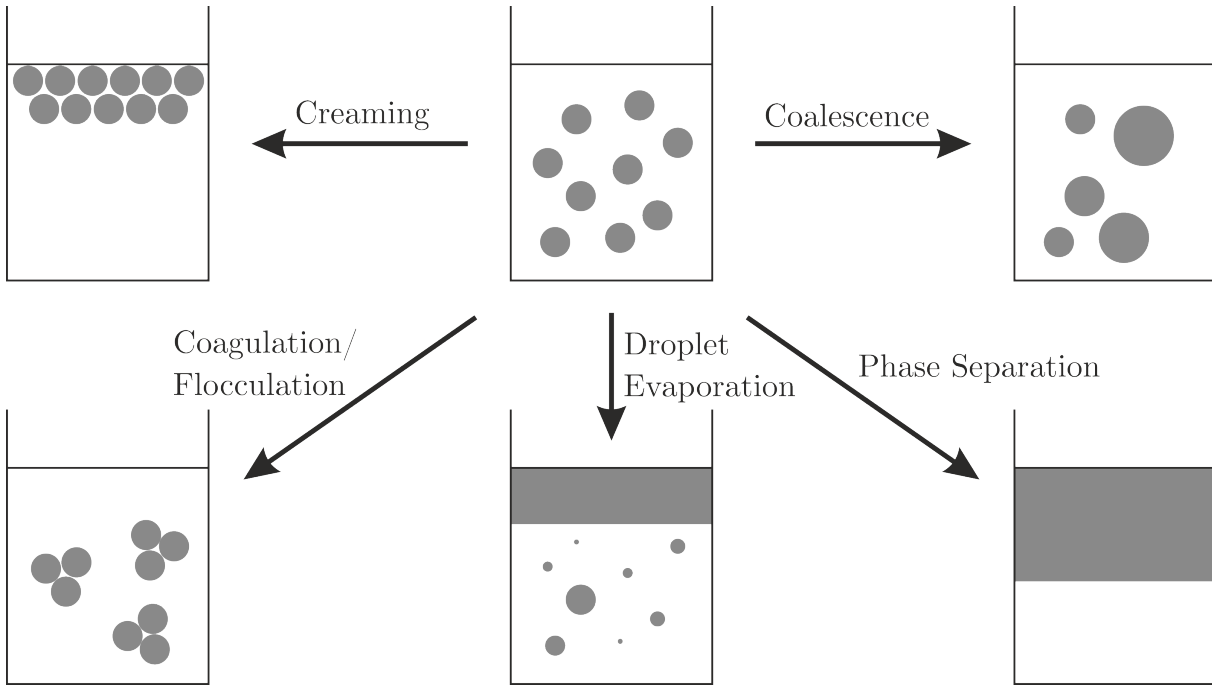


Figure 1.1: The various pathways to emulsion destabilisation.

discussed in depth here.

There is clearly a delicate balance of keeping an emulsion stable, and this must be managed while using droplets as micro-reactors to avoid the many pathways to destabilisation (Figure 1.1).

1.1.3 Droplet Production

There are many methods to produce an emulsion, from simply shaking a mixture of oil and water [35] to a wide range of microfluidic devices designed specifically for droplet generation [36]. Single cell assays use the latter method for a wide range of reasons, mostly as microfluidic devices can create droplets of monodisperse size reproducibly, and the concentration of reagents can be well controlled [37]. Provided size homogeneity of droplets, droplet microfluidic technologies can also ensure each droplet contains a maximum of one cell (controlled by the Poisson distribution [38], see Section 1.1.5), an important feature for single cell resolution.

Droplet microfluidics uses a microfluidic device consisting of microchannels on a glass and PDMS chip (described in Chapter 2) to produce droplets [27]. Fluid flow exists in one of two regimes, turbulent (chaotic) or laminar (smooth), quantified by the dimensionless Reynolds number:

$$Re = \frac{\rho U D_h}{\mu}, \quad (1.3)$$

which describes the ratio of inertial to viscous forces. Here, ρ is the fluid density, U is the characteristic velocity, D_h is the hydraulic diameter (and depends on channel geometry) and μ is the dynamic viscosity. The Reynolds number originates from the incompressible Navier-Stokes equation,

$$\rho \frac{D\mathbf{u}}{Dt} = -\nabla p + \mu \nabla^2 \mathbf{u} + \mathbf{f} \quad (1.4)$$

which describes the flow of viscous fluids, where $\mathbf{u}$ is the flow velocity, p is the pressure exerted on the system, $\mathbf{f}$ is an external force per unit volume, and $\frac{D}{Dt} = \frac{\partial}{\partial t} + u_j \frac{\partial}{\partial x_j}$ [39]. The Navier-Stokes equation can be solved for a particular system to obtain the flow velocity profile. It contains an inertial term $\frac{D\mathbf{u}}{Dt}$ and a viscous term $\mu \nabla^2 \mathbf{u}$. Both sides have units of $m^2 s^{-1}$. This is non-dimensionalised by setting

$$\mathbf{u}^* = \frac{\mathbf{u}}{U}, \quad p^* = \frac{p}{\rho U^2}, \quad \nabla^* = \nabla L, \quad \frac{\partial}{\partial t^*} = \frac{L}{U} \frac{\partial}{\partial t^*} \quad \text{and} \quad \mathbf{f}^* = \mathbf{f} \frac{L}{U^2 \rho} \quad (1.5)$$

where U and L are characteristic velocity and length scales respectively. This simplifies the Navier-Stokes equation to

$$\frac{D\mathbf{u}^*}{Dt^*} = -\nabla^* p^* + \frac{\mu}{\rho U L} \nabla^{*2} \mathbf{u}^* + \mathbf{f}^*, \quad (1.6)$$

where the coefficient of the viscous term is $\frac{1}{Re}$ (once the characteristic length scale is defined as the hydraulic diameter) [39]. A large Reynolds number (> 5000) shows inertial forces dominate and flow is turbulent [40]. In microchannels, the characteristic diameter is small and therefore the Reynolds number is very small ($Re \ll 1$), and typically flow is assumed to be laminar (although there is some evidence of the contrary [41]).

A droplet microfluidic device typically consists of an aqueous stream being interrupted by an oil stream to produce droplets [42]. Various different channel arrangements can be used, T junctions [43], flow focusing junctions (FFJ), [44] and co-flow arrangements of channels [45] (Figure 1.2). T junctions can produce hundreds of droplets per second so would be high throughput, but do not typically have a high homogeneity in droplet size [46], so maintaining single cell resolution would be difficult. Flow-focusing junctions are shown to have high monodispersity of droplet sizes, and are reliable to use. Therefore they are the dominant droplet generation technique in the field [46]. This thesis uses flow-focusing junctions.

There are many different regimes of immiscible fluid flow in microfluidic devices, which can be

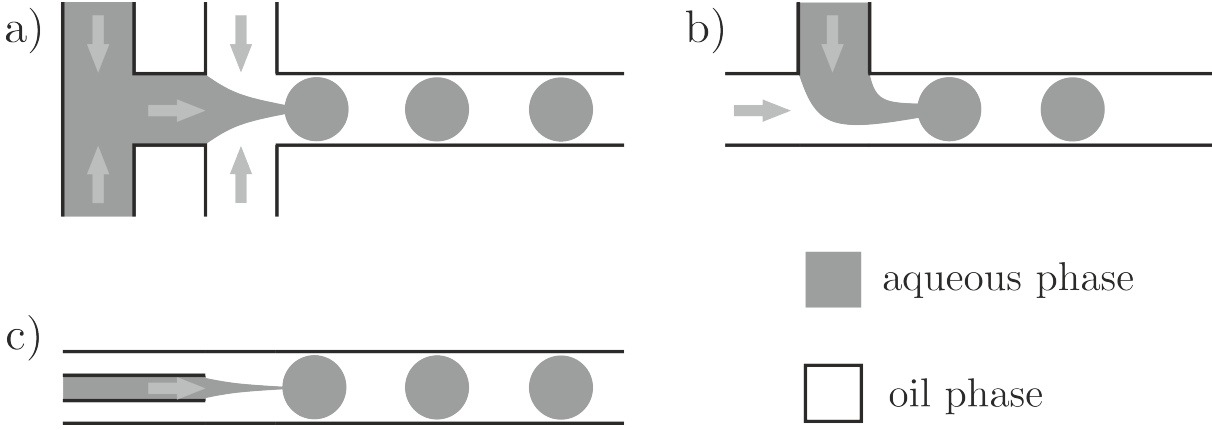


Figure 1.2: Various junction designs for droplet production: a) a flow-focusing junction, b) a T junction and c) a co-flow junction.

understood by observing dimensionless numbers [47]. Flow-focusing junctions have three main regimes of flow: dripping, jetting, and stable co-flow (Figure 1.3a-c). Dripping produces a steady stream of droplets, whereas jetting produces a stream of the disperse phase flowing alongside the continuous phase, generating erratically sized droplets or no droplets at all. Stable co-flow produces no droplets. Which regime dominates depends on the flow rates, channel dimensions, and viscosities of the fluids [49, 50], and is described by the Rayleigh-Plateau instability [51]. The effect of these variables can be described by two dimensionless numbers - the capillary number:

$$Ca_{oil} = \frac{\mu_{oil} u_{oil}}{\gamma}, \quad (1.7)$$

and the Weber number:

$$We_{aq} = \frac{\rho_{aq} u_{aq}^2 l}{\gamma} \quad (1.8)$$

where ρ is the density, μ is the dynamic viscosity, u is the characteristic velocity, l is the characteristic length, and γ is the surface tension. We showed earlier how the Reynolds number describes the ratio of inertial to viscous forces. Similar arguments can be made to derive the capillary and Weber numbers. The capillary number describes the ratio of viscous forces to surface tension forces, and the Weber number describes the ratio of inertial to surface forces. This can be confirmed by simple dimensional analysis. If the capillary number is the ratio of viscous to surface forces from the Navier-Stokes equation (Equation 1.4), then

$$Ca = \frac{\text{Viscous forces}}{\text{Surface forces}} = \frac{\mu \nabla^2 \mathbf{u}}{-\nabla p}. \quad (1.9)$$

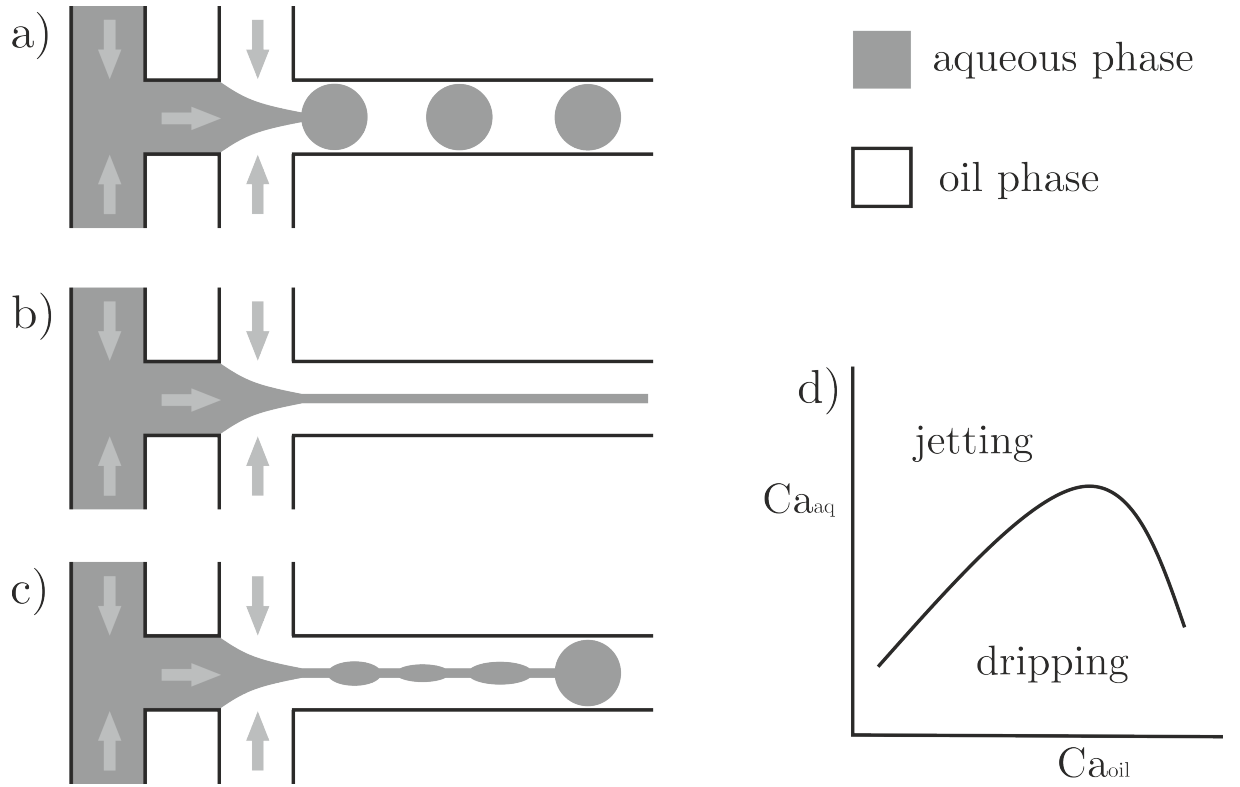


Figure 1.3: Regimes of flow in a flow-focusing junction: a) dripping, b) stable co-flow and c) jetting. d) A simplified graph of the Kovalchuk model showing how capillary number affects the regime of flow [48].

Replacing ∇ , ∇^2 and $\mathbf{u}$ with characteristic physical quantities $\frac{1}{l}$, $\frac{1}{l^2}$ and u respectively, and using the definition of surface tension $\gamma = \frac{F}{l} = \frac{pA}{l} = pl$ gives

$$Ca = \frac{\mu \frac{u}{l^2}}{\frac{\gamma}{l}} = \frac{\mu u}{\gamma}, \quad (1.10)$$

as in Equation 1.7. Similar arguments comparing inertial to surface forces produce the Weber number as follows. The Weber number is the ratio of of inertial to surface forces,

$$We = \frac{\text{Inertial forces}}{\text{Surface forces}} = \frac{\rho \frac{D\mathbf{u}}{Dt}}{-\nabla p}. \quad (1.11)$$

Replacing $\frac{D\mathbf{u}}{Dt}$ with the characteristic physical quantity $\frac{u}{t}$ and $\nabla p = \frac{\gamma}{l^2}$ as above gives

$$We = \frac{\rho \frac{u}{t}}{\frac{\gamma}{l^2}} = \frac{\rho u^2 l}{\gamma}, \quad (1.12)$$

as shown in Equation 8.

Utada et al showed that a small capillary number for the oil phase (Ca_{oil}) and a small Weber

number for the aqueous phase (We_{aq}) are required for successful dripping [52]. When there is a large We_{aq} , jetting is driven by the inertia of the aqueous phase, producing large droplets. This is fixed by reducing flow rate of the aqueous phase. When there is a large Ca_{oil} , jetting is driven by viscous drag, producing small droplets. This is fixed by reducing the flow rate of the oil. Note that fluids in microfluidic channels are typically driven at a controlled rate of flow (though they can be driven by pressure pumps). Flow rate is a volume pushed per unit time rather than a characteristic velocity, but the conversion is simple once dimensions of the channels are known. Kovalchuk et al showed that for a specific Ca_{aq} , increasing the Ca_{oil} can move from a jetting regime to a dripping regime and back to a jetting regime (Figure 1.3d) [48]. A concern for bioassays is the presence of DNA increasing the viscosity of the aqueous phase, increasing the Ca_{aq} which according to the model proposed by Kovalchuk et al moves towards the jetting regime [48].

1.1.4 Droplet Manipulation

For droplets to be used as microscopic reaction vessels, they need to be able to be manipulated in similar ways to macroscopic reaction vessels. Typical reaction techniques involve adding reagents at controlled time intervals, heating of reagents, the purification of products via washes, and the selection of successful reactions for subsequent experiments. Some of these are more easily achieved in droplets than others. There are many ways to stimulate coalescence of two droplets to allow time controlled addition of some aqueous reagents to a droplet bioreactor. This can be done by the merging of two droplets driven by use of an electric field to reduce surface tension at the point they meet in a microchannel [53, 54], or other passive methods such as hydraulic pressure [55]. Alternatively, pico-injection devices inject into a passing droplet from a separate channel under pressure, again using an electric field to disrupt surfactant layers [56, 57, 58].

To do the opposite, and selectively remove one component from a droplet is a considerably more difficult feat. While there are microfluidic technologies to successfully split a droplet into two or more smaller droplets [59, 60], selectively choosing which components go where is considerably trickier. In macroscopic reactions, this is typically done by use of a filtration system, or by chromatography. This thesis proposes that a network structure such as a hydrogel could act as a trap for the contents of a droplet. Therefore it could be possible to discriminate between what remains in the micro-reactor and what does not based on size. Large molecules would remain

trapped within the network, while smaller reagents and waste can be washed in and out. Section 1.2 discusses how hydrogels are a potential solution to this.

As discussed in Section 1.1.2, the heating of droplets causes destabilisation of the emulsion. This can be avoided by using a thermostable oil phase, and the presence of a thermostabilising surfactant such as Igepal. This thesis uses a droplet heating method developed by Dr Matt Jones (previous member of the Frater group, University of Oxford) and Dr Lucia Parolini (Aarts and Frater groups, University of Oxford) [8], described in Section 2.2.5.

Selecting droplets based on successful reactions is also already a widespread technique. It is possible to sort droplets based on a fluorescent signal using a laser. A fluorescent droplet is detected by the laser, which then triggers an electric field across the channel which diverts the path of flow of the fluorescent droplet. This is often called fluorescent activated droplet sorting (FADS) [61]. If a successful reaction can be identified by fluorescence, then these droplets can be selected for further reactions. There are many uses for this, such as the use of a probe to detect antibodies secreted by cells [62], enzyme evolving [63] or bacteria screening [64]. There are many other methods of droplet sorting using electric, acoustic, pneumatic, thermal and magnetic properties of droplets which are the subject of several comprehensive review articles [65, 66], and therefore not discussed in depth here.

1.1.5 Encapsulation of Cells

We have briefly mentioned (and will discuss in more detail in Section 1.4.2) why encapsulation of a single cell per droplet is important. Here we will discuss the difficulties in achieving single cell encapsulation and how the work in this thesis aims to reduce these.

This thesis uses passive encapsulation methods for cell capture. We replace an aqueous inlet in our flow-focusing microfluidic device with a suspension of cells and produce an emulsion as normal. By controlling the concentration of cells and the size of the droplets produced, we can control the number of cells encapsulated in each droplet.

Assuming cells arrive at the flow-focusing junction randomly distributed (i.e. there is no clumping of cells), and the production of droplets is uninterrupted, then the cell occupancy of droplets can be predicted by the Poisson distribution [67]. The probability of k cells being encapsulated within a droplet is given by

$$p(k, \lambda) = \frac{\lambda^k e^{-\lambda}}{k!}, \quad (1.13)$$

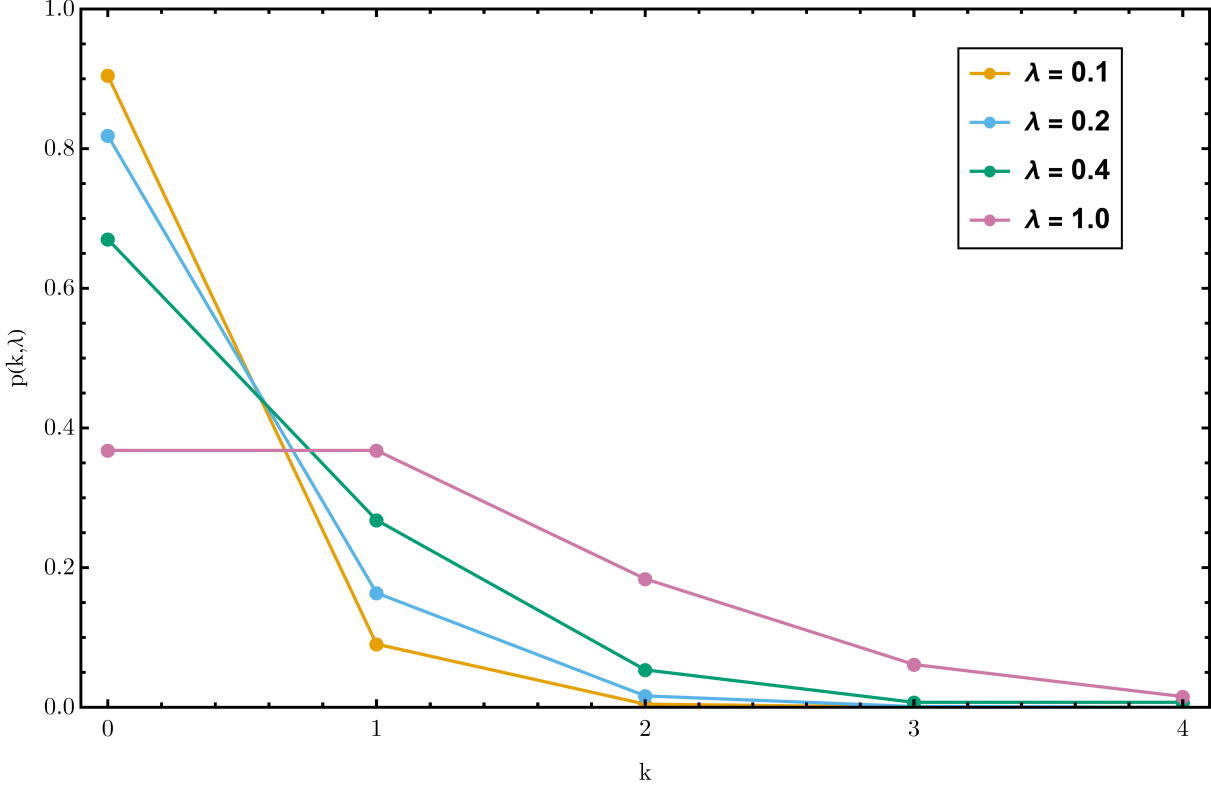


Figure 1.4: The Poisson probability distribution for $0.1 < \lambda < 1.0$. The probability of a droplet containing k cells is shown as a function of k .

where λ is the average number of cells per the volume of a droplet in the initial pre-encapsulated solution. This is the same as the ratio between the volume fraction of cells in the pre-encapsulated solution (ϕ_s) and the volume fraction of a droplet that a cell would occupy (ϕ_d) [67],

$$\lambda = \frac{\phi_s}{\phi_d}. \quad (1.14)$$

The occupancy probability of given cell type (with a specified size) is therefore controlled by droplet size and the initial cell concentration.

Figure 1.4 shows the Poisson probability distribution for $0.1 < \lambda < 1.0$, with $p(k, \lambda)$ displayed as a function of k . In the interests of maintaining single cell resolution, the number of droplets containing two cells should be minimised as much as possible (by reducing λ). In order to reduce the probability of a doubly occupied droplet to below 0.01, the probability of a single occupied droplet is ≈ 0.10 and most droplets must be empty (≈ 0.9). We now see another reason to develop efficient high throughput single cell sequencing. As discussed earlier, to sequence one HIV intact provirus, we could have to sequence 10^7 cells. If 90 % of droplets are unoccupied, then typical droplet single cell sequencing (without sorting for occupancy) could involve attempting

to sequence 10^8 droplets to obtain intact sequences from one latently infected cell at a single cell resolution. We see here the benefit of developing a platform to sort the desired population prior to sequencing - to reduce the inefficiency of sequencing up to 10^8 droplets not containing latently infected cells containing intact provirus.

1.2 Hydrogels

1.2.1 Hydrogel Structure and Properties

There are many definitions of a gel. Almdal proposes a gel consists of two or more components (of which one is a liquid) and that they are soft, solid or solid-like materials [68]. IUPAC however define a gel as a "non-fluid colloidal network or polymer network that is expanded throughout its whole by a fluid", and further define a hydrogel as a gel where the swelling agent is water [69]. The pore size of a gel is a characteristic length related to the gaps between chains.

This thesis works with hydrophilic polymeric gels, where polymer chains are held together by a cross-linking force and surrounded by water molecules. Cross-linking causes a phase transition from a sol (colloidal suspension) to a gel. Cross-linking can take many forms, chemical cross-links are typically covalent bonds, whereas physical cross-links can be ionic or weaker intermolecular forces such as van der Waals or hydrogen bonds [70]. Which one of these bonds is involved in cross-linking often dictates the properties of the sol-gel transition. The mechanical properties of hydrogels can be probed by rheology [71, 72], a technique described in more detail and used in Chapter 3.

For example, agarose is a polysaccharide gel, where the cross-links are hydrogen bonds. Therefore the sol-gel transition is controlled by temperature as thermal energy is able to break hydrogen bonds. Poly(vinyl) alcohol (PVA) hydrogels also use hydrogen bonds as cross-links, but here the sol-gel transition is driven by repetitive freeze-thaw cycles. These can also be modified to allow for agent-induced or irradiative cross-linking [73].

Other hydrogels utilise ionic bonds. Salts produced from alginic acid form hydrated ions in solution, and so a counter ion can act as a bridging cross-link between two polymeric charges. Alginate anion chains can be held together by divalent cations, such as calcium ions. Increasing the temperature or pH allows neutral polyacrylamide (PAA) chains to become anionic and can then also be cross-linked by multivalent cations [74].

Hydrogel applications are widespread in the modern world, ranging from production of contact

lenses [75] and wound dressings [76], to preservation of quantum dots characteristics [77]. There are many uses for hydrogels in biomedical applications too, including biosensors, drug delivery and scaffolds for tissue engineering [78, 79, 80]. Agarose is particularly well known for use in gel electrophoresis for the determination of molecular weight of DNA and RNA [81, 82]. Polyacrylamide gel electrophoresis (PAGE) is used for the sorting of proteins smaller than 30 kDa [83]. Alginate microspheres are often used for drug delivery and cell culture [84, 85]. PVA hydrogels are used primarily for tissue scaffolds and artificial cartilage [86].

The use of agarose gel matrices to sort DNA based on size immediately suggests the use of hydrogels as the previously proposed micro-sieve to sort contents of a droplet based on size. If a droplet micro-reactor contained a hydrogel which could be induced to cross-link on demand, then the emulsion could be broken and desired material could be trapped within an individual hydrogel microsphere. This process would have to be designed to prevent cross-linking of microspheres to each other post emulsion-breaking, in order to keep the micro-reactors separate from each other.

1.2.2 Choosing a Hydrogel

This thesis chose to investigate using hydrogels as micro-sieves using agarose and alginate microspheres. As agarose gel matrices are well known to act as a DNA filter, they were the obvious first hydrogel to test. However the temperature dependence of the sol-gel transition suggests potential difficulties in flowing agarose solutions in tubing and microfluidic devices, so it would be useful to contrast this with a hydrogel which cross-links using a different variable. Alginate was chosen, having a cation concentration dependence on the sol-gel transition due to previous literature showing successful cell culture in alginate microspheres [85]. This means that alginate gels are bio-compatible and able to trap cells. PVA hydrogels were discounted as the cross-linking techniques have reduced biocompatibility. Freeze-thawing would destabilise an emulsion [87], and the covalent cross-linking agents are largely bio-toxic [73]. They are also considered to have poor cell adhesion ability [73]. Although PAA hydrogels are beginning to be used for cell culture [88], they have a complex gelation system [74], and so were discounted in favour of alginate gels which have a large literature of use in cell culture [85, 89, 90].

The structures of agarose and alginic acid (the precursor of alginate salts) are shown in Figure 1.5 [91, 92]. Note that alginic acid is a copolymer and can have many different arrangements of the two repeating units [91].

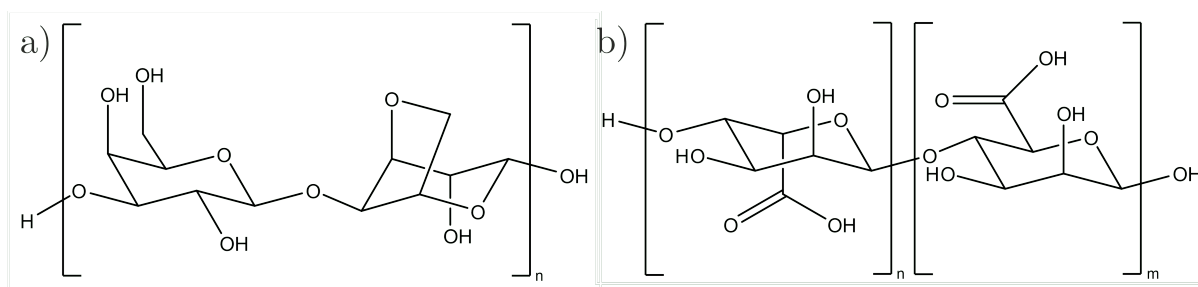


Figure 1.5: The skeletal formulae of a) agarose and b) alginate polymer chains.

Agarose is extracted from red seaweed [93], and forms a thermoreversible gel - often changing states at two different temperatures. It will have a high melting temperature (T_m) at which it transitions from a gel to a sol (and above which samples are heating for preparation from solute) a lower gelling temperature (T_g) at which the sol to gel transition happens [94]. Therefore most agarose solutions are made by heating the sol above T_m and mixing for a time period, then cooling below T_g [94], often requiring refrigeration. Chemical modification of side groups can be used to change the gelling and melting temperatures, as well as other properties of the gel [95, 96].

Alginate is extracted from brown algae, and forms salts with many metal ions. The time controlled cross-linking of alginate gels is used for purposes such as dental impressions [97]. The creation of alginate gels is a multi-component system. It involves at least an alginate salt and a cationic cross-linking agent, but often includes chelating ligands to control when cross-linking occurs [98]. Chapter 3 investigates the effect of these many components on the sol-gel transition of alginate gels, and their mechanical properties using rheology.

The creation of suspensions of agarose and alginate microspheres, and the investigation into whether their properties allow them to act as a size filter is the subject of Chapter 3.

1.3 HIV and the Latent Reservoir

1.3.1 The Global HIV Pandemic

In June 1981 the Centers for Disease Control and Prevention (CDC) in the United States of America documented five cases of men who have sex with men being diagnosed with Pneumocystis Pneumonia [99], a fungal infection usually found in people with a medical condition that weakens their immune system [100]. By September 1982 this had risen to 593 cases and was now being referred to as acquired immune deficiency syndrome (AIDS), 243 of these cases resulted in death

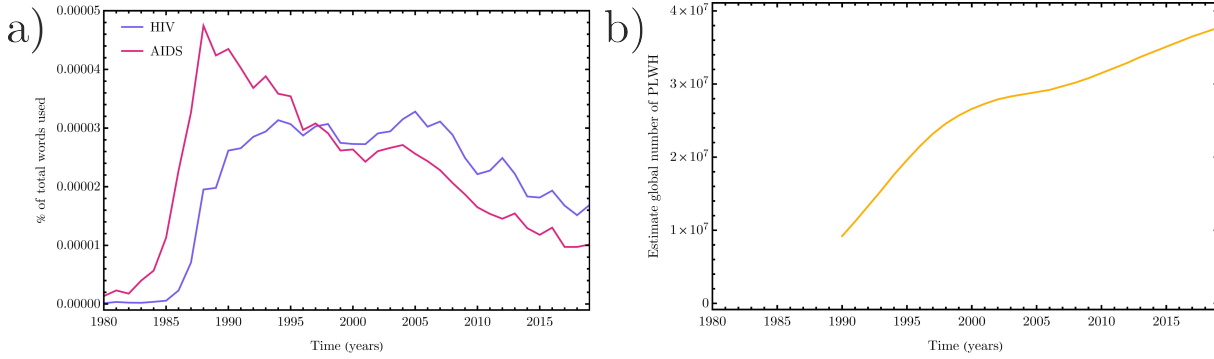


Figure 1.6: a) Google Ngram data showing the frequency of use of the terms "HIV" and "AIDS" (as a percentage of all words used) over time using their english 2019 corpus database [112] and b) The WHO estimates of the number of PLWH globally between 1990 to 2022 [113].

[101]. In 1983, the CDC identified the main causes of disease transmission as being men having sex with men, the use of intravenous (IV) drugs, or being a sexual partner of or a recipient of blood transfer from members of these groups [102]. The retrovirus recognised as causing AIDS was identified as human T-lymphotropic virus type III [103], though was later renamed the human immunodeficiency virus (HIV) [104].

The antiretroviral drugs azidothymidine (also known as zidovudine or AZT) [105] and saquinavir [106] were among the first to be used as antiretroviral therapy (ART), and it was quickly discovered that a combination of multiple antiretroviral drugs was the most effective way to treat AIDS [107, 108]. This combination treatment (known as highly active ART or HAART) had the downside of requiring **people living with HIV** (PLWH) to take multiple pills a day, so in the late 90s Combivir, a single tablet containing AZT and lamivudine (a nucleoside/nucleotide reverse transcriptase inhibitor) was approved by the FDA. Over the years since then many combination therapy drugs have been created [109]. In 2010 it was discovered that giving a combination of antiretroviral drugs to people at risk from HIV infection prior to exposure reduced transmission [110] and this became known as pre-exposure prophylaxis (PrEP). ART reduces HIV symptoms and transmission by suppressing HIV replication [111].

Despite a reduction in use of the terms "HIV" and "AIDS" in general public use (Figure 1.6a [112]) since their respective peaks in 2005 and 1988 respectively, HIV infection is not a problem that has gone away. The world health organisation (WHO) have published data estimates showing the global number of PLWH has increased every year since 1990 (See Figure 1.6b) [113]. In the 2023 world AIDS day data sheet, the Joint United Nations Programme on HIV/AIDS (UNAIDS) published the following epidemiological estimates [1] - That globally in 2022:

- 39 million people were living with HIV

- 1.3 million people became newly infected with HIV
- 86 % of all PLWH knew their HIV status
- 9.2 million PLWH do not have access to ART

In particular they estimate that 24 % of people living with HIV do not have access to ART highlights that in 2022 the response to AIDS was still ongoing.

The current UNAIDS targets for 2025 to "put the world on course to end AIDS" [114] are that:

- less than 10 % of PLWH experience stigma and discrimination
- less than 10 % of PLWH, women and girls experience gender based inequalities and violence
- less than 10 % of countries have punitive laws and policies
- 95 % of PLWH use combination treatment
- 95 % of PLWH know their HIV status, 95 % of PLWH diagnosed have access to ART and 95 % of those receiving ART have achieved viral load suppression (this is known as 95-95-95 % HIV treatment)
- 95 % of women access sexual and reproductive health services
- 95 % coverage of services for eliminating vertical transmission
- 90 % of PLWH receive preventative treatment for tuberculosis
- 90 % of PLWH and people at risk are linked to other integrated health services [114]

While ART and PrEP are known to be able to improve health and reduce the risk of HIV transmission, they inhibit HIV replication rather than eradicating HIV from PLWH. To this date there have only been a few PLWH who have been "cured", and via unique stem cell transplantations [115, 116]. This means ART is an ongoing treatment rather than a cure, and asks the question "is a replicable cure possible?" [111]. It is well established that the largest barrier to a cure for HIV is the latent reservoir [117, 118], a subject discussed in more detail in Section 1.3.3.

1.3.2 The Structure and Pathogenesis of HIV

HIV is a retrovirus, a type of virus that integrates a DNA copy of its RNA into the DNA of a host cell. The integrated form of a virus is known as a provirus. HIV is further classified as a lentivirus due to having a long incubation period and causing chronic infection. There are two species of HIV, HIV-1 and HIV-2, and while they share general properties (such as transmission pathway and progression to AIDS), they also exhibit significant differences in behaviour. In

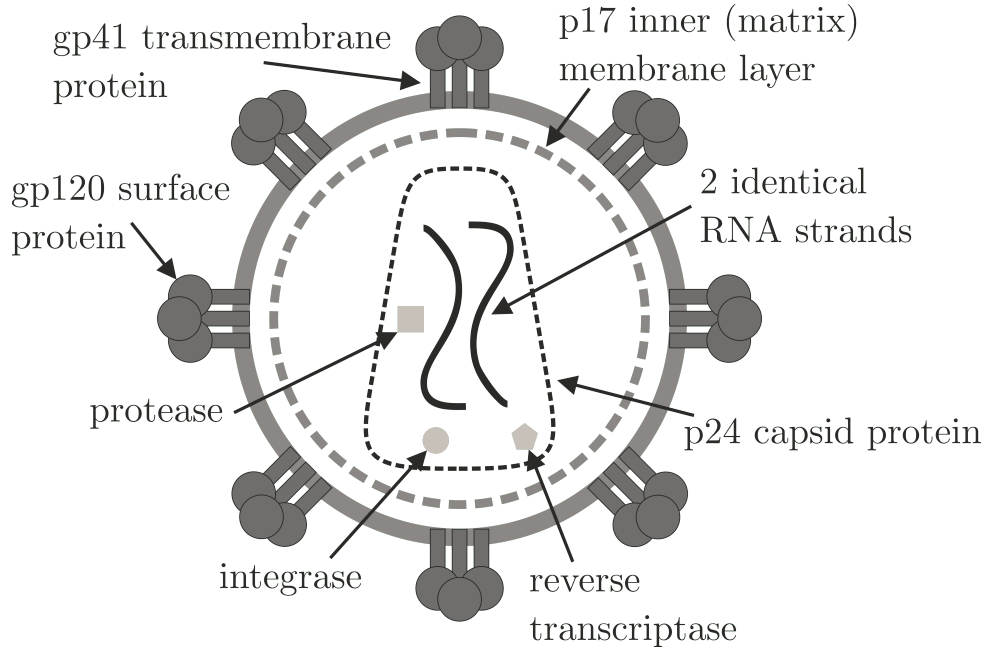


Figure 1.7: The structure of a HIV-1 virion.

particular, HIV-1 is found worldwide whereas HIV-2 is mostly located in West Africa, and HIV-2 has a lower transmissibility and slower progression to AIDS than HIV-1 [119]. HIV infection progresses to AIDS by reducing the ability of CD4⁺ T cells (white blood cells involved in the adaptive immune response) to act as part of the adaptive immune system, and also by destroying them [120, 121].

Typically viruses consist of RNA or DNA contained within a capsid (a protein shell). As viruses do not contain organelles, they rely on a host cell to produce energy, and synthesise proteins for replication [122].

Electron microscopy of the HIV virion reveals a spherical structure (approximately 50 nm in radius) containing an electron dense ribonucleoprotein nucleocapsid core with 70-80 "complex surface knobs". Immuno-electron microscopy showed that the capsid contained a viral protein called p24, the viral envelope contained gp41 and the surface knobs contained gp120 (a gp, or glycoprotein, is a protein containing oligosaccharide chains covalently linked to amino acid side chains) [123]. Further work showed the viral envelope could be described as trimers of gp120 [124] on a gp41 transmembrane. Like other retroviruses, the capsid encloses two identical RNA strands, alongside protease, reverse transcriptase and integrase enzymes required for viral replication [125]. Within the capsid, the RNA strands are bound to nucleocapsid proteins [126]. The structure of a HIV-1 virion is shown in Figure 1.7. The HIV-1 provirus genome contains nine genes (gag, pol, env, tat, rev, nef, vpr, vif and vpu) which code for 15 distinct proteins

[127]. These genes are enclosed at either ends of the proviral DNA with long terminal repeats (responsible for integration of the DNA into the host cell's DNA). The three major genes (gag, pol and env) are common to all retroviruses [127] and are broken down to produce multiple proteins, each with their own function during viral replication. Gag consists of the structural proteins responsible for the capsid (p24), matrix or inner membrane layer (p17), nucleocapsid (p7) and p6 (a protein involved in virus particle release). Pol contains three enzymes required for replication that are contained in the nucleocapsid; protease, reverse transcriptase and integrase. The two env glycoproteins are structural components of the viral envelope, gp120 (surface) and gp41 (transmembrane). Vif, nef and vpr are three accessory proteins found in the virion, and tat and rev regulate transcription and RNA splicing. Finally vpu aids virion assembly [125, 127]. HIV-1 virion replication occurs as follows (and is shown in Figure 1.8) [127]:

1. The gp120 surface protein on a mature HIV-1 virion binds to CD4 receptors and other co-receptors on CD4+ T cells
2. The gp41 transmembrane proteins undergo a conformational change to allow the virus and host T cell membranes to fuse
3. The contents of the virus particle including the capsid, nucleocapsid, and genome enter the host T cell
4. The reverse transcriptase enzyme converts the single stranded RNA genome into double stranded DNA and the original viral RNA is degraded
5. The integrase enzyme integrates this double stranded DNA into a host chromosome (where it becomes proviral DNA)
6. When the host T cell is activated or divides, it transcribes unspliced viral RNA genome and new viral enzymes
7. The newly transcribed HIV-1 proteins assemble at the host T cell membrane and assemble into a new virus particle (a process known as budding)
8. The immature virus particle is released from the host cell. After release, proteolytic activity of HIV Gag-Pol results in the formation of mature virus particles

1.3.3 The Latent Reservoir

Reservoirs of HIV-1 were first hypothesised when it was theorised that only 2 to 3 years of HAART treatment would be required to eradicate a patient's HIV viral load, assuming no viral reservoirs existed [128]. Replication competent HIV was later discovered in the resting (inactivated) CD4+

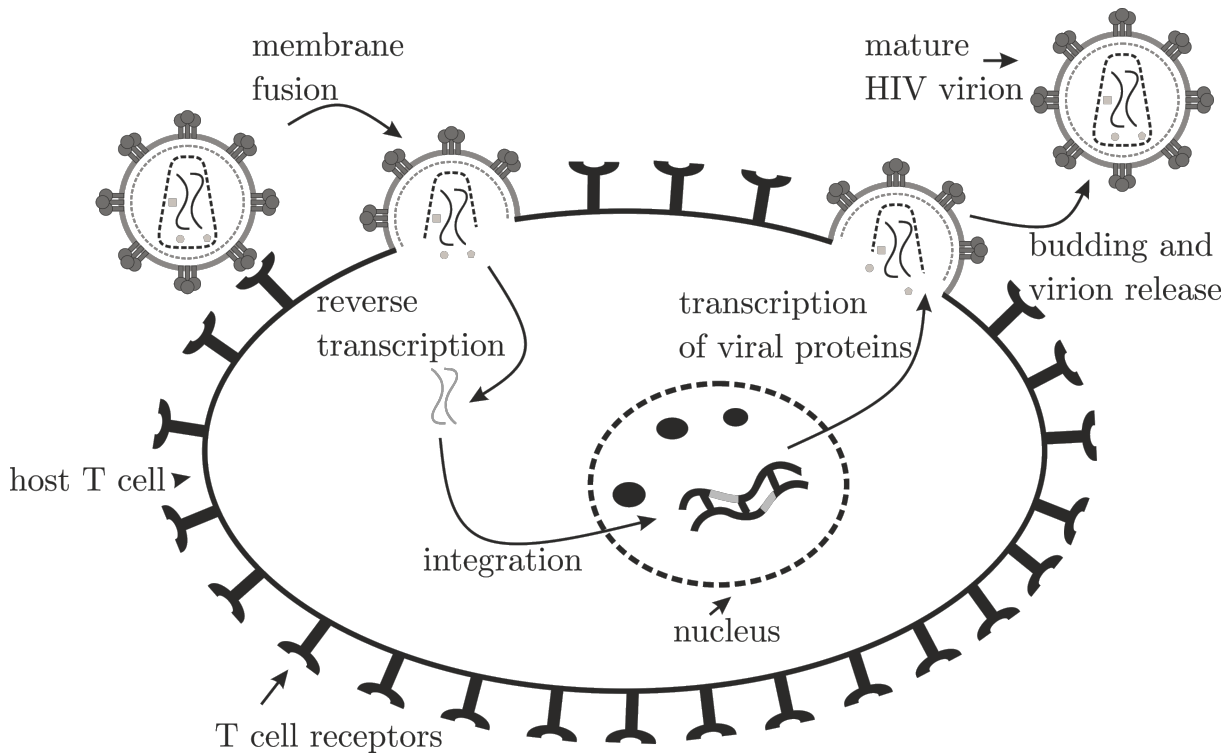


Figure 1.8: A simplified summary of HIV-1 virion replication in a host CD4+ T cell.

T cells in patients treated with HAART for 30 months and in patients whose viral load was less than 50 copies per mL for up to 2 years [129, 130]. While there are many other methods by which HIV evades innate [131] and adaptive host immunity [132, 133], the largest barrier to a cure is often named as the latent reservoir [134].

The latent reservoir consists of transcriptionally inactive provirus DNA integrated in host resting CD4+ T cells, present even in patients with undetectable viral loads (as well as non-integrated HIV DNA) [129, 135]. This means that ART cannot eliminate the latent reservoir, only reduce the viral load in patients to below the detectable limit. If ART is interrupted, the resting memory CD4+ cells will eventually activate, producing a new unspliced viral RNA genome and new viral enzymes, leading to the formation of new HIV virion particles - hence a rebounding of the viral load. There are many proposed mechanisms contributing to the persistence of the integrated HIV DNA [136]. Integrated HIV in resting CD4+ T cells is considered stable with respect to time, with a half-life of up to 25 years [137, 138], which is too long to hope for a cure by self-degradation. Therefore a key milestone in the development of a cure to HIV and the AIDS pandemic is to accurately detect and measure the size of the latent reservoir.

1.3.4 Quantifying the HIV Reservoir

The current "gold standard" technique for quantifying the size of the HIV reservoir is the virus outgrowth assay (VOA) [139], but there are many other assays in development [140].

The quantitative VOA (QVOA) activates resting CD4⁺ T cells (obtained from the blood of PLWH on ART treatment) and induces the production of virus particles. Cells susceptible to infection are added and cultured alongside these activated T cells to allow exponential growth of virus to a viral concentration detectable by a viral antigen ELISA (an enzyme linked immunosorbent assay) or other assay. Detection using p24 antigen ELISA takes 14 to 21 days, or comparable RT-qPCR takes 7 days, mostly in order to show virus detected is intact, or replication competent (only around 12% of proviral DNA detected is intact [141]). While there are modifications to QVOA to make it less labour, time and resource intensive, it still underestimates the size of the reservoir and requires a large number of cells [2, 142]. This is attributed to either activated cells proliferating without producing virus, or not all latent cells being activated upon initial stimulation and therefore require further activation [143, 144].

There also exist a number of polymerase chain reaction (PCR) based assays for reservoir detection. A set of cells suspected to contain latent provirus can be lysed to release the genomic DNA. PCR then detects and amplifies a particular sequence of bases in a DNA sequence through cycles of heating and cooling (this thermocycling methodology is discussed in more detail in Chapter 4) and can be designed to target a region of the HIV genome. Quantitative PCR (qPCR) uses a fluorescence probe to quantify the number of successful amplifications, and therefore quantifies the reservoir. Digital PCR (dPCR) reduces noise by dividing the sample into thousands of micro-reactions on a plate, or using micro-droplets (droplet digital PCR - ddPCR) [145, 146]. In contrast to the QVOA, these PCR methods overestimate the size of the reservoir as they cannot tell intact and defective provirus apart [3]. The intact proviral DNA assay (IPDA) is a ddPCR method that can distinguish between intact and defective provirus by using two target sequences rather than just one. 97 % of proviruses shown to be defective by near full genome sequencing are excluded by IPDA [138].

Another measurement of the latent reservoir is the frequency of CD4⁺ T cells which produce tat/rev multiply-spliced HIV RNA upon activation. This is done by the tat/rev induced limiting dilution assay (TILDA), which estimates a reservoir size between that of the QVOA and PCR based assays [142].

It becomes obvious that there is currently not a single assay that can be used to quickly, easily

and accurately determine the size of a PLWH's latent reservoir [147]. An alternative to the assays described above is full genome sequencing, as this is a method that tells us all the available information about a provirus and avoids the loss of information from the sub-genomic sequencing methods described above [2]. Single molecule genome sequencing can provide information about the source of persistence of the latent reservoir and the transmission dynamics of HIV [148]. Section 1.4 investigates current sequencing technologies, looking at the differences between DNA and RNA sequencing, how HIV proviruses are currently sequenced, and the difficulties in doing so. This is continued in Chapter 4, where we will propose a new single cell sequencing assay using a combined hydrogel and microfluidic platform for latent reservoir characterisation.

1.4 Sequencing Technologies

1.4.1 DNA and RNA Analysis

DNA (deoxyribonucleic acid) is a long chain polymer which carries the genetic information of most organisms. This information controls the development and activity of cells in the organism required to be functional. DNA consists of a sugar-phosphate backbone with a sequence of bases (any of A, T, C or G). Each base has a complementary base it can form hydrogen bonds to (A with T or C with G), and this causes DNA to exist as a double helix of two polymer chains with complementary sequences held together by hydrogen bonds [149]. DNA is contained within the nucleus of human cells, so nuclear lysis (breakdown of cell membranes) is required to release DNA strands for sequencing. There are many methods of cellular lysis [150], a topic discussed in more detail in Chapter 4.

Ribonucleic acid (RNA) has a similar structure to DNA. RNA is usually single stranded, has a ribose backbone and contains base U in place of T. Most RNA in a cell does not reside in the nucleus [149]. While DNA stores the genetic information in a cell nucleus, RNA molecules copy this information, leading to the generation of proteins that are responsible for the structure and function of cells [149].

It is the sequence of bases that encodes the biological function of DNA and RNA, so studying the sequence of bases in a specific strand is important for understanding the biological function. There are many methods of next generation sequencing (NGS), DNA NGS usually first involves DNA fragmentation and a library preparation (modifying DNA segments to have an identifiable index and adding sequencing adaptors). Massive parallel sequencing of all DNA segments is then

achieved using a variety of methods such as chips or flow cells. Finally, the data is analysed using an array of bioinformatic approaches to collate the data, and generate sequences for the original full-length DNA. This also allows identification of mutations [151]. Typically, the detection of bases is done by methods such as sequencing by synthesis, where a polymerase is observed copying a DNA strand using fluorescence [152] or electrical charge [153].

The length and purity of a DNA fragment can be determined by gel electrophoresis. A DNA sample is stained with an intercalating dye, then moved through a gel matrix by an electric field (as DNA is negatively charged). Shorter strands move further in a given time period. PAA gels are used for small fragments (< 100 s nucleotides) and agarose gels for larger (< 50 kb) [149]. The result is compared with a ladder of DNA fragments of known length to allow estimation of the lengths of DNA fragments in the sample. This is also used as method of separation of different length DNA strands for purification [82, 154]. The ability of a DNA strand to move through a gel matrix was shown to depend on the ratio of radius of gyration of the DNA strand and the pore size of the gel [155]. The radius of gyration of a random coil polymer:

$$R_g = \frac{R}{\sqrt{6}} \quad (1.15)$$

depends on the mean end-to-end distance ($R = \sqrt{Na^2}$), which itself depends on the number of statistical segments in the chain (N) and the length of these segments, otherwise known as the Kuhn length (a) [156].

The concentration of a DNA or RNA sample can be found using a Qubit fluorometer. A dye is added which binds to the polymer of interest, fluorescing only on binding. The fluorescence intensity is detected by a fluorometer and is compared to a standard sample to calculate a DNA concentration, usually in units of mass per unit volume [157]. This technique is discussed in more detail in Chapter 5.

1.4.2 HIV Sequencing

The full genome sequencing of a provirus is interesting due to the diversity of the HIV-1 genome [158, 159]. By sequencing the provirus it is possible to show whether it is intact or defective, though different methods may show different results [160]. HIV sequencing can be used to monitor transmission dynamics and drug resistance [161]. There are many methods for full genome sequencing of the provirus contained in a latent T cell, but the largest obstacle to overcome is the "rarity" of a latently infected cell. It is estimated that for a PLWH on ART,

around 300 per 10^6 resting CD4+ T cells contains provirus [2], of which only 0.1 to 10 per 10^6 contain intact provirus [3]. This would suggest that to sequence one intact provirus sequence, you would have to sequence up to 10^7 cells. While there are methods to sequence rare targets like these, they tend to be limited by sensitivity and cost. They also are unable to preserve the information which could link specific mutations to other mutations on the same template genome, and to other specific characteristics of host cells (such as transcriptomes or protein expression) [162, 163].

Instead a set of T cells obtained from a PLWH on ART is lysed in bulk, PCR amplifies only near full-length provirus which then minimises the noise from non-proviral DNA. The amplified DNA is purified, and undergoes next generation sequencing (NGS). For example, the full-length individual proviral sequencing (FLIPS) assay does this by quantifying the DNA concentration post amplification, and diluting this down on a plate so that 80 % of wells contain a single provirus strand [164]. Similar techniques include STIP-seq [165] and MIP-seq [166], but these methods typically miss intact provirus or cannot distinguish between intact and defective provirus [167]. The advantage of this methodology over the assays discussed in Section 1.3.4 is that near full-length genomic data is obtained rather than sub-genomic. The problem caused by the rarity of HIV infected latent cells is reduced by only amplifying the sequences containing HIV DNA. However it only processes one strand of proviral DNA per well, and therefore is not high-throughput. As each strand of PCR product is analysed with equal weight, and independently, it is impossible to tell which products came from the same cell, so a dimension of the information is lost. If there was a method that retained single cell resolution, we could keep all the amplified proviral DNA from one cell together. Here errors in PCR could be detected as such rather than as genetic variation (if every DNA strand was assumed to be unique), and we could be confident we are observing a representative HIV proviral genome. The desire for such an assay prompted the development of many single cell approaches to investigate HIV, mostly through RNA sequencing [162, 163].

1.4.3 Single-Cell Sequencing Technologies

There are many high throughput single cell RNA sequencing platforms. The general mechanism involves cells being sorted into individual bioreactors. There they are lysed and undergo reverse transcription to select mRNA, and the cDNA is amplified and sequenced [168]. Cell sorting typically is done by fluorescence activated cell sorting (FACS), a technology which labels surface

markers on cells with fluorescent labels. The cells are then flowed past a laser beam, and those tagged emit fluorescence. This is detected by a computer and the cell's path is diverted using an electric field [169, 170]. Drop-Seq, DroNc-Seq and 10x Genomics use droplets [168, 171, 172] and Nx1-Seq and Seq-Well and BD RhapsodyTM use microwells [168, 173, 174] as the micro-reactors. inDrop (Illumina) uses a combined hydrogel and droplet approach to deliver oligonucleotides [175]. Even the most high-throughput of these, 10x Genomics, can only sequence up to 80,000 cells per chip at a single-cell resolution [4].

To study the HIV latent reservoir, interest lies in sequencing the proviral DNA rather than the free RNA. The desired information is the position of the provirus integrated into host cell DNA, and this cannot be detected from RNA sequencing. DNA single cell sequencing is a more tricky problem than RNA sequencing. Releasing nuclear DNA requires stronger lysis reagents than to release RNA from a cell, and these can inhibit subsequent amplification [9]. Therefore the droplet micro-reactors need to be able to remove cell lysates while retaining trapped genomic DNA at a single cell resolution.

Another problem is that full genome sequencing of all cells in a population at a single-cell resolution would be time and resource inefficient due to the rarity of proviral DNA. So there would be considerable interest in the development of a technique to sort latently infected cells from healthy cells to reduce the cost of such methods. Therefore there is a need to develop a method to only sequence proviral DNA - a way to sort infected cells from uninfected cells prior to sequencing while keeping nuclear material from each individual cell separate. While there is some evidence of surface markers unique to latently infected cells, there is not yet a method that can reproducibly find all latent cells in a sample [176], so FACS is not yet a viable method for this.

The ideal solution to these problems would be an assay in which cells can be singly encapsulated into droplets and lysed. Small cell lysates and excess lysis reagents can then be removed, while keeping genomic DNA trapped before PCR reagents are added to form new droplets. Successful PCR amplification of HIV provirus can then produce a fluorescent signal for the droplets which are of interest to be sequenced. These can be sorted and sent for sequencing. The development of such an assay was the motivation behind this thesis, where we produced hydrogel microspheres for the trapping of nuclear DNA at a single cell resolution for further PCR and sequencing. We proposed that the trapping of the relatively large genomic DNA while washing with smaller reagents could be achieved using hydrogel microspheres with tunable pore sizes.

As discussed in Section 1.1.4, droplet encapsulation [42] and droplet sorting based on fluorescence [65] are well studied techniques which would be required in this assay. The bulk of the work presented in this thesis therefore instead investigated how to produce and utilise hydrogel microspheres for DNA trapping, and to discover how effective they were at trapping DNA and other material.

1.5 Thesis Outline

The rest of this thesis is split into one methods and three results chapters. Each of the latter is designed to answer several related questions required to see how feasible a combined microfluidic and hydrogel assay is for the detection and quantification of the HIV latent reservoir. Each results chapter discusses the theory and methods required to answer these questions, and presents the outcome of the investigative work, highlighting where further work is required.

Chapter 2 - Experimental Techniques - describes the reagents and equipment relevant for the thesis as a whole. It describes how to use soft lithography to make masters of microfluidic chips, and how the chips are fabricated using these. It then describes the typical set up for droplet formation and manipulation, and ends by discussing the culturing and staining of the Jurkat cell line used throughout this thesis.

Chapter 3 - Hydrogel Properties and Microsphere Formation - questions how agarose and alginate hydrogels behave in bulk with varying conditions, using rheology to characterise their mechanical properties. It then discusses the use of a microfluidic platform to produce both agarose and alginate microspheres, showing the advantages and disadvantages of each. Finally, the properties of the microspheres relevant for the assay to be successful are studied, in particular their swelling, stability (with respect to heating, centrifugation, time, storage method and irreversible coagulation), and reversibility.

Chapter 4 - The HIV Latent Reservoir Assay - outlines and tests the three separate technologies required for the overall assay to be successful, comparing agarose and alginate hydrogels. First we discuss cell lysis of cells encapsulated within droplets and within gel beads, looking at how the order of reactions affects lysis ability, and the variation between cell lysis techniques in droplets. The second technology investigated is the re-encapsulation of hydrogel beads inside new, larger droplets for eventual PCR, looking at how this is effected by channel geometries, washing steps and strength of gel bead. Chapter 4 closes by developing techniques for the PCR of DNA inside droplets, bulk gels and hydrogel beads.

Chapter 5 - The Trapping Ability of Microspheres - compares and contrasts different agarose and alginate beads' ability to trap DNA, colloidal particles and cells. It aims for a quantification of the trapping abilities demonstrated in Chapter 4, and ends by using microscopy techniques to characterise microspheres of different compositions.

Chapter 6 - Conclusions and Outlook - summarises the findings found in the other chapters, and details further work required on the assay, as well as highlighting other potential applications of the technology developed in this thesis.

Chapter 2

Experimental Techniques

This chapter details experimental procedures that are relevant to two or more later chapters. Any techniques related to only one chapter will be discussed in the relevant chapter. We begin by describing how soft lithography is used to produce masters of microfluidic designs, which themselves are then used to create microfluidic devices. We then discuss the general setup to flow a microfluidic device (using a device designed to make a single emulsion of water-in-oil droplets as an example) to produce a collection of droplets which we then show breaking and heating protocols for. We finish by describing the model cell system used in this thesis, detailing cell culture, collection and staining.

2.1 Reagent Information, Preparation, and Storage

Here we detail extra information about reagents and consumables used in Chapter 2. We also discuss preparation of any materials used in multiple of the following chapters.

Silicon Wafers were obtained from Si-Mat Silicon Materials. They were single side polished with a diameter of 100 mm and thickness of 525 μm . They were grown by the Czochralski method [177] and doped with boron.

Oils - The two oils used in this thesis are HFE-7500 (Acota Ltd) and FluorinertTM FC-40 (Merck). Where HFE-7500 is used to produce a 1 % EA solution in HFE-7500 it was first filtered using Minisart[®] filter unit 0.2 μm .

EA-008 - The fluorosurfactant used in this thesis is obtained from RAN Biotechnologies under the name 008-FluoroSurfactant (also known as E2K0660 [178, 8]). It was either obtained as a 2 % w/w solution in HFE-7500 or as a neat (un-dissolved) solid. This thesis used a 1 % EA solution in HFE-7500 or 1 % EA solution in FC-40 made by mixing the above samples with

relevant volumes of filtered HFE-7500 or FC-40 oils.

Alginate powder (medium viscosity, ≥ 2000 cP for a 2 % w/w at 25 °C) and low viscosity (4-12 cP for a 1 % w/w at 25 °C) alginate samples were obtained from Merck (alginic acid sodium salt from brown algae) and were stored at 5 °C. An alginate sample tagged with fluorescein was obtained from Creative PEGWorks with a viscosity of 100-300 cP, 1 MOL % degree of substitution and 95.3 % purity. This alginate fluorescein has excitation maxima of 494 nm and emission maxima at 518 nm.

Water - MilliQ ultrapure (type 1) water from a Direct-Q® 3UV water purification system was used for preparation of all solutions in this thesis, unless otherwise stated. UltraPure™ distilled water (DNase/RNase-free) was used for any samples involved in PCR.

AlgEDTA Mixes - In chapter 3 we discuss using a mixture of sodium alginate and ethylenediaminetetraacetic acid calcium disodium salt (hereafter referred to as calcium EDTA) as a precursor to a crosslinked alginate hydrogel. These samples were made by mixing equal volumes of sodium alginate and calcium EDTA solutions of double the required concentration (themselves made using MilliQ distilled water and by stirring overnight). For simplicity these are referred to as AlgEDTA mixes in this thesis. For example a 2.5 % AlgEDTA mix was made by mixing equal volumes of 5 % w/w sodium alginate and 0.5 M calcium EDTA. This ratio of % w/w sodium alginate to molar concentration of calcium EDTA solution is maintained throughout this thesis. New samples were made every month to avoid degradation.

Agarose - Two types of agarose were used throughout this thesis. D1-LE molecular biology grade agarose (Alfa Aesar) was used with a gelling temperature of 36 ± 1.5 °C. We also used ultra low gelling agarose (A5030, Merck) with a gelling temperature of 8 -17 °C, and melting temperature of 50 °C for a 0.8 % w/w sample. Samples are produced either in an Eppendorf tube using a thermomixer or in a beaker covered by parafilm on a hotplate while heating the sample at 90 °C for 1 h. These are referred to as D1-LE or ULG (ultra low gelling) agarose throughout this thesis. Agarose powder is stored at 5 °C.

Tubing - All polytetrafluoroethylene (PTFE) tubing was obtained from Masterflex™. Unless otherwise mentioned, all tubing is 30 AWG (with an internal diameter of 0.32 mm).

Needles - Blunt QuantX™ precision dispense tips (27-gauge, 1.37 cm in length) were used for the bulk of this thesis. Any other tips used are discussed when relevant. All tips were obtained from Fisnar.

SYBR™ Gold Nucleic Acid Gel Stain - A cyanine dye obtained from ThermoFisher

Scientific which fluoresces upon binding to single or double stranded DNA or RNA. It is used in Chapters 4 and 5. It has excitation maxima at 495 nm and 300 nm and emission maxima at 537 nm.

CellTracker™ CM-Dil Fluorescent Dye - Obtained from ThermoFisher Scientific™, this dye had excitation maxima at 553 nm and emission maxima at 570 nm respectively. This was used for cell staining.

R10 Culture Media - R10 consists of 10 % fetal calf serum (FCS) and 1 % Penicillin-Streptomycin in RPMI-1640 (which contains biotin, vitamin B₁₂, para-aminobenzoic acid, inositol and choline).

2.2 Microfluidic Techniques

The microfluidic chips used to create and manipulate droplets consist of a polydimethylsiloxane (PDMS) layer bonded to a glass slide. The PDMS layer itself is made using a master wafer with the device design as an embossed pattern (then creating a PDMS layer with the device design as a debossed or indented pattern). A chip contains multiple individual microfluidic devices.

2.2.1 Soft Lithography

This section describes the creation of a master wafer using soft lithography. Master wafers for a single emulsion device and a bead re-encapsulation device are shown in Figure 2.1a and Figure 2.1b respectively. This protocol is adapted from the MicroChem SU-8 2000 series processing guidelines [179] and is summarised in Figure 2.3. The conditions that vary between different masters are labelled with greek letters α to ϵ in the protocol and are summarised in Table 2.1.

Substrate Pre-heat - A silicon wafer (Silicon Materials) was heated at 200 °C for 2 h to dry the substrate.

Spin-Coat - The wafer was held in place on a spin-coater (WS-650MZ-23NPP, obtained from Laurell Technologies) by a vacuum and then covered with 4 mL SU-8 2015 epoxy negative photoresist (Kayaku Advanced Materials). The wafer was accelerated at 100 rpm/s to 500 rpm and held there for 10 s. It was then accelerated at 300 rpm/s to α rpm and held there for 30 s. This final rotational speed varied depending on the height desired for the features on the master wafer.

Soft Bake - The wafer was removed from the spin-coater and heated on a hot plate at 65 °C for 2 min then at 95 °C for β min and finally at 65 °C for 2 min.

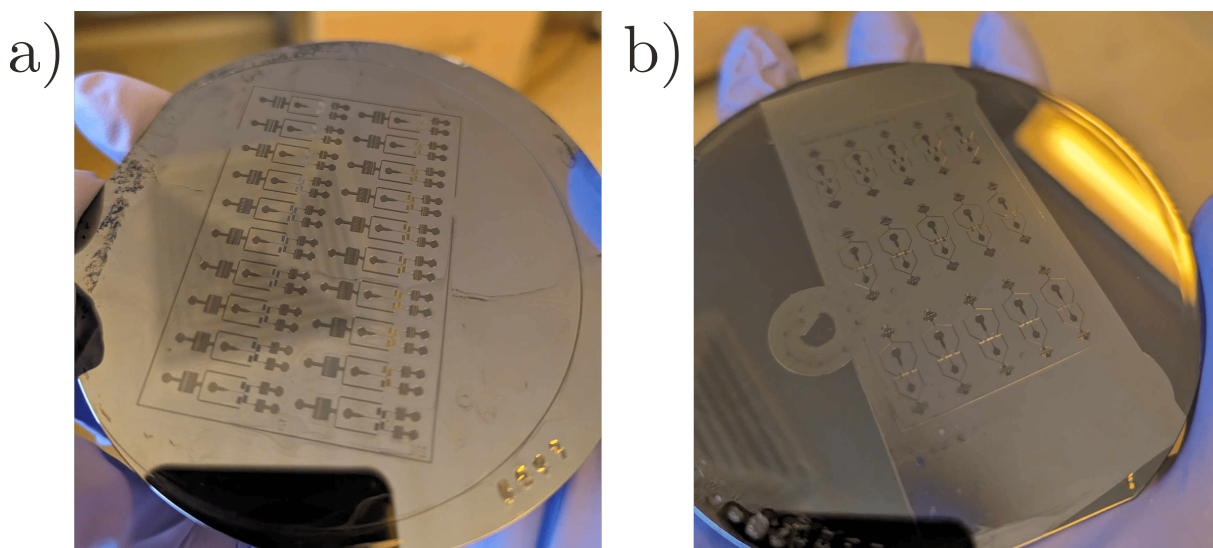


Figure 2.1: Master wafers for a) a single emulsion device and b) a re-encapsulation device.

Expose to UV - The wafer was placed on an inverted Petri dish atop three magnets. A photo-mask of the features (where the parts of the substrate covered from UV light will not cross-link) was placed on top of the wafer, covered with a long pass filter and held in place with another three magnets. This is exposed to UV light from a spot light source (LightningCure™, LC8 L9588) for γ s. A simplified diagram of this is shown in Figure 2.2.

Post Exposure Bake - The wafer was heated on a hot plate at 65 °C for 1 min then 95 °C for δ min and finally 65 °C for 1 min.

Develop - The wafer was sprayed with propylene glycol methyl ether acetate (PGMEA - a developing agent, Merck) for 2 min ensuring all features are well sprayed. It was then sonicated while submerged in PGMEA for ϵ s before spraying with PGMEA for another 2 min.

Rinse and Dry - The wafer was rinsed with isopropanol to remove all residual PGMEA and allowed to air dry.

Hard Bake - The wafer was heated on a hot plate at 65 °C for 1 min, then 150 °C for 5 min, and finally 65 °C for 1 min.

Coat - The wafer was left overnight in an air-tight container with 20 μ L of a silanizing agent (1H,1H,2H,2H-perfluorooctyltrichlorosilane, Alfa Aesar) to reduce sticking to the PDMS layer.

Note - All device patterns were designed and drawn in AutoCAD by Dr Lucia Parolini with the exception of the single emulsion device which was designed by Alice Russell in CorelDraw.

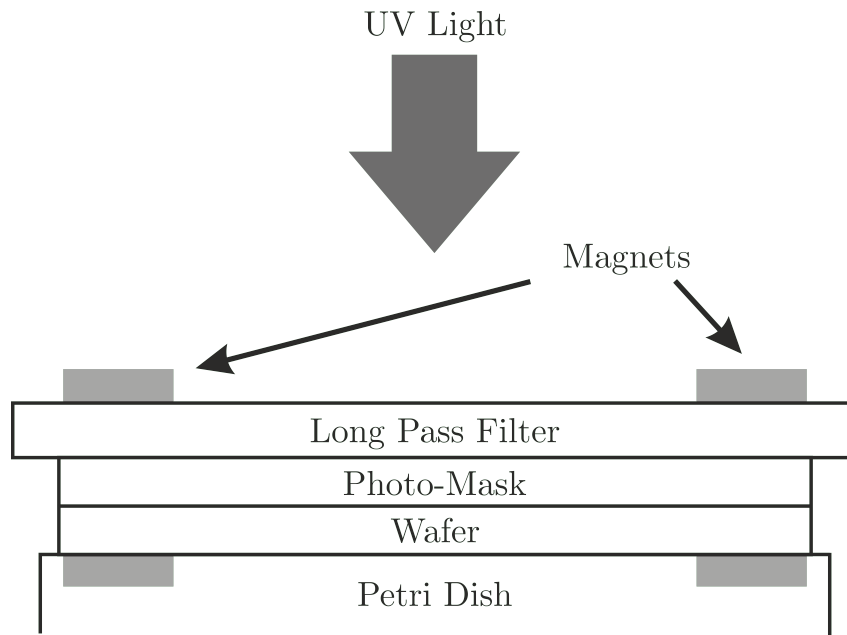


Figure 2.2: A simplified summary of how to expose a substrate to UV light for photo-curing.

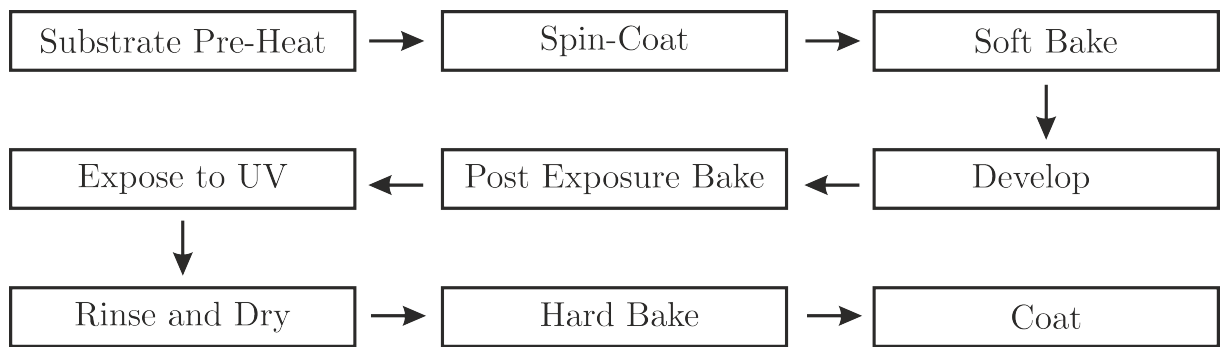


Figure 2.3: A simplified summary of the creation of a master wafer.

2.2.2 Microfluidic Chip Production

Microfluidic chips consist of a PDMS layer bonded to a glass slide as shown in Figure 2.4. The PDMS layer is made using a silicon wafer master made as described in Section 2.2.1. The protocol is as follows, and summarised in Figure 2.5. The relevant silicon master was cleaned using an air supply and wrapped in tape to form a mould. SYLGARD™ 184 silicone elastomer base and SYLGARD™ 184 silicone elastomer curing agent were mixed in a 10:1 weight ratio (of a volume large enough to give a chip depth of 0.5 cm) thoroughly. This mix was poured onto the mould (Fig. 2.5a) and left in a desiccator under vacuum until all bubbles are removed (around 2 h). The mould was then heated in an oven at 75 °C until cured (around 2 h). The PDMS layer was peeled away from the silicon master and cut to shape and size (Fig. 2.5b). Holes were then punched using a WellTech Rapid-Core sampling tool (0.75 mm) through the PDMS to form the entry and

Table 2.1: A summary of the varying conditions used to produce the different master wafers used throughout this thesis.

Device	Spin-Coat Rotational Speed /rpm (α)	Soft Bake Duration /min (β)	Exposure Time /sec (γ)	Post Exposure Bake Duration /min (δ)	Developing Duration /sec (ϵ)
Single Emulsion	1250	6	90	6	60
Single Emulsion with Acid Inlet (20 μm)	2100	4	60	4	30
Single Emulsion with Acid Inlet (30 μm)	1250	6	90	6	60
Re-encapsulation (25-45)	1250	6	90	6	60
Re-encapsulation (35-50)	1250	6	90	6	60
Re-encapsulation (40-55)	1125	6	90	6	60

exit points (inlets and outlets) for various liquids into the device. The PDMS layer and glass slide were washed with ethanol and dried, before being plasma cleaned and bonded together (Fig. 2.5c). The device was then left in an air tight container with 1 mL hexamaethyldisilazane (Merck) overnight in order to apply a hydrophobic coating.

2.2.3 Droplet Production

We produced a water-in-oil emulsion (W/O) by flowing various liquids through a single emulsion (SE) microfluidic device containing a flow-focusing junction (FFJ). This process takes place on a microscope stage to allow viewing of the droplet formation in real time, this is necessary to make any alterations to conditions required for stable droplet formation. The process of making droplets containing certain reagents within a water-in-oil droplet is referred to as encapsulation of said reagents throughout the rest of this thesis. The layout of channels in the typical single emulsion droplet making device is shown in Figure 2.6 and was designed by Alice Russell and Mathew Jones [180]. This is referred to as the SE device in any further work. Any other devices used will be discussed in their relevant chapters.

The SE device contains two aqueous inlets, one oil inlet and an outlet, and uses a FFJ to create droplets. This allows for a droplet to be created consisting of two different aqueous samples that will not mix until droplet formation. The general protocol for making droplets is as follows and

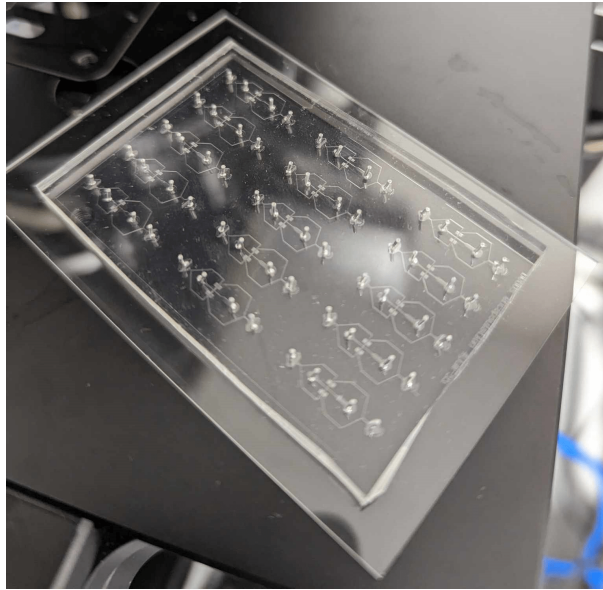


Figure 2.4: A microfluidic chip used for the re-encapsulation of hydrogel beads, it contains 15 individual devices.

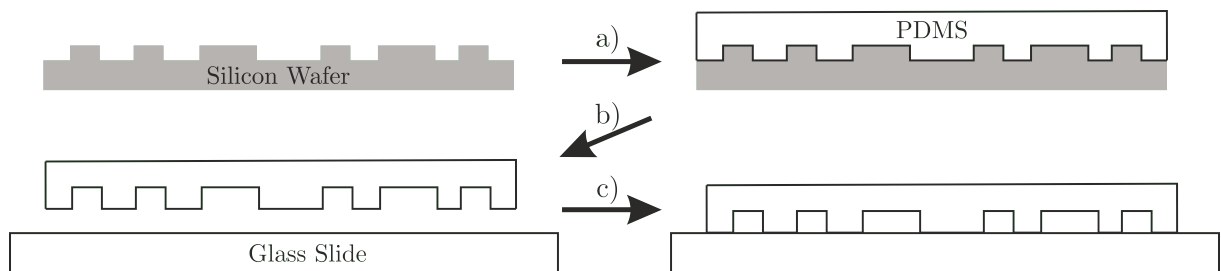


Figure 2.5: A simplified summary of the creation of a microfluidic chip.

summarised in Figure 2.7.

PTFE tubing was attached to a precision tip which was placed on top of a syringe (as shown in Figure 2.7c). For the aqueous inlets, the syringe was first filled with 300 μL HFE-7500 oil. The oil was pushed to the end of the tubing and then backfilled first with a small amount of air, and then with the relevant aqueous sample (at 60 $\mu\text{L min}^{-1}$, or 30 $\mu\text{L min}^{-1}$ if cells or hydrogel microspheres were part of the aqueous sample). The oil inlet was a syringe filled with 1% EA in HFE-7500), attached to PTFE tubing by a QuantXTM precision dispense tip and flowed directly into the device. The syringes were then pushed by separate controlled flow rate syringe pumps (Harvard Apparatus Pump 11 Elite), with the oil syringe typically pushed at 7 $\mu\text{L min}^{-1}$ and the aqueous syringes typically pushed at 1 $\mu\text{L min}^{-1}$ each. These flow rates were adjusted to form droplets of around 30 μm in diameter, but any large deviations from this will be mentioned when relevant. The droplets were collected via PTFE tubing into an Eppendorf container (Figure 2.7d).

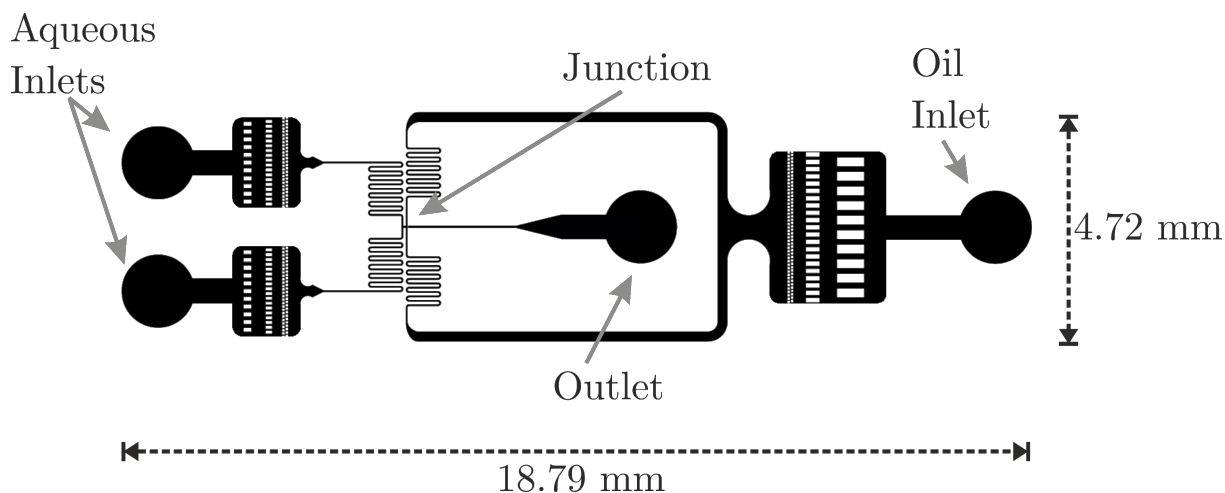


Figure 2.6: A schematic of the Alice Russell single emulsion device.

2.2.4 Droplet Breaking

A frequently required step in multi-step droplet assays is breaking of the water-in-oil emulsion to form aqueous and oil layers. This was done by the addition of 1H, 1H, 2H, 2H-perfluoro-1-octanol (PFO, Merck) to a sample of droplets and water in a 1:1:0.25 volume ratio. Assuming the initial creamed droplets are close packed, this should produce an aqueous layer of the same volume as the initial volume of droplets collected. The sample was left to stand for a couple of minutes until complete phase separation had occurred. The oil layer at the bottom was then removed from the sample. This is the method used for emulsion-breaking unless otherwise stated.

2.2.5 Droplet Heating

Emulsions are thermodynamically unstable (with respect to liquid-liquid phase separation - see Chapter 1) so are kinetically stabilised with surfactants. Upon heating, W/O emulsions are destabilised further so steps are required to stabilise these. Any droplets to be heated were formed containing 0.5 % v/v Igepal C-360 (ChemCruz™). When droplets are heated, the 1 % EA in HFE-7500 continuous phase was replaced by 1 % EA in FC-40. Also a layer of mineral oil (0.833 g mL^{-1} , Merck) was placed above the droplets. This increased thermostability and reduced aqueous evaporation [8].

2.2.6 Imaging

Two separate microscope setups were used to image droplet formation, and the other samples imaged in this thesis. Both setups consisted of an inverted microscope with various 10x and 20x

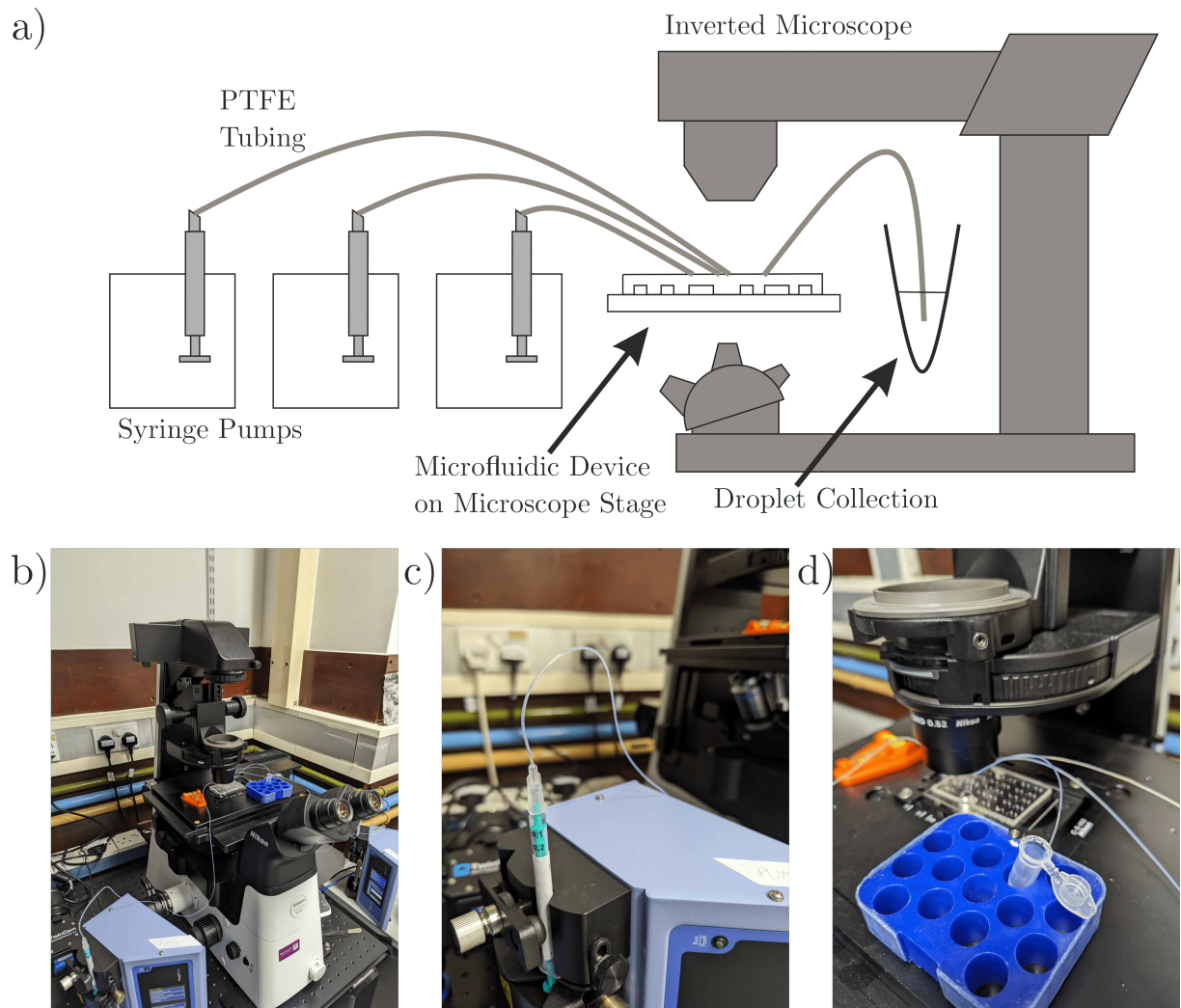


Figure 2.7: a) A schematic of the whole droplet making setup. Photographic images showing b) an overview of the setup, c) the syringe and tubing connection and d) the Eppendorf container collecting droplets.

objectives attached to a fast camera for taking videos of droplet formation and a separate camera for taking photos. They both had a LED source for imaging fluorescence, and full capacity for imaging in phase-contrast. The precise details of the microscope setups are detailed in Table 2.2. Any samples involving water-in-oil droplets were imaged in a sample chamber made of a glass slide, double-sided sticky tape (used as a spacer) and a glass cover slip. Any part of the chamber left free to air was covered with vacuum grease if longevity of the sample was required. Other samples were either imaged similarly, or simply with a glass slide and cover slip. Glass slides and cover slips were washed with ethanol and blown dry prior to use.

Table 2.2: Details of microscope setups used in this thesis.

Setup	Microscope	Fast Camera	Image Camera	Fluorescence Source
1	Leica DMi8	Phantom Miro LAB 3a10	QImaging Exi Blue	CoolLED pE-300lite
2	Nikon Eclipse Ti2-U	Phantom VEO-E 310L 18GB	Ximea xiQ USB3	Nikon D-LEDI
Setup	Objectives		Filters	
1	10x N Plan PH 1		FITC (EX 480/40) DSRed (EX 546/11)	
	20x N Plan PH 1			
	20x HC PL FLUOTAR			
	40x N Plan PH 2			
2	10x CFI Plan Fluor DLL		FITC-3540C (EX 482/35) TxRed-4040C (EX 562/40)	
	20x CFI Plan Fluor DLL			
	20x CFI Plan Apochromat Lambda			
	60x CFI Plan Apochromat Lambda Oil			

2.3 Biological Procedures

This thesis uses the Jurkat cell line as a model. The Jurkat cell line consists of immortal human T lymphocytes, meaning that a mutation has allowed the cells to continue dividing indefinitely rather than undergoing senescence. The Jurkat cell line originated from a 14 year old boy with T cell leukaemia [181].

2.3.1 Cell Culture

The Jurkat cell line was cultured in R10 culture medium. The cells were kept incubated at 37 °C and 5 % carbon dioxide content (required to regulate the pH of the culture medium [182]). To passage the cells, half of the volume of cells in culture media were discarded and replaced with new R10 culture medium.

To determine the cell density, 10 μ L of the cell culture was mixed with 10 μ L of trypan blue solution. 10 μ L of the resulting solution was imaged on a Fast-Read® 102 counting slide (Biosigma). The volume required to collect roughly 2×10^6 cells was collected and pelleted. To pellet cells, the media was centrifuged at 1600 RPM (200 x g) for 4 min in an ALC® PK130 centrifuge. The supernatant was then removed and the cells resuspended in an appropriate volume of R10 culture media to generate a solution of 2×10^6 ($\pm 5 \times 10^5$) cells in 85 μ L.

2.3.2 Cell Staining

For several experiments, to better locate cells and to determine how successfully they had been lysed, cells were stained with the CellTracker™ CM-Dil fluorescent dye. The protocol for staining Jurkat cells is as follows:

Jurkat cells were counted as described above and an appropriate volume of cells in culture medium was collected. This sample was pelleted and resuspended in phosphate buffered saline (PBS) with 1 mL for every 10^6 cells in the original sample. 0.5 μ L CellTracker™ CM-Dil dye was added for every 10^6 cells and this sample was then incubated at 37°C for 20 min. For every 10^6 cells, 5 mL R10 media was added and incubated at 37°C for another 5 min. This sample was again pelleted and resuspended in 1x phosphate buffered saline (PBS) with 1 mL for every 10^6 cells before finally being pelleted and the supernatant removed. This resulted in a suspension of around 2×10^6 ($\pm 5 \times 10^5$) stained Jurkat cells in 85 μ L PBS.

2.3.3 DNA Staining

Alternatively to cell staining, successful lysis and trapping of DNA was also demonstrated by staining a DNA sample with the intercalating fluorescent dye SYBR™Gold (Thermofisher). Unless otherwise stated, when used in this thesis, any samples imaged with stained DNA had a final concentration of 5x SYBR Gold and 1x TE Buffer.

Chapter 3

Hydrogel Properties and Microsphere Formation

In this chapter we start by developing a method to use microfluidic devices to produce hydrogel microspheres, investigating the limits of the developed methods. We focus on agarose and alginate networks, as discussed in Section 1.2.2. Rheological techniques were then used to characterise the viscoelastic properties of bulk agarose and alginate hydrogels. The results from this were then used to predict the best composition of hydrogel components to give the required properties for our purposes. Finally, the relevant properties for the use of these microspheres as micro-reaction-vessels were tested, ending with the study of microsphere swelling upon de-emulsification.

3.1 Hydrogel Microsphere Formation

Hydrogel microspheres in this thesis were produced by utilising time controlled sol-gel phase transitions, such that microfluidic devices can be used to produce water-in-oil (W/O) droplets of sol-phase hydrogels, which can then be cross-linked to produce gel-phase hydrogel microspheres (after breaking of the emulsion). We started with the development of a method to produce agarose microspheres, showing how the temperature dependence of the sol-gel transition discussed in Section 1.2.1 causes issues for droplet formation and how these are overcome using slide and tubing heaters to keep the microfluidic devices warm. Then, in Section 3.1.2, various multi-component alginate gelation techniques were surveyed, from direct cross-linking with calcium chloride to ligand exchange systems. We attempted the most promising with microfluidic devices designed for droplet formation, eventually choosing a calcium EDTA induced gelation process. In

Section 3.1.3, we investigated the many variables within this alginate microsphere method (such as cross-linking time and acetic acid concentration), choosing a standard method that will be built upon in the later chapters of this thesis. Finally in Section 3.1.4, we summarise the different microsphere production techniques, highlighting where agarose or alginate have advantages over each other.

3.1.1 Agarose Microsphere Formation

There are two main methods to fabricate agarose microspheres from micro-droplet reactors, (i) using a microfluidic device [183, 184] or (ii) the creation of an emulsion by vortexing an oil and aqueous mixture [185, 186]. The former method produces a high size monodispersity of beads, a quality useful for subsequent re-encapsulation of beads [183], so it is the method used in this thesis.

Methods

Prior to encapsulation using the single emulsion (SE) device described in Section 2.2.3, agarose solutions were made by weighing the appropriate masses of agarose powder and water, mixing in a 1.5 mL Eppendorf tube and heated at 60 °C for 1 h in an Eppendorf Thermomixer compact. This produced a colourless and non-viscous solution, which appeared homogeneous when flowing through a microfluidic device. Droplet production as described in Figure 2.7 typically produces 1 - 4 $\mu\text{L min}^{-1}$ of droplets. Usually 40- 120 μL of droplets are collected per experiment. This thesis uses two different agarose samples, D1-LE and ULG (See Section 2.1). Normally droplet production is performed at room temperature. However the D1-LE agarose with a gelling temperature of 36 ± 1.5 °C will gel at room temperature in the tubing and in the microchannels of the device, preventing droplet formation. Despite the ULG agarose having an advertised gelling temperature of 8 - 17 °C, ULG solutions of 2 % w/w were also found to cross-link in the tubing.

A method to heat the device and tubing was developed. As shown in Figure 3.1, a slide heater (AmScope TMTCS-200 Temperature Controller) was placed in-between the microfluidic device and the microscope stage, and the tubing containing the agarose solution was surrounded by a tubing heater (manufactured by the electrical workshop staff in the Physical and Theoretical Chemistry Laboratory at the University of Oxford). The slide heater was set at 45 °C and the tubing heater at 60 °C.

This setup enabled the flow of up to 2 % w/w ULG and D1-LE agarose solutions. Any

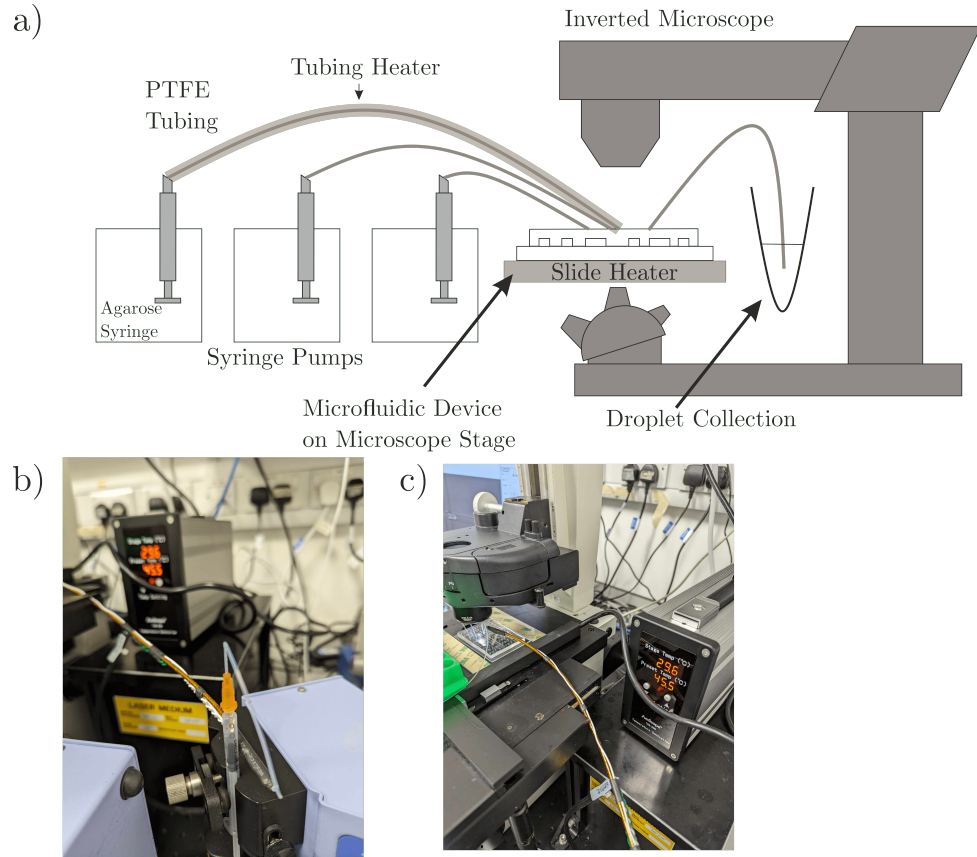


Figure 3.1: a) A schematic of the adapted droplet production method using tube and device heaters. Photographic images of b) the tubing heater and c) the slide heater.

concentrations higher than this would result in regular blockage of the microchannels every 20 min, requiring droplet production to be paused and moved to a new device. Note that the internal diameter of the tube heater was considerably larger than the 30 AWG tubing used for typical droplet formation (Section 2.2.3), so would inefficiently heat the 30 AWG tubing. Instead the larger 24 AWG PTFE tubing was used for flowing of agarose solutions, alongside a 23-gauge needle. As shown in Section 2.2.3, the SE device has two aqueous inlets, which when flowed at equal flow rates would produce a droplet containing material from each inlet, at half the concentration of the initial samples. Here we used one aqueous inlet for flowing in agarose and the other to flow in water. In future chapters this water inlet will be replaced by the cells, colloids or DNA to be trapped within the hydrogel microspheres. This allowed for the production of 1 % w/w agarose beads. Flowing 2 % agarose solutions in both inlets would result in a maximum final bead w/w concentration of 2 %. After collection of the resulting emulsion, the droplets were cooled at 5 °C for 1 h to allow cross-linking to occur. Droplets were then broken (as described in Section 2.2.4) to produce a suspension of beads-in-water shown in Figure 3.2. Note that as

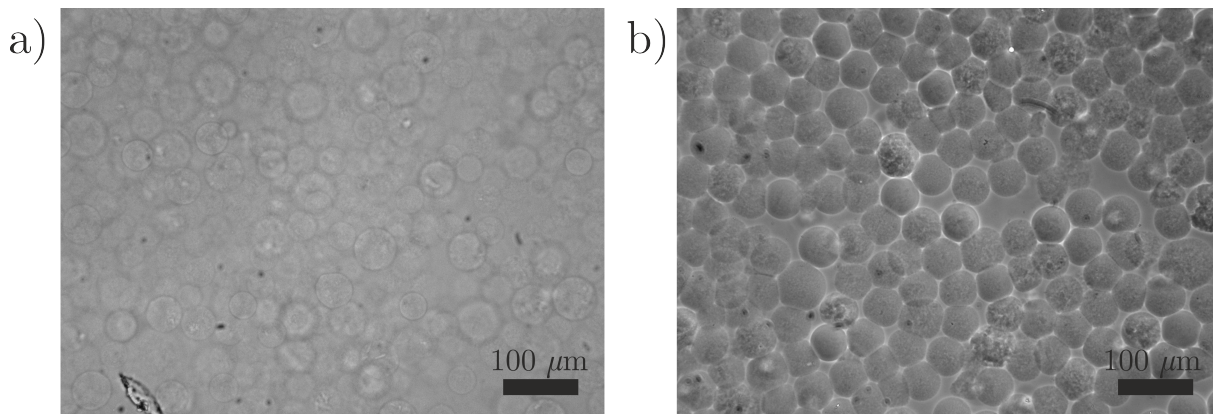


Figure 3.2: A suspension of 1 % w/w agarose microspheres imaged via a) bright-field microscopy and b) phase-contrast microscopy. These are not the same field of view.

these beads have a similar density to water, the contrast in bright-field microscopy is poor, and therefore phase-contrast microscopy was used to enhance visibility in images. A combination of these microscopy techniques is used throughout this thesis.

3.1.2 Alginate Microsphere Formation

As with the agarose microspheres, we chose to use droplet microfluidics to produce alginate microspheres for reasons of size monodispersity discussed above. Unlike agarose, which cross-links upon cooling, alginate cross-links in the presence of divalent cations. This allows a wide range of cross-linking approaches to be taken. Typically the cation of choice for controlled cross-linking of alginate is calcium [187, 98].

Methods

The simplest method to cross-link alginate would be to mix a sodium alginate solution with a calcium salt solution. The SE device has two aqueous channels, so it would be possible to have the alginate mix with the calcium ions at the junction, and then cross-linking would occur post droplet formation. A wide range of combinations of sodium alginate and calcium chloride concentrations were trialled with this method (listed in Table 3.1).

Despite previous literature showing this as a successful method [187], all the concentration pairings attempted resulted in the cross-linking of alginate chains happening within a much shorter time-scale than that of droplet formation (droplet formation typically occurs on the order of 1000 Hz). Cross-linking would occur at the flow-focusing junction and block the path of flow, preventing stable droplet formation (shown in Figure 3.3).

To combat this, most reliable methods to form alginate microspheres using a microfluidic device

Table 3.1: All combinations of sodium alginate and calcium chloride concentrations tested in the SE device. These all resulted in premature cross-linking.

#	Sodium alginate concentration (% w/w) of inlet 1	Calcium chloride concentration (M) of inlet 2
1	2.0	0.2
2	2.0	0.4
3	4.0	0.05
4	4.0	0.1
5	4.0	0.2
6	4.0	0.4
7	4.0	0.8
8	4.0	1.0

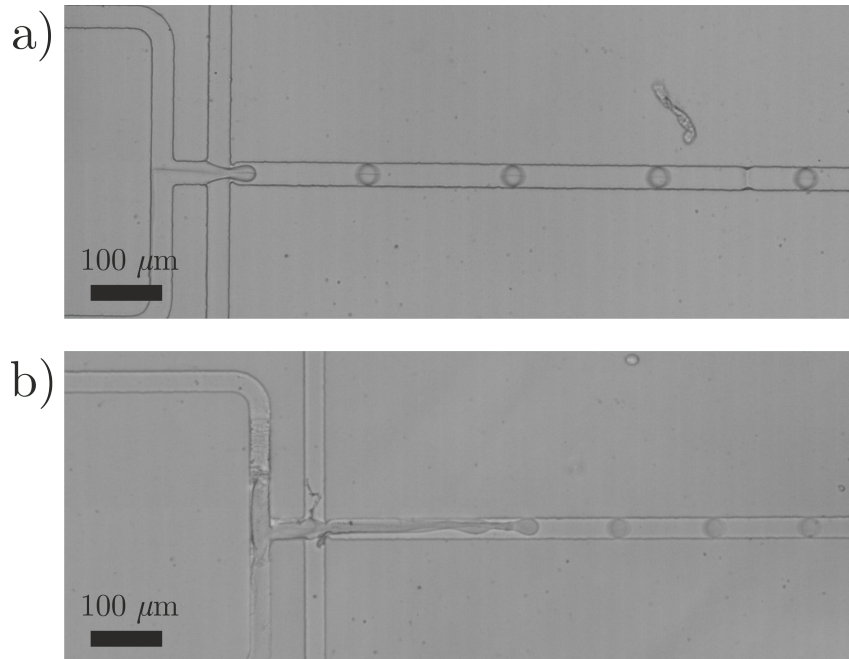


Figure 3.3: Bright-field microscopy images of a SE device showing a) two unreactive aqueous phases meeting at the flow focusing junction and b) calcium and alginate ions meeting at the flow focusing junction, causing cross-linking and blockage of the channel.

instead delay cross-linking until after droplet formation. Typically this is done by use of a calcium salt that will not spontaneously dissociate into hydrated conjugate ions in solution, such as calcium disodium ethylenediaminetetraacetic acid (EDTA) [188] or calcium carbonate [189, 190]. Cross-linking is then induced by methods such as reducing the pH of the emulsion [98] or exposing the emulsion to ultraviolet light [190]. This releases the calcium ions from the complexes and allows the formation of ionic cross-links between alginate chains. These methods are often referred to as competitive ligand exchange cross-linking (CLEX) [98]. This thesis chose to use the calcium EDTA approach, on the basis that EDTA salts are used in a wide range of DNA amplification techniques and are therefore unlikely to inhibit PCR used later in the assay (Chapter 4).

EDTA is a polydentate ligand, typically forming six ligand-ion bonds, and therefore the chelate effect allows preferential binding of metal ions to EDTA over multiple ligands with a lower coordination ability. The chelate effect refers to the entropic driving force of this coordination. For example, a six coordinate metal ion bound to one EDTA ligand with six monodentate water ligands free to move in solution is more entropically favoured than the metal ion bound to six monodentate ligands and one EDTA molecule free. Simply, the more molecules free to move about, the higher the entropy of the system. This overall preference for the calcium ion to form a calcium EDTA complex is reversed upon reducing the pH of solution, as the EDTA molecule can be considered protonated, reducing the affinity of metal-EDTA bonding until it is preferential for the calcium ions to bind to the alginate chains instead. In practice this is achieved by adding acetic acid to the oil phase of the water-in-oil emulsion after successful droplet formation, thus avoiding cross-linking occurring at the flow focusing junction (FFJ) [98]. A summary of this process is shown in Figure 3.4. This process is referred to as internal gelation [191], contrasted with external gelation where micro-droplets of alginate are ejected into the air phase through a nozzle and fall into a bath of divalent cations. This would be unsuitable for subsequent droplet manipulation with microfluidics as the droplets produced are too large (on the millimetre scale) and often inhomogeneous in network structure [191].

The alginate hydrogels in this thesis are therefore a three component system (dependent on the concentration of alginate, EDTA and acetic acid). So to simplify the large range of combinations, this thesis maintains a x % w/w alginate to $\frac{x}{10}M$ calcium EDTA ratio, described as a x % w/w AlgEDTA mix (see Section 2.1). This ratio ensured that the number of calcium EDTA ions in solution were in excess when compared to the number of potential cross-linking sites on the

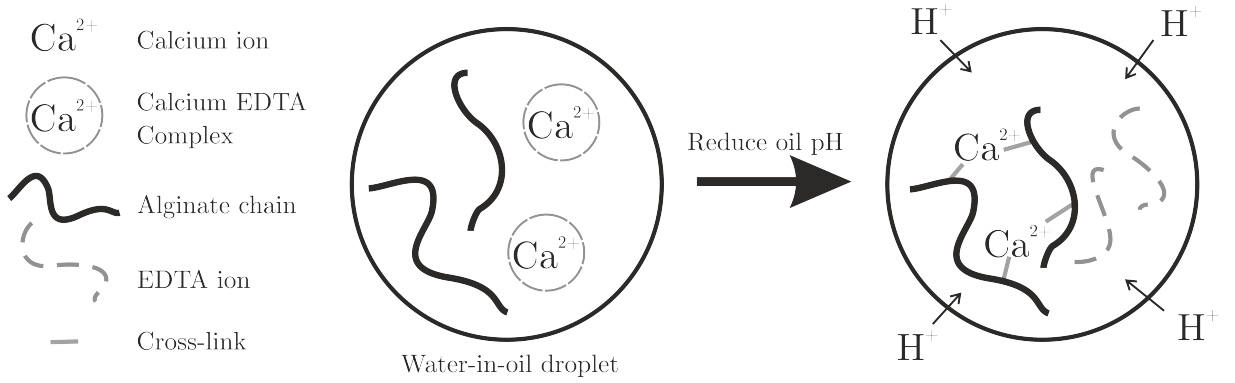


Figure 3.4: A schematic showing cross-linking of sol-phase alginate within a water-in-oil droplet using calcium EDTA and acetic acid.

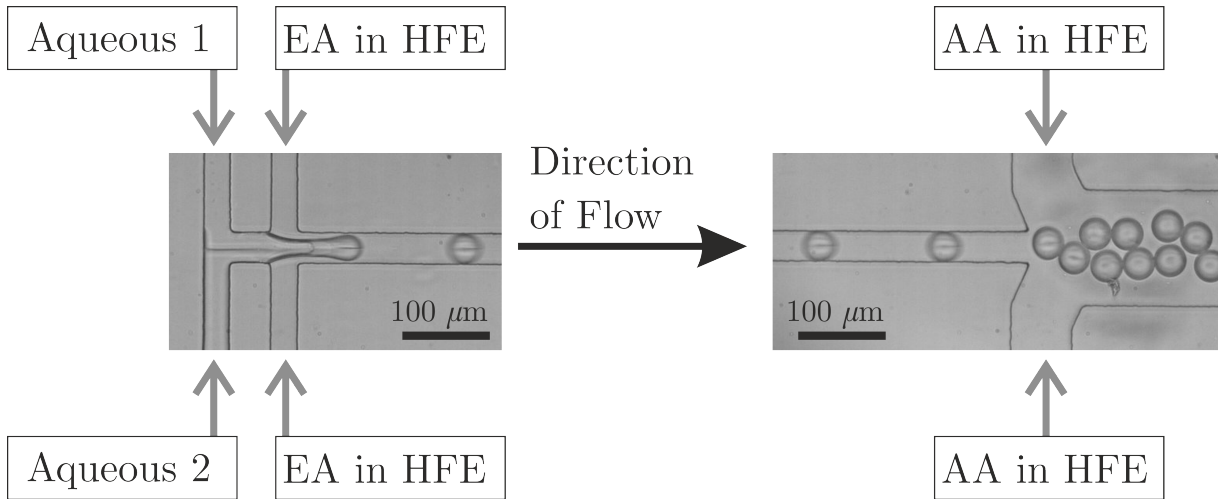


Figure 3.5: Top down bright-field images showing the creation of water-in-oil droplets containing two different aqueous phases using a flow focusing junction, with later addition of acetic acid (AA) into the continuous phase to allow cross-linking of alginate chains.

alginate chains. Therefore the effective calcium concentration can be completely controlled by the concentration of acetic acid, without the calcium EDTA concentration ever becoming the limiting factor (assuming a high enough acetic acid concentration could release all the calcium ions from coordination to EDTA ligands). Dr Lucia Parolini (Aarts and Frater groups, University of Oxford) drew a mask for a microfluidic device similar to the SE device, with an extra inlet channel for oil mixed with acetic acid to mix with the droplets after droplet formation (shown in Figure 3.5). This is referred to as the acetic acid single emulsion (aaSE) device throughout the rest of this thesis, and is used in conjunction with the SE device to create alginate microspheres. To test this system, an emulsion of 1 % alginate droplets was made as described in Section 2.2.3. DI water (Section 2.1) and a 2 % AlgEDTA mix were flowed at equal flow rates ($1\mu\text{L min}^{-1}$) through the aaSE device. The droplets were then met with a stream of 0.5 % v/v acetic acid

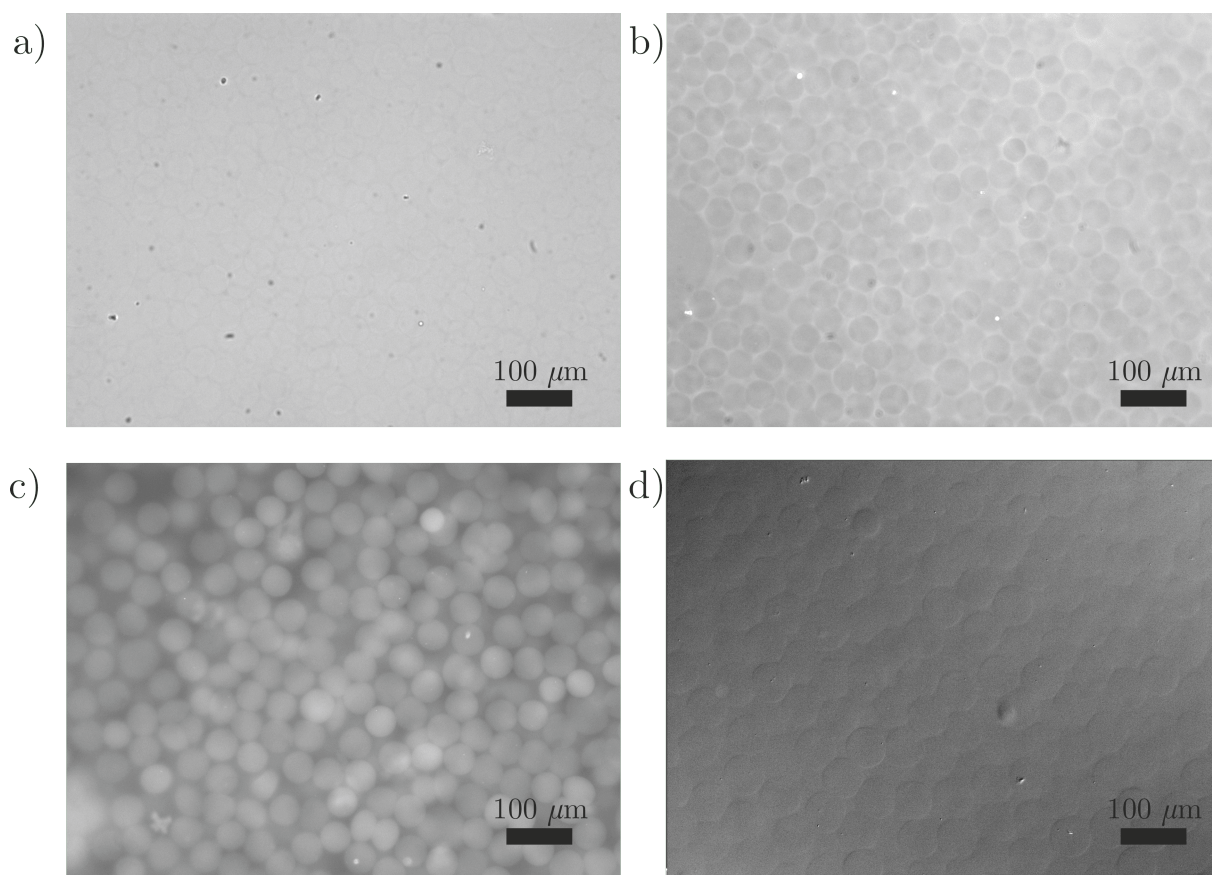


Figure 3.6: 1 % w/w Alginate microspheres imaged via a) bright-field microscopy, b) phase-contrast microscopy, c) fluorescent imaging and d) DIC microscopy.

and 1 % v/v EA in HFE (referred to hereafter as 0.5 % AA in HFE) flowing at $1 \mu\text{L min}^{-1}$. As the droplets exit the device, they were bubbled through $50 \mu\text{L}$ 0.5 % AA in HFE. This was run for 40 minutes, generating roughly $80 \mu\text{L}$ of droplets. The resulting emulsion was left overnight to cross-link. This emulsion was then broken by adding perfluorooctanol (PFO) as described in Section 2.2.4. Figure 3.6a shows a bright-field image of the alginate microspheres. Similarly to the agarose microspheres above, they have a similar refractive index to that of water, so bright-field images show poor contrast. Figure 3.6b shows a phase-contrast image of the same field of view. Differential interference contrast (DIC) images were taken by Dr Jack Holland (Aarts group, University of Oxford) and are shown in Figure 3.6d.

Finally we improved visibility of microspheres by mixing low viscosity alginate and fluorescein labelled alginate. When the process described above to produce alginate microspheres was repeated with a 1:9 volume ratio of low viscosity alginate and alginate fluorescein (Section 2.1) made to a final concentration of 2 % AlgEDTA, fluorescent microspheres were produced. These are shown in Figure 3.6c (imaged using a FITC filter).

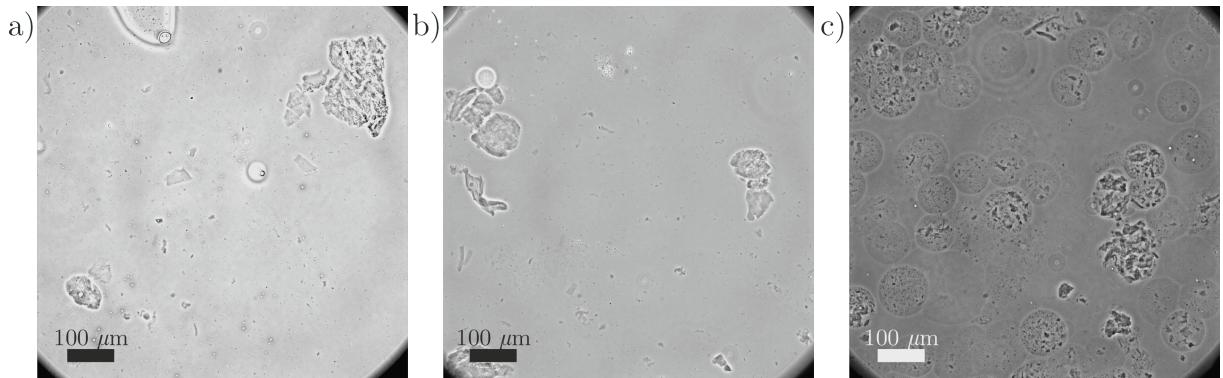


Figure 3.7: An emulsion of 2.5 % alginate droplets was broken a) 5 minutes, b) 2 hours and c) 6 hours after addition of 50 μ L 0.5 % acetic acid in HFE.

The microscopy techniques used in the rest of this thesis are bright-field, phase-contrast and fluorescence imaging, depending on the appropriate images required for the application. Clearly this multi-component system is a more complicated formation process than for the agarose microspheres. There are three key variables here, the AlgEDTA concentration, the acetic acid concentration and the time allowed for cross-linking. We next explore how changing these affects microsphere formation.

3.1.3 Variables in Alginate Microsphere Formation

As the method of production of alginate microspheres is a higher component system than for agarose, it involves a number of variables such as concentration of AlgEDTA, acetic acid and cross-linking time. Therefore investigated how these affect the structure and successful formation of microspheres.

Alginate concentration

First, we examined the effect of varying AlgEDTA concentration. The highest % solution that could be made was a 5 % AlgEDTA mix, any higher than this would not dissolve to a homogeneous solution after 24 hours stirring at room temperature. Alginate microspheres were made with a final w/w concentration of 1 %, 2.5 % and 5 %. We will test whether these microspheres have different diffusion properties in Chapter 5.

Time to cross-link

Next we investigated the time period in which the cross-linking occurs. A set of droplets containing 1 % alginate microspheres were made as described above. At each time point over a 6 hour period, a subset of these were taken and broken (using the protocol described in Section 2.2.4). Images of the resultant suspensions are shown in Figure 3.7. Emulsions broken 5 minutes

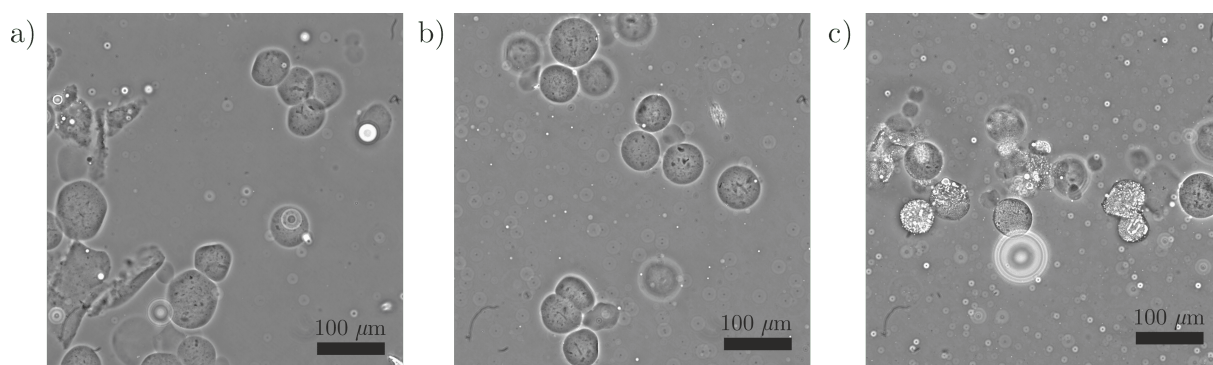


Figure 3.8: 10 μL 2.5 % alginate mix encapsulated in water-in-oil droplets added to a) 0.5 μL acetic acid, b) 1 μL acetic acid or c) 5.0 μL acetic acid immediately before breaking, then imaged by phase-contrast microscopy.

or 2 hours after droplet formation resulted in unsuccessful microsphere formation, any clumps of gel produced did not show spherical structure. Allowing 6 hours for cross-linking after droplet production resulted in successful microsphere production, although there were still non-spherical gel structures present, and the sample is not as clean as shown in Figure 3.6. Therefore, for the optimal formation of alginate microspheres with 0.5 % AA in HFE, the emulsion should be allowed to cross-link for at least 6 hours.

Acetic acid concentration

This 6 hour wait would decrease the time efficiency of any assay using these microspheres. We therefore investigated whether increasing the % AA in HFE could decrease the time interval. Alginate microspheres with a final concentration of 2.5 % w/w were made using the SE device, such that the exact concentration of acetic acid added could be controlled. 10 μL of the droplets were mixed with either 0.5 μL , 1.0 μL or 5.0 μL acetic acid to make solutions that were 4.8 %, 9.0% or 66 % acetic acid respectively. These were broken 5 min after the addition of acetic acid to produce the suspensions pictured in Figure 3.8. In this case the alginate has cross-linked after 5 min, which it did not originally. Increasing acetic acid concentration accelerates the cross-linking rate. However, the microspheres from 66 % and 9.0% acetic acid emulsions appeared deformed and bound to each other. By eye, the acetic acid appeared to destabilise the emulsion. Some phase separation was observed, due to coalescence of droplets, and therefore there would be subsequent cross-linking between microspheres driven by present acetic acid. This deformation is likely due to hydrogen ions disrupting surfactant adsorption at the interface. To test this, we imaged water-in-oil droplets with 66 %, 9.0% acetic acid composition. These show droplet deformation and coalescence when compared to water-in-oil droplets not containing acetic acid (Figure 3.9). Although this cross-linking is fast, it is unreliable for stable suspension formation.

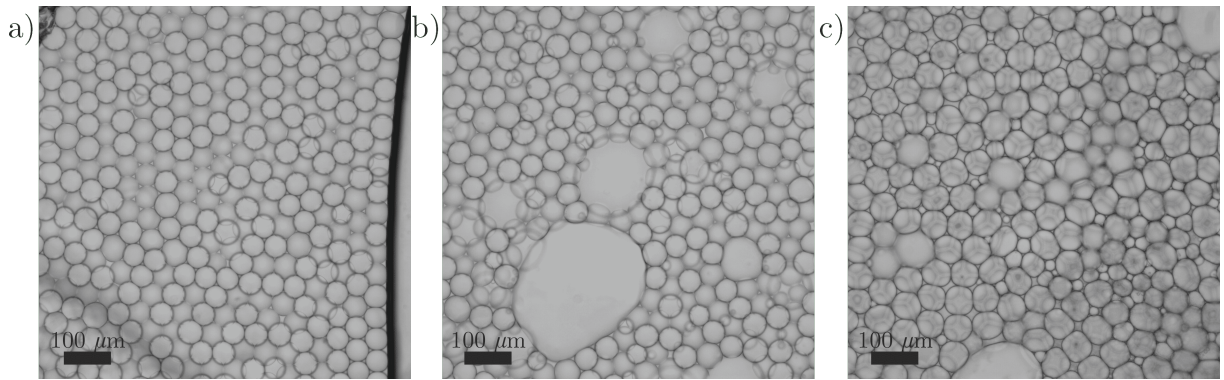


Figure 3.9: Water-in-oil droplets encapsulating a) 0 % acetic acid, b) 9.0 % v/v acetic acid or c) 66 % v/v acetic acid imaged by bright-field microscopy.

The microspheres produced from 4.8 % acetic acid emulsions seem deformed from the spherical shape, instead appearing ovular or even showing some flat edges. This suggests that at this timescale cross-linking of the whole volume of a spherical droplet had not occurred, therefore risking the loss of genomic material upon de-emulsification. It was decided that for reliable alginate microsphere formation, emulsions would be formed and bubbled through 50 μ L 0.5 % AA in HFE, and allowed to stand overnight.

3.1.4 Comparison of Agarose and Alginate Microsphere Production

There are clear benefits and downsides to the production of both alginate and agarose microsphere. The stable formation of agarose microspheres varies depending on temperature: Any accidental cooling of tubing or device results in channel clogging which can usually only be fixed by starting microsphere production again. Also, by having a maximum flowable concentration of 2 % w/w, keeping an inlet free to flow cells in limits the variation in microsphere concentration achievable. This could potentially be improved in future by the creation of a three inlet device. A similar effect is also present in alginate microsphere formation where the maximum flowable concentration is 5 % AlgEDTA mix. Note that cells can be resuspended in the 5 % AlgEDTA mix upon collection (Section 2.3.1), resulting in a maximum concentration of 4.8 % w/w alginate microspheres. Resuspending cells in agarose has the potential to cause premature lysis, as the agarose is heated at 60 °C up until the moment of backfilling into the tubing. Premature lysis will result in the loss of single cell resolution and should be avoided. Formation of alginate microspheres is much more reliable than agarose, as all samples involved flow reliably without causing blocked channels, ensuring simple droplet formation. The main limitation of alginate microsphere formation is the overnight waiting period for cross-linking to complete. This long

Table 3.2: A summary of the advantages and disadvantages of agarose and alginate microsphere formation.

	Advantages	Disadvantages
Agarose	Short cross-linking time (1 Hr)	Erratic droplet formation on variable temperatures Limited by maximum flowable concentration
Alginate	Easy droplet formation Wide variety of microsphere compositions possible	Long cross-linking time (overnight)

time period could result in unwanted diffusion of trapped material out of the microspheres. The advantages and disadvantages of each are summarised in Table 3.2.

3.2 Viscoelastic Behaviour of Agarose and Alginate Hydrogels

In this section, we will discuss the use of rheology and frequency sweeps to characterise the bulk viscoelastic properties of agarose and alginate hydrogels. These are used to discuss the mechanical behaviour of the eventual hydrogel microspheres, commenting on possible relations to pore size and diffusion ability. We vary the compositions of bulk hydrogels using different concentrations, viscosities and gelation techniques, in order to predict the viscoelastic properties of microspheres produced using similar conditions.

3.2.1 Introduction

Hydrogels are expanded polymer networks with solid like properties (See Section 1.2.1) [68, 69]. A particular hydrogel is capable of showing both "solid-like" (elastic) or "liquid-like" (viscous) behaviour in different conditions. A mixture of these two behaviours is termed viscoelasticity. Rheology translates literally as the "study of flow" but is now more broadly defined as the "science of the deformation and flow of matter" [192]. Rheology is therefore often used to investigate hydrogel properties such as viscoelasticity [193]. The stress-strain relationship can be used to characterise a material's yield stress and ultimate tensile strength [194], determine the viscosity of a liquid or polymeric solution [195] or investigate time dependent properties in the food industry [196]. Oscillatory shear tests examine the viscoelasticity properties of materials, as they can investigate the effect of varying conditions such as frequency and temperature [197, 198]. While there is a large amount of data available in the literature on hydrogels and their rheological

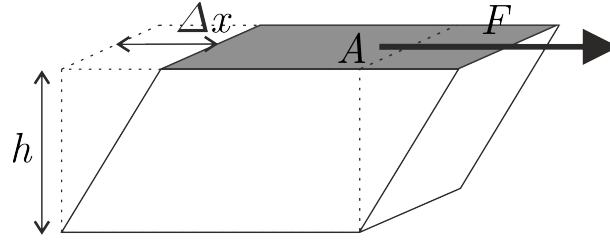


Figure 3.10: A model of shear flow.

properties, these properties are sensitive to variations in specific conditions (such as temperature, time and relative molecular weight [194]). Therefore, this thesis investigates the compositions of agarose and alginate hydrogels used for microsphere formation (Section 3.1).

3.2.2 Theory

Flow of a liquid can be visualised as layers of fluid moving over each other: If a force is exerted on the top layer, the layers beneath it will begin to move with decreasing velocity. Next we introduce some terminology: a force applied on a sample (perpendicular to gravity) per unit area is a stress ($\sigma = F/A$), and the displacement of the top layer of fluid (Δx) relative to the thickness of the sample (h) is the strain ($\gamma = \Delta x/h$). This is shown for a simple model in Figure 3.10. In practice we apply an oscillatory stress upon our samples.

The fundamental model for an elastic solid is Hooke's Law,

$$F = kx, \tag{3.1}$$

where extension of a spring (x) is proportional to exerted force, with the proportionality constant defined as the force constant (k) [199]. A purely elastic material will relax with no delay once the force is removed (Figure 3.11a). A viscous liquid can be described by a dashpot model (Figure 3.11b). This is illustrated by a plunger moving through a viscous liquid as a result of a force acting on the plunger. Here the plunger will continue to move as the force is applied, only stopping when the force is removed - the deformation now depends on time (t) as well as applied stress,

$$\sigma = \frac{\gamma\eta}{t}. \tag{3.2}$$

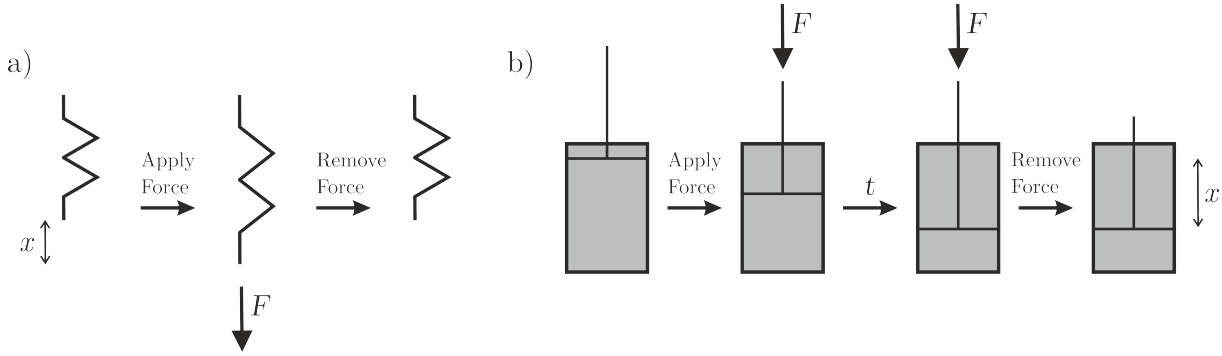


Figure 3.11: a) The fundamental model for Hooke's Law for an elastic solid, and b) A dashpot model describing Newton's Law of viscosity.

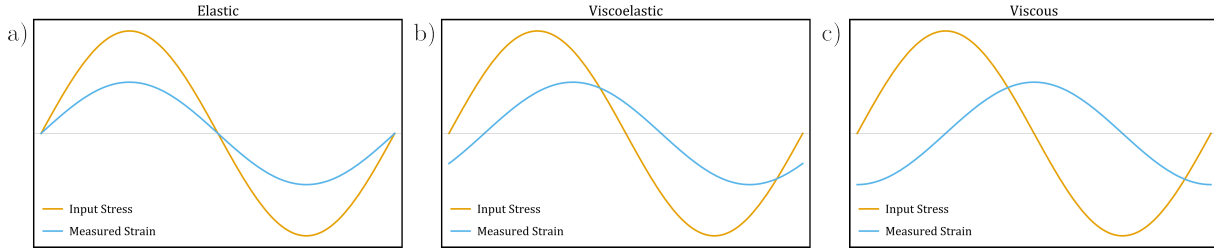


Figure 3.12: The relationship between stress and strain as a function of time for a) an elastic solid (in phase), b) a viscoelastic material and c) A viscous liquid (anti-phase).

Here the proportionality constant is the viscosity (η) [200].

When we apply an oscillatory stress to a material, the time delay between applied stress and measured strain shows whether elastic or viscous behaviour dominates. If stress and strain are in phase (phase difference $\delta = 0$) the sample shows purely elastic behaviour, and if stress and strain are in anti-phase ($\delta = \frac{\pi}{2}$) the sample shows purely viscous behaviour. A phase difference in-between these two extremes ($0 < \delta < \frac{\pi}{2}$) implies a mixture of behaviours, i.e. the sample is viscoelastic (Figure 3.12).

To quantify how "solid-like" or "liquid-like" a material is, the complex modulus,

$$|G^*| = \frac{\sigma_{max}}{\gamma_{max}}, \quad (3.3)$$

is converted into the storage modulus

$$G' = G^* \cos \delta, \quad (3.4)$$

and the loss modulus

$$G'' = G^* \sin \delta, \quad (3.5)$$

using the relation

$$G^* = G' + iG'', \quad (3.6)$$

If the storage modulus dominates then the material is considered to be dominated by elastic forces (as $0 < \delta < \frac{\pi}{4}$) and is therefore "solid-like" under the conditions. If the loss modulus dominates ($\frac{\pi}{4} < \delta < \frac{\pi}{2}$) then the material is considered to be "liquid-like". This is the measure we will use to characterise the behaviour of hydrogels in this section.

It is important to note that this oscillatory testing is only valid while within the linear viscoelastic region (LVR) where complex stress and oscillatory strain are proportional. Otherwise the microstructure of the gel will be changed and the data collected is no longer representative of the original gel. The LVR is determined by performing a stress sweep at a constant frequency. Any region with a constant storage modulus lies within the LVR (at high stress the storage modulus will begin to decrease, this is the limit of the LVR) [195].

De Gennes was able to estimate the mesh size (or pore size) of a polymer using

$$G^0 \cong \frac{k_B T}{\xi^3}, \quad (3.7)$$

where G^0 is the plateau modulus and ξ is the characteristic mesh size [201]. This relation allows estimate calculations of pore size from an observed plateau modulus.

3.2.3 Methods

An oscillatory rheometer consists of a peltier plate upon which the sample rests and a geometry which is lowered onto the sample and rotated by a spindle, controlling either the stress or strain of oscillation. The temperature of the peltier plate can be controlled to allow for temperature sweeps.

All rheological tests were ran on an AR-G2 stress controlled rheometer (TA Instruments) with a 60 mm cone geometry (0:59:24 deg:min:sec). Samples were loaded onto the peltier plate, and the geometry was lowered onto the sample as shown in Figure 3.13. Two types of test were

run, stress sweeps were used to determine the LVR, while frequency sweeps characterised the viscoelastic behaviour of the gel.

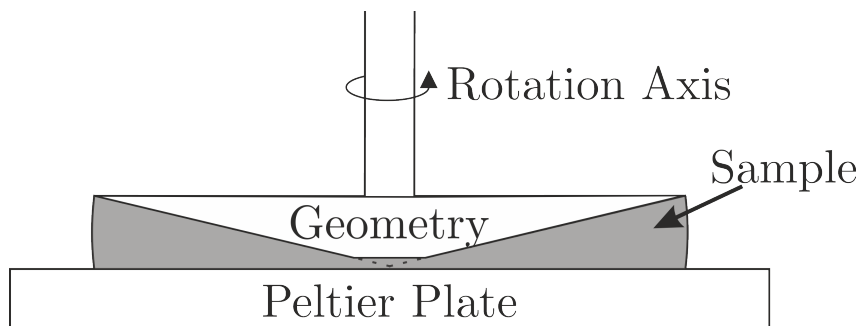


Figure 3.13: A schematic showing a sample correctly loaded between the peltier plate and geometry in the rheometer.

Stress Sweep Protocol - The stress sweep was performed at 21 °C with an equilibration time of 5 min. Stress was applied at a continuous oscillation, ranging from 0.2 to 20 Pa taking 10 measurements per decade. This was run with a 3 s conditioning time and 3 s sampling time. This procedure was run for each sample at 1 Hz, 10 Hz and 100 Hz to determine the LVR.

Frequency Sweep Protocol - The frequency sweep was run within the LVR. The peltier plate equilibrated at 21 °C with an equilibration time of 2 min. The frequency of continuous oscillation was varied from 0.01 to 10 Hz taking 10 measurements per decade. The applied oscillatory stress remained constant at 3 Pa. For a few samples these conditions contained regions outside the LVR so were instead run at 1 Pa, or with a reduced frequency range of 0.01 to 1 Hz. These are clearly marked in the results section.

Samples - In total 15 different samples of bulk hydrogels were characterised, split into five groups for comparison purposes; two agarose and three alginate (summarised in Table 3.3). Agarose gels are a simple two component system (agarose and water) so only the agarose concentration was varied. The first group (samples 1-3) consisted of three D1-LE agarose samples with concentrations of 1.0%, 1.8% and 2.5% w/w respectively. The second group compared the 1.8 % D1-LE sample with 1.0%, 1.8% and 2.5% w/w ultra low gelling (ULG) agarose samples (samples 2 and 4-6).

As described in Section 3.1.2, cross-linking sodium alginate in droplets is a multi-component system. Sodium alginate is mixed with calcium EDTA (disodium) inside the droplet and then acetic acid is mixed with the continuous oil phase of the emulsion which reduces the pH inside the droplets, reversing the affinity of the calcium ions from with the EDTA chelating ligand to the with alginate chains. This was simulated in bulk by adding varying volumes of acetic acid to

Table 3.3: A summary of the agarose and alginate hydrogel samples tested with rheology.

#	Precursor	% w/w agarose
1	D1-LE Agarose	1.0
2	D1-LE Agarose	1.8
3	D1-LE Agarose	2.5
4	ULG Agarose	1.0
5	ULG Agarose	1.8
6	ULG Agarose	2.5

#	Precursor	% alginate mix	Volume of acetic acid (μL)	% of total alginate that is medium viscosity
7	Sodium alginate	1.0	25	0
8	Sodium alginate	2.5	25	0
9	Sodium alginate	5.0	25	0
10	Sodium alginate	2.5	0	0
11	Sodium alginate	2.5	5	0
12	Sodium alginate	2.5	100	0
13	Sodium alginate	2.5	25	0.4
14	Sodium alginate	2.5	25	2
15	Sodium alginate	2.5	25	100

a fixed volume with specified concentrations of calcium EDTA and sodium alginate (AlgEDTA mix - Section 2.1).

Three groups of alginate samples were tested. The first group (samples 7-9) controlled the volume of acetic acid added (25 μL acetic acid to 1995 μL AlgEDTA) and varied the % AlgEDTA (1.0%, 2.5% and 5%). The second group (samples 8 and 10-12) controlled the % AlgEDTA at 2.5 % and varied the volume of acetic acid added (0 μL , 5 μL , 25 μL and 100 μL). The final group (samples 8, and 13-15) controlled both % AlgEDTA at 2.5 % and the volume of acetic acid added at 25 μL acetic acid to 1995 μL AlgEDTA. Instead we varied type of alginate used, and ran on a sample of low viscosity alginate, a medium viscosity alginate and two mixtures of 0.4 % and 2.0 % medium viscosity-in-low-viscosity respectively (see Section 2.1 for details of the different alginate samples).

3.2.4 Results and Discussion

First we examine the stress sweeps. Example stress sweeps are shown in Figure 3.14 for a 2.5 % D1-LE agarose sample and a 2.5 % alginate mix with 25 μL acetic acid sample. Here we see that the agarose sample remains inside the LVR for all frequencies and stresses tested, as G' remains constant throughout. For the alginate sample, the 1 Hz and 10 Hz sweep show a non constant G' around $>5\text{ Pa}$, and the 100Hz sweep has non constant G' throughout the whole

domain. Stress sweeps for all the samples are shown in the Appendix in Figures 6.1 and 6.2. If the above frequency sweep protocol uses regions outside the LVR, the protocol is adapted to avoid this and will be discussed with the relevant sample.

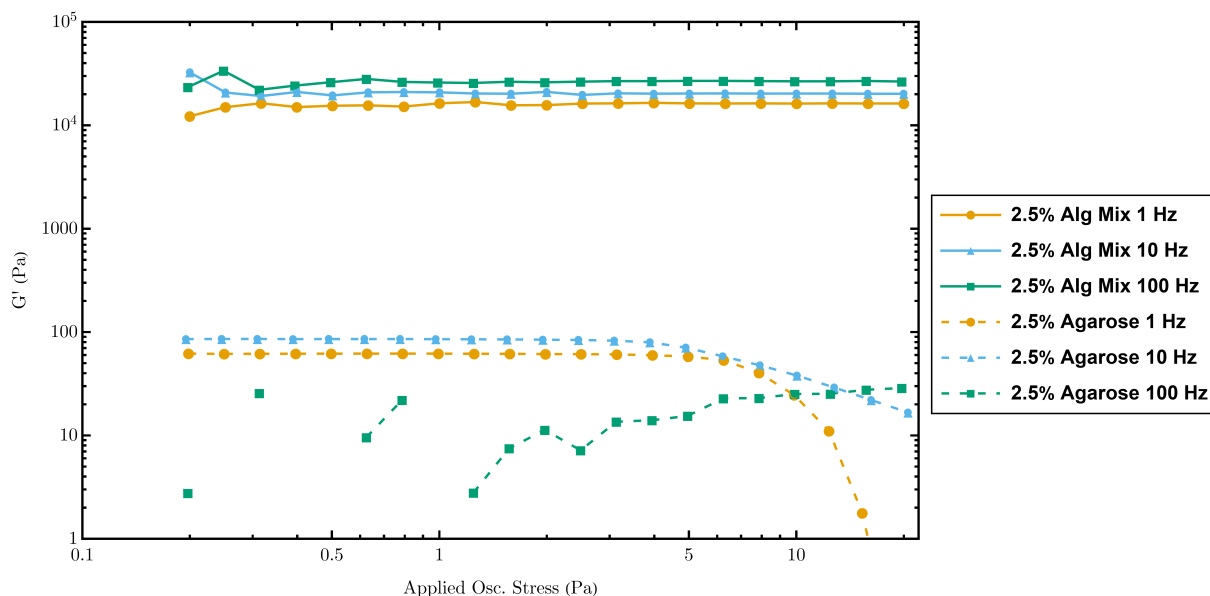


Figure 3.14: **Stress sweeps** - Plots showing storage modulus as a function of applied oscillatory stress used to determine the linear viscoelastic region for a 2.5 % w/w D1-LE agarose sample (#3) and a 2.5 % AlgEDTA mix with 25 μ L acetic acid sample (#6).

The focus is to determine whether each sample is dominated by G' or G'' . When G' dominates the sample is said to be more "solid-like" and when G'' dominates the sample is said to be more "liquid-like" (Section 3.2.2). In the agarose networks (samples 1 to 4) (Figure 3.15), G' dominates for all samples and at all frequencies which fits with the bulk observations that cross-linked agarose does not flow, and instead predominantly deforms like an elastic solid. As % w/w agarose increases so does G' and G'' indicating an increase in strength of the gel. This is consistent with theorising that an increased concentration of polymer chains increases the frequency of cross-linking van der Waal bonds.

When comparing the D1-LE agarose to the ULG agarose samples (Figure 3.16), we see that the above trend is maintained for the ULG samples; increasing % w/w concentration increases the storage modulus of the gel. The 1.8 % ultra low gelling agarose has a decreased G' and G'' compared to the 1.8 % D1-LE agarose. Instead the 2.5 % ULG agarose is more comparable with the 1.8 % D1-LE sample. This suggests that the ultra low gelling agarose has a structural property that reduces the degree of cross-linking, or the strength of the cross-links. This is consistent with the melting temperature (and inversely the gelling temperature) being lowered,

as less energy is required to break all cross-linking and separate the chains. Reducing the degree of cross-linking or cross-link strength will reduce the strength of the gel.

Figure 3.17 shows the frequency sweeps for the first group of alginate samples, where the % alginate mix was varied (Note the 1.0 % sample was run at 1 Pa in order to stay within the LVR). As expected, by increasing the alginate % w/w we see a transition from entirely viscous dominated behaviour to a regime where the samples have a crossover point between G' and G'' . for both the 2.5 % and 5.0 % mix, at higher frequencies the samples behave more "solid-like" with the 5.0 % crossover point occurring at a lower frequency.

Figure 3.18 shows the frequency sweeps for the second group of alginate samples (samples 6, 8, 9 and 10). Here the volume of acetic acid added to the "alginate mix" is varied (Note the 0 μ L and 5 μ L samples were run at 1 Pa in order to stay within the LVR). For the 0 μ L and 5 μ L samples G'' dominates, suggesting "liquid-like" behaviour dominates. This is as expected of a sample with little or no cross-linking. As more acetic acid is added, the degree of cross-linking increases and the sample become more "solid-like". For the 100 μ L sample, G' instead dominates for the whole range investigated. The 25 μ L sample however behaves as "liquid-like" under low frequency oscillations and "solid-like" under frequencies above 1 Hz. In general, the more acetic acid added, the more "solid-like" the sample behaves, this is consistent with a higher volume of acetic acid reducing the pH by a larger amount, releasing more calcium ions from the EDTA complexes and creating more cross-links. This shows that while our estimation in Section 3.1.2 shows the alginate cross-linking sites was the limiting factor, and the CaEDTA ions are in excess, in the pH conditions shown here, not enough of the calcium ions are released for the calcium ions to be surplus to the alginate cross-linking sites.

The frequency sweeps for the final group of alginate samples (6, 11, 12 and 13), characterising mixtures of low and viscosity alginate for a constant alginate mix % and acetic acid volume, are shown in Figure 3.19. The medium viscosity sample is dominated by G' , as is the 2 % medium viscosity, albeit with a lower magnitude of storage and loss moduli. As the % content of medium viscosity decreases further, the behaviour of G' and G'' tends more towards the low viscosity sample. Higher viscosity gels can result from longer polymer chains, a feature which also causes a greater amount of cross-linking ability. This predicts the apparent increase in mechanical strength of the hydrogels as the viscosity increases, as seen in the frequency sweeps.

We can also estimate the pore sizes of these hydrogels from their plateau moduli using Equation 3.7. The results of these are displayed in Table 3.4. Sample number refers to the

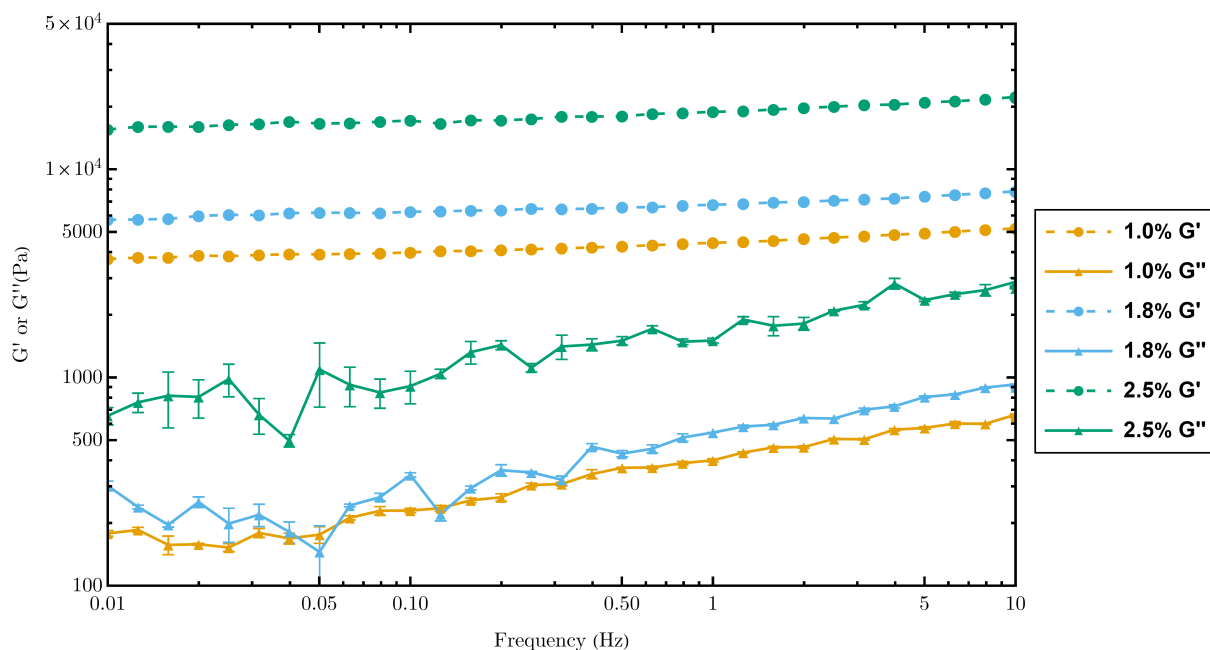


Figure 3.15: **Frequency Sweep of Agarose Samples** - Plots showing storage and loss moduli as a function of oscillatory frequency for 1.0 %, 1.8 % and 2.5 % w/w D1-LE agarose samples.

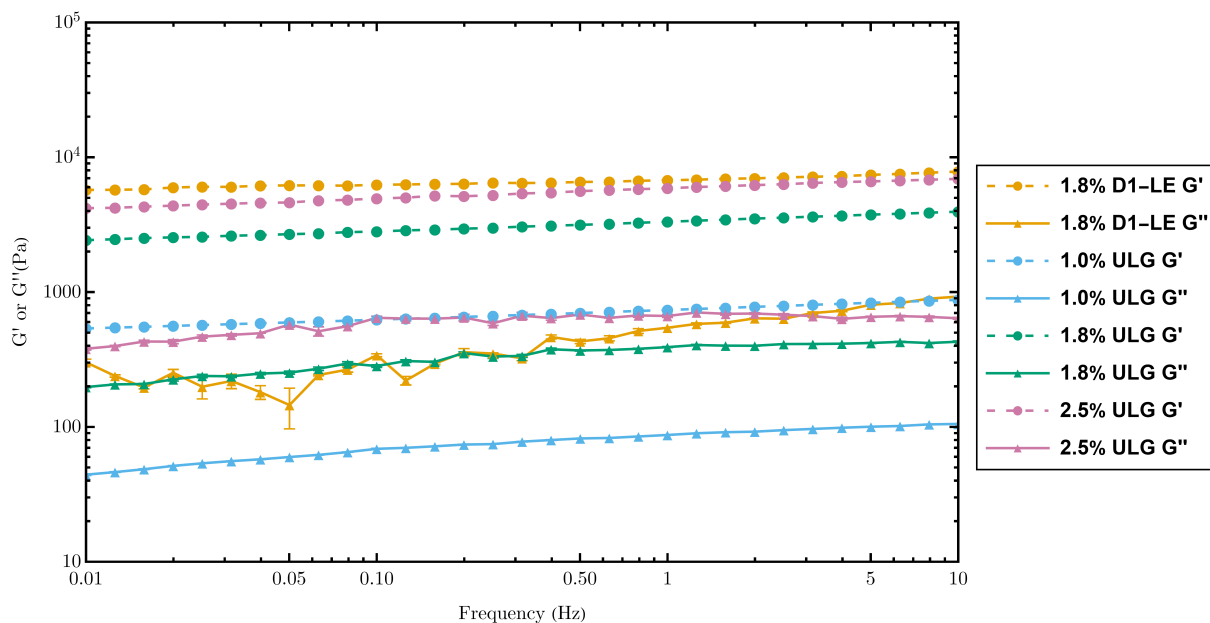


Figure 3.16: **Frequency Sweep of Agarose Samples** - Plots showing storage and loss moduli as a function of oscillatory frequency for 1.8 % w/w D1-LE, and 1.0 %, 1.8 % and 2.5 % w/w ULG agarose samples.

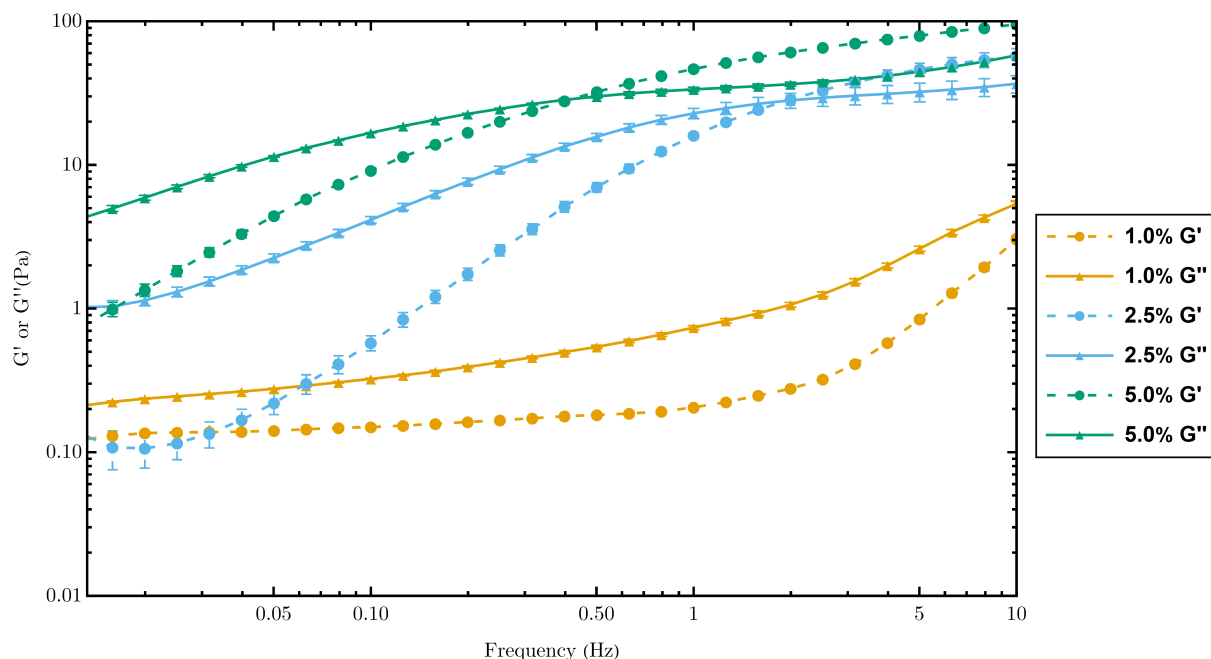


Figure 3.17: **Frequency Sweep of Alginate Samples** - Plots showing storage and loss moduli as a function of oscillatory frequency for 1.0 %, 2.5 % and 5.0 % AlgEDTA samples, conserving acetic acid concentration.

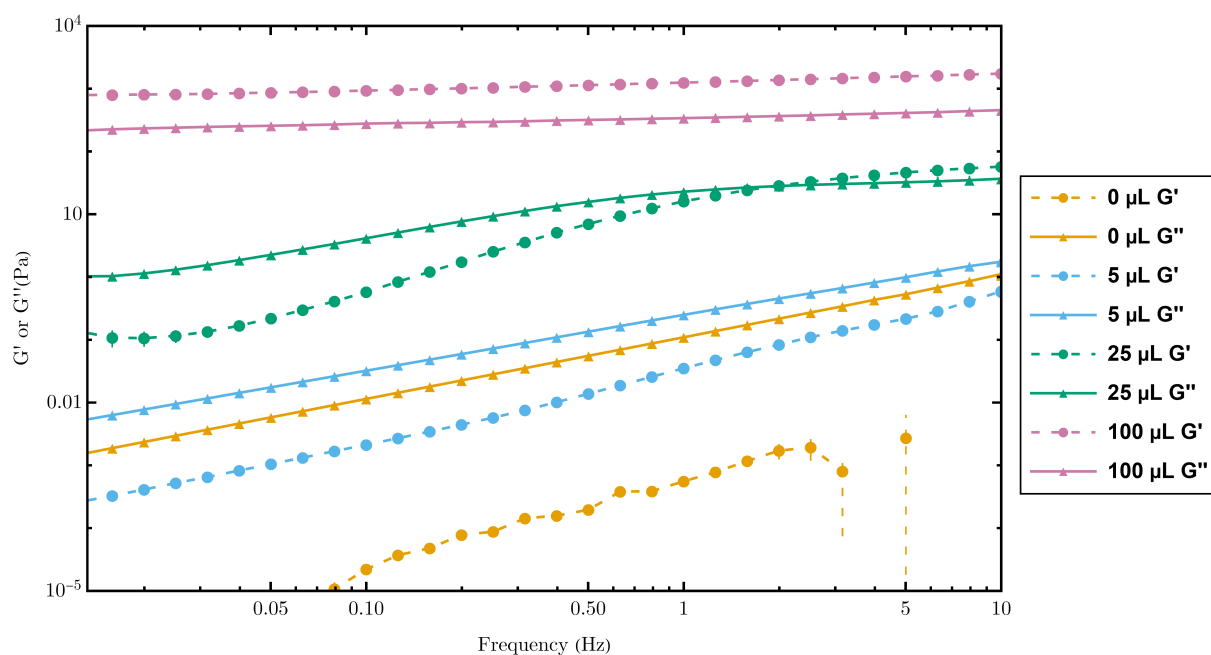


Figure 3.18: **Frequency Sweep of Alginate Samples** - Plots showing storage and loss moduli as a function of oscillatory frequency for 0 μ L, 5 μ L, 25 μ L and 100 μ L acetic acid added, conserving initial 2.5 % AlgEDTA concentration.

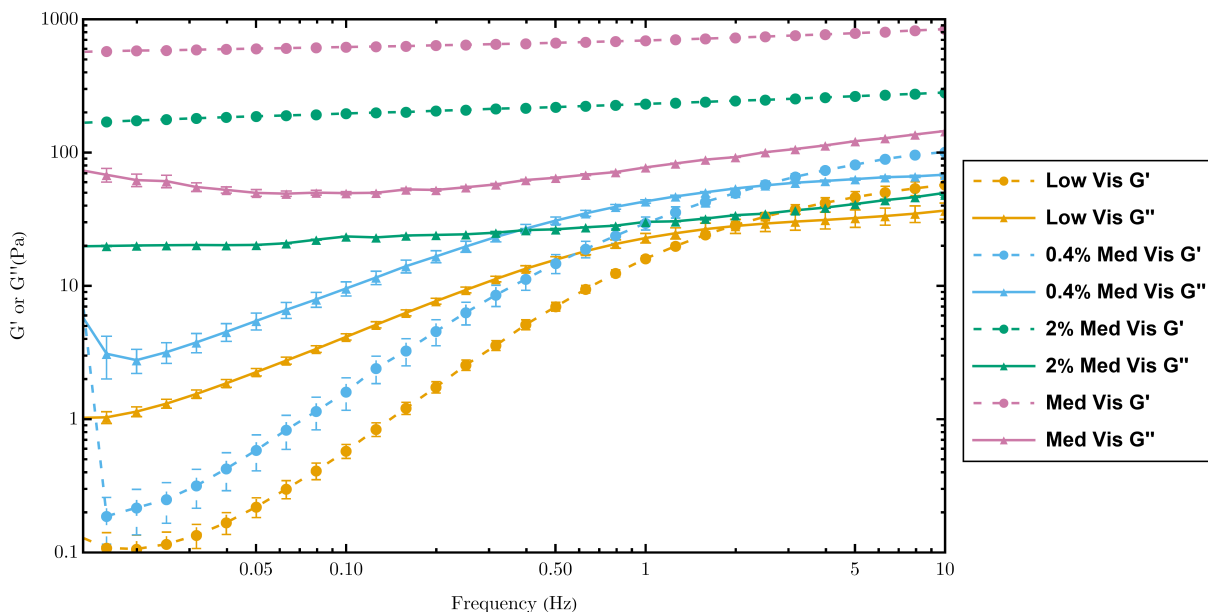


Figure 3.19: **Frequency Sweep of Alginate Samples** - Plots showing storage and loss moduli as a function of oscillatory frequency for low viscosity alginate, a medium viscosity alginate and two mixtures of 0.4 % and 2.0 % medium viscosity-in-low-viscosity conserving 2.5 % AlgEDTA and 25 μ L acetic added.

hydrogel composition described in Table 3.3. Note that in the case of samples 7, 10 and 11, there was no plateau or the plateau did not lie enough within the domain surveyed for an estimate to occur. In the case of sample 9 the plateau may increase from the value stated outside of the domain measured. We have stated here the maximum value observed. Agarose pore sizes are reported to range from several to hundreds of nanometres [202]. Our estimates are consistent with these. The estimate alginate mesh sizes are consistent with literature showing pore sizes in the 10 - 100 nm scale [203, 204, 205]. The trends here mirror those of mechanical strength discussed above. We return to these estimated mesh sizes in Chapter 5, where we compare these to other estimates of pore sizes in hydrogel microspheres.

In summary, the mechanical strength of cross-linked hydrogels seems to increase with increasing the number of cross-links between chains. This can be achieved by increasing the w/w % of polymer in alginate or agarose gels, decreasing the pH or using alginate gels of higher viscosities, or using normal gelling temperature agarose gels. Mechanical strength of gel matrices is intrinsically linked to the pore size of the gel [206], which as we saw in Section 1.2.1, determines the ability of DNA to be constrained by or pass through a gel matrix. Controlling the timescale on which DNA is retained inside a gel matrix is key for the assay to work, as we must ensure DNA remains trapped until subsequent re-encapsulation of gel beads in water-in-oil droplets for PCR

Table 3.4: Estimated plateau moduli and mesh size for each hydrogel sample described in Table 3.3

Sample Number	Plateau Modulus (Pa)	Estimated Mesh Size (nm)
1	4500	9
2	7000	8
3	20000	6
4	700	18
5	3300	11
6	5500	9
7		
8	60	41
9	100	34
10		
11		
12	1000	16
13	100	34
14	200	27
15	700	18

detection and amplification. In Chapter 5, where we test the ability of different compositions of hydrogels to trap DNA, we will link the mechanical properties to the diffusion properties of hydrogels.

3.3 Properties of Hydrogel Microspheres

In order to use hydrogel microspheres as part of biological assays, we should investigate their compatibility with the individual reactions involved in these (often multi-step) assays. In this Section we investigated their general properties in order to understand how these microspheres can be utilised and where their limitations would lie.

In Section 3.3.1, we tested whether the microspheres can be stored for a time interval (rather than being made whenever required), and whether they should be stored as emulsions or suspensions. In Section 3.3.2 we investigated their thermal stability, as reaction steps such as PCR or other denaturation processes will require heating. In Section 3.3.3 we developed a method to cause the microspheres to sediment, in order to allow washing steps to take place. Finally in Section 3.3.4 we investigated the swelling in volume of hydrogel microspheres upon de-emulsification. This is useful for any re-encapsulation of microspheres into water-in-oil droplets for further compartmentalised reactions.

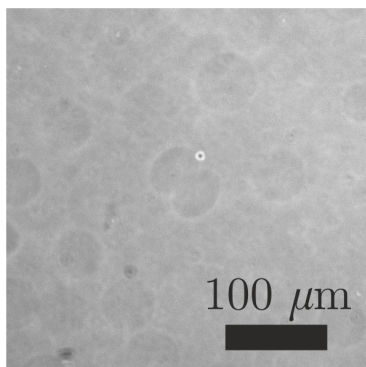


Figure 3.20: Two alginate microspheres cross-linked together after partial cross-linking, resulting in one double-sphere shaped particle.

3.3.1 Time Stability

For use in multi step assays, or assays that will be repeated on many samples, it will be useful to know in which conditions they can be stored. This will allow the production of a large suspension of microspheres at once, rather than creation of many small volume suspensions.

Alginate microspheres were stored at room temperature in emulsion and suspension form (as water-in-oil droplets and as microspheres-in-water) for a ten day period. At each time point a subset of the emulsion was broken and imaged alongside a subset of the microspheres stored as a suspension. When storing microspheres as an emulsion where there is still acetic acid present in the oil phase, there is a risk of loss of control over when cross-linking occurs. Over time, droplets coalesce together (and acetic acid was shown to reduce droplet stability in Section 3.1.3, which would exacerbate this issue). This could form a larger droplet containing two alginate microspheres, that could then be cross-linked together by the acetic acid to form a large, misshapen micro particle (Figure 3.20). Breaking the emulsion, and storing microspheres as a suspension avoids this as the acetic acid is removed in the breaking procedure.

3.3.2 Thermal Stability

Heating agarose microspheres disrupts intermolecular bonds, leading to deformation. Heating bulk 1 % w/w ULG or D1-LE agarose to 60 °C for 1 h was shown to reduce the bulk gel to a colourless and non-viscous liquid (Section 2.1). Therefore all microsphere structure was lost when heating to any temperature above this.

Alginate microspheres are cross-linked instead by stronger ionic bonds. When a suspension of these was heated to 65 °C for 1 h and allowed to cool, the microsphere structure was still intact. Heating these while still an emulsion (under the same conditions) resulted again in droplet

coalescence and an increased frequency of the misshapen micro-particles seen in Figure 3.20.

3.3.3 Microsphere Sedimentation and Washing

For the microspheres to act as micro-sieves, they need to be washed to dilute out small undesired molecules without allowing the diffusion of the larger molecules trapped within the hydrogel. The quickest way to wash a suspension of bead is to dilute the suspension, then use centrifugation to quickly sediment the microspheres, allowing the supernatant to be removed by pipetting. The microspheres will remain at the bottom of the suspension so are not lost, and can subsequently be resuspended. In order to do this the ability of microspheres to survive centrifugation is desirable. 1 % w/w agarose microspheres were produced as in Section 3.1.1, and diluted by a factor of ten. In work done by Devinder Srari (Aarts and Frater groups, University of Oxford), they were found to conserve their spherical structure upon centrifugation at 2300 RPM (390 x G) for 3 min. Figure 3.21a shows an uncentrifuged sample of 1 % w/w agarose microspheres while

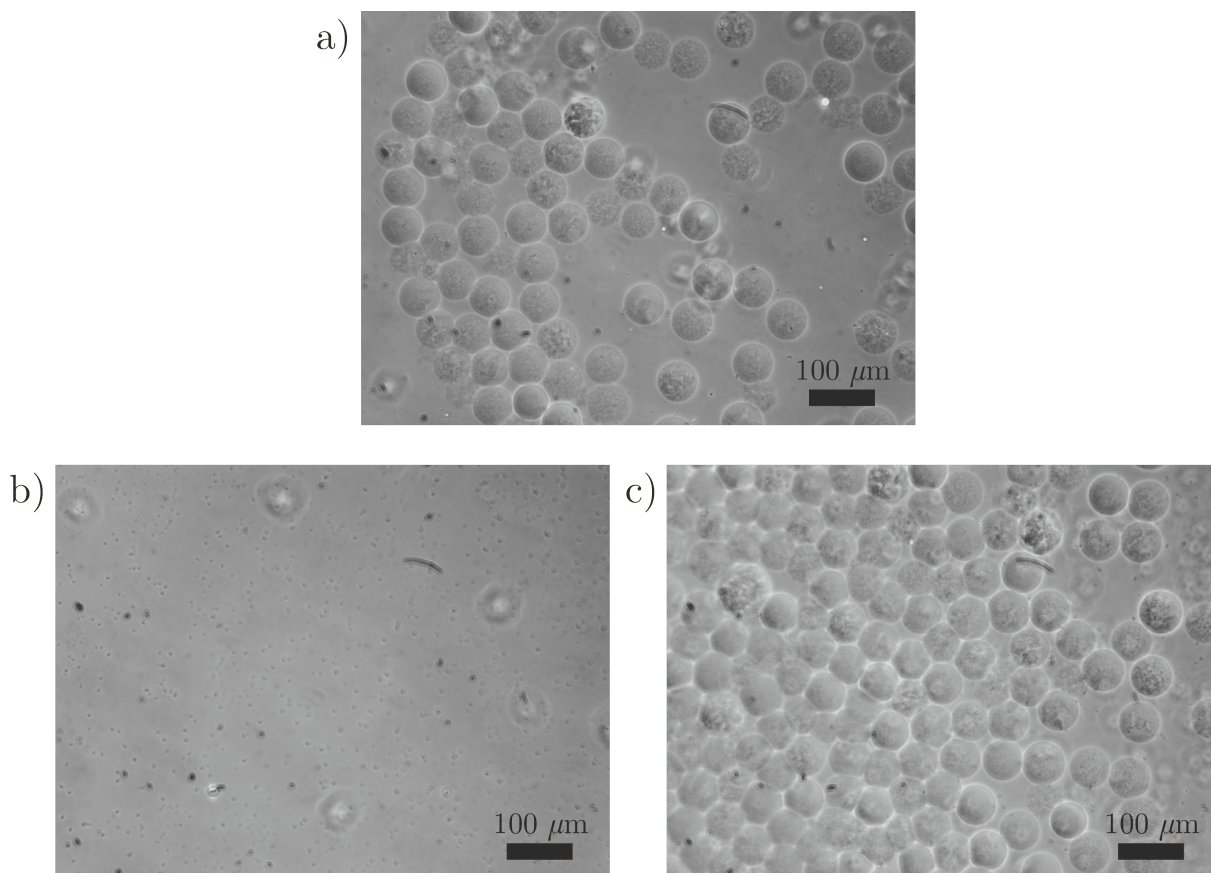


Figure 3.21: Phase-contrast images of a) uncentrifuged 1 % w/w agarose microspheres are compared with the b) supernatant and c) sediment of a 1 % w/w agarose sample after centrifugation at 2300 RPM (390 x G) for 3 min.

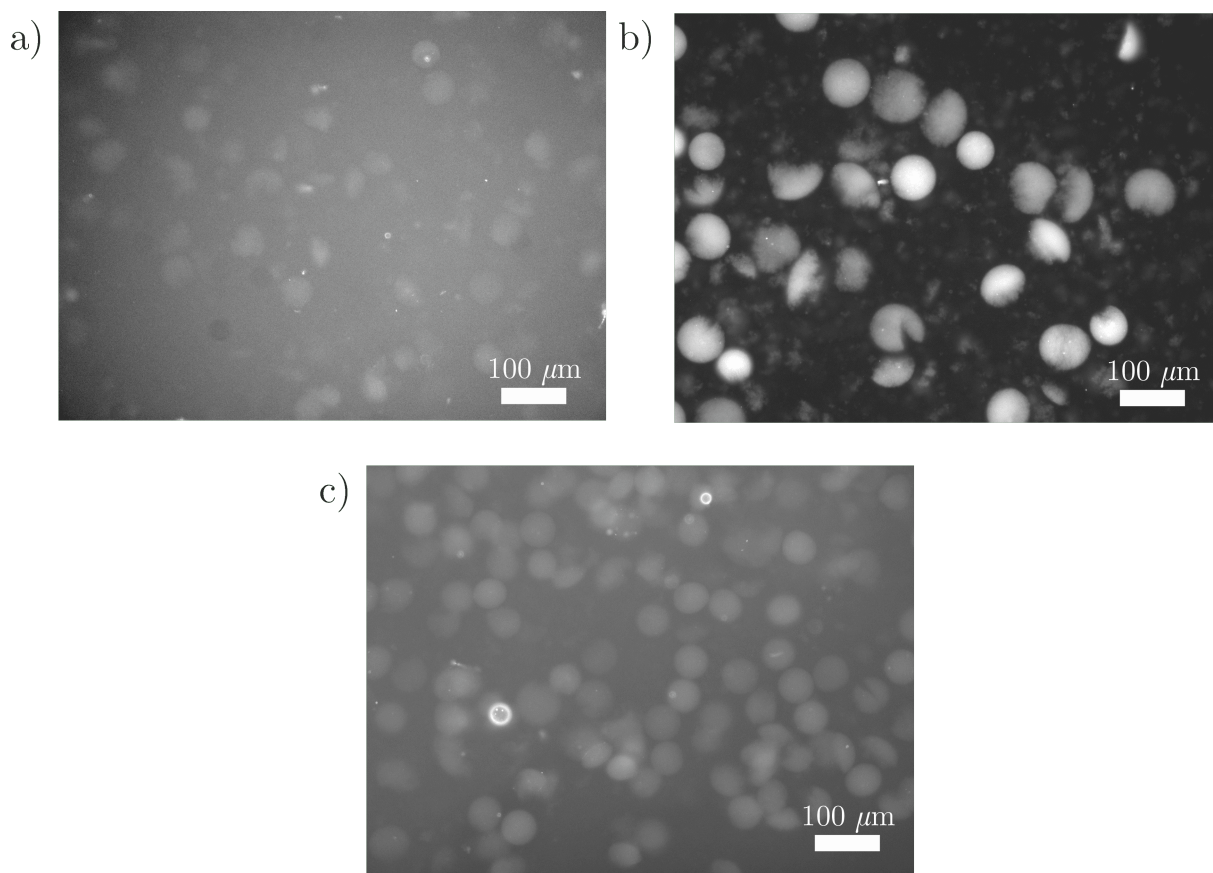


Figure 3.22: Fluorescent 1 % w/w alginate microspheres were diluted by a factor of 10 in DI water and centrifuged at a) 1000 RPM (74 x G) for 10 min, b) 300 RPM (6.6 x G) for 3 min or c) 500 RPM (18 x G) for 5 min. They were then imaged using a FITC filter.

Figures 3.21b and 3.21c show the supernatant and sediment of the post centrifugation sample respectively. This method results in clear sedimentation, and allows for easy washing.

When 2.5 % alginate microspheres (containing alginate fluorescein as in Section 3.1.2) were also diluted by a factor of ten and centrifuged. Regardless of the force exerted on them, and the time period, the microsphere structure was lost (some of these are shown in Figure 3.22). Therefore any washing procedures involving alginate microspheres will require allowing the microspheres to sediment without an external force. Instead alginate beads must be allowed to sediment using gravity. A suspension of fluorescent 1 % alginate microspheres was diluted by a factor of ten, and allowed to stand. The top and bottom 10 μ L were imaged. Figure 3.23 shows 2 h was long enough for the beads to sediment. For all future washing of alginate microspheres, they were broken, diluted by a factor of ten, and allowed to sediment for 2 h.

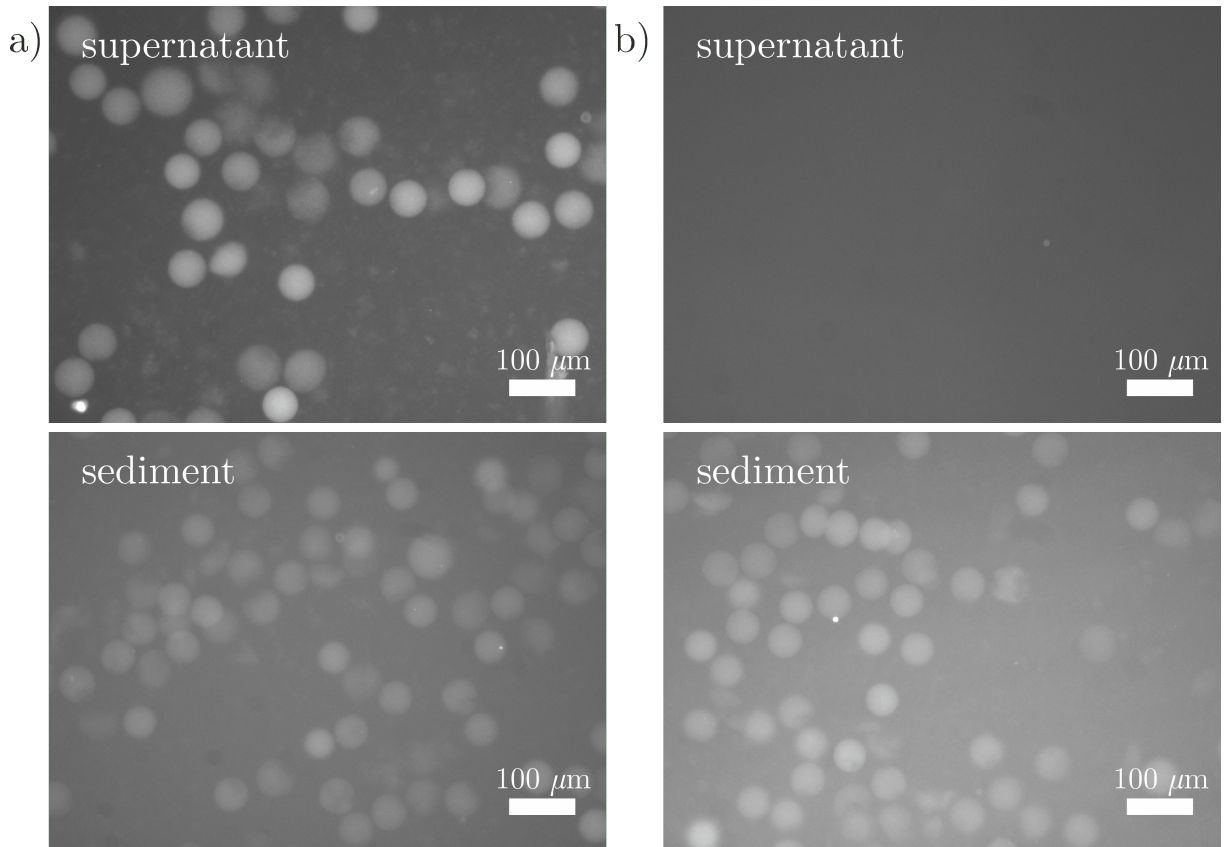


Figure 3.23: The sediment and supernatant of a suspension of fluorescent 1 % alginate microspheres, diluted by a factor of ten in DI water, and allowed to stand for a) 0 h and b) 2 h. They were then imaged using a FITC filter.

3.3.4 The Swelling of Hydrogel Microspheres

For successful re-encapsulation of microspheres into new droplets for further reaction steps, such as PCR, their size needs to be controlled. Droplet size can be easily controlled by the size of the microfluidic channels, and by modifying the flow rates of the inlets in real time to create droplets of a desired volume. Hydrogels however, are known to swell when the networks are added to water, behaviour taken advantage of for applications such as tissue regeneration, but less useful for use as micro-reaction vessels [207]. Therefore it is important to investigate whether the size of a microsphere correlates with the initial droplet size.

The simplest way to conduct this investigation was to produce a set of microspheres as detailed in Section 3.1 and take images of the emulsion and resulting suspension. A script was then written in Mathematica 13 which located circular objects in an image and calculate their radii in pixels. These radii were converted into micrometers. A summary of the droplet identification process is shown in Figure 3.24. While this image analysis worked very well for images of emulsions, it was less successful at identifying alginate microspheres when imaged with phase-contrast microscopy

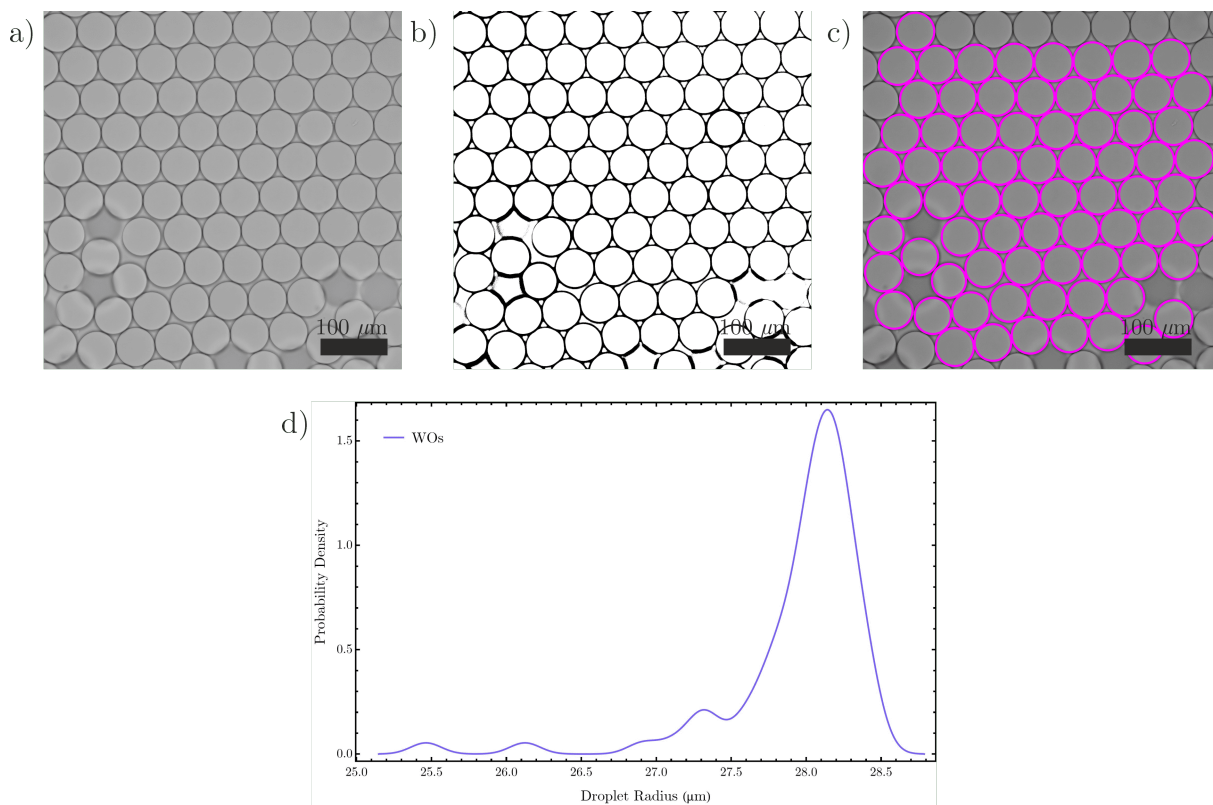


Figure 3.24: An overview of the Mathematica script to generate a histogram of droplet radii: a) the input image of water-in-oil droplets, b) the input image is binarized, c) disks are located in the image, and d) a histogram of the equivalent disk radii is generated.

(Figure 3.25b). This is due to the large amount of noise in phase-contrast images. Instead fluorescent alginate beads are created as these are more homogenous (Figure 3.25c). Agarose phase-contrast images (Figure 3.25a) were less noisy so these were used for this analysis. This script was designed to not include microspheres where they are deformed, touching another microsphere, or on the boundary of the image. This was necessary to ensure only correct radii of microspheres are collected. This script was run for samples of fluorescent 2.5 % w/w alginate microspheres. The same process was completed for a 1 % w/w ULG agarose sample using phase-contrast images.

The script detected 3983 droplets and 446 microspheres for the 2.5 % w/w alginate sample, and 1057 droplets and 308 microspheres for the 1 % w/w agarose sample. Figure 3.26 shows histograms of the distribution of radii for the droplets and microspheres of both alginate and agarose hydrogels. The alginate microspheres showed a clear increase in volume upon demulsification, consistent with previous results [208, 209]. In contrast agarose microspheres do not seem to show similar swelling in water, again consistent with previous results [210]. These differences will be discussed again when looking at re-encapsulation in Chapter 5.

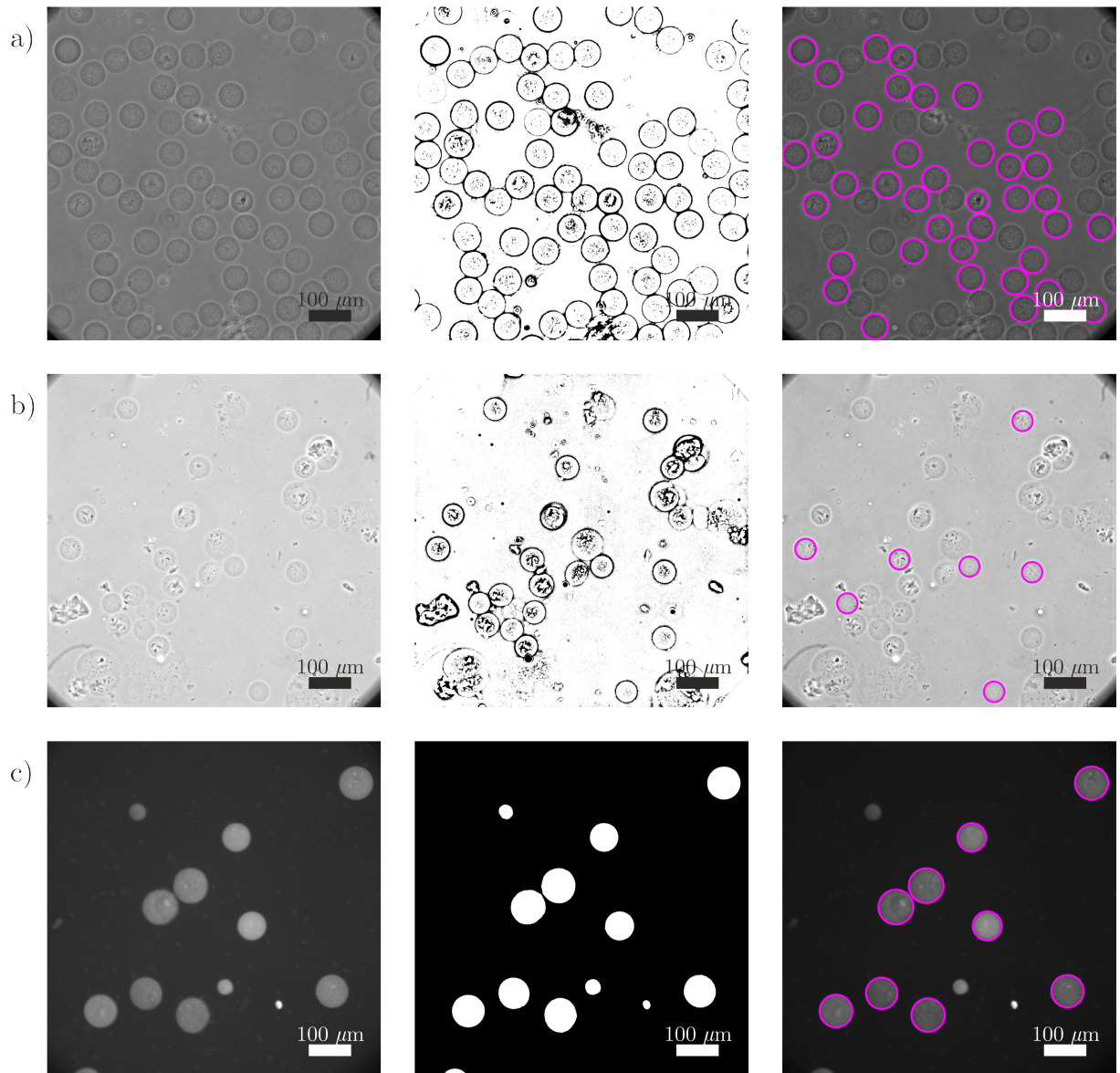


Figure 3.25: The disk locating script was applied to images of a) agarose microspheres taken with phase-contrast microscopy and of alginate microspheres taken with b) phase-contrast microscopy and c) fluorescence imaging. For each of the three types of image, the input image, binarized image, and image highlighted with located disks are shown.

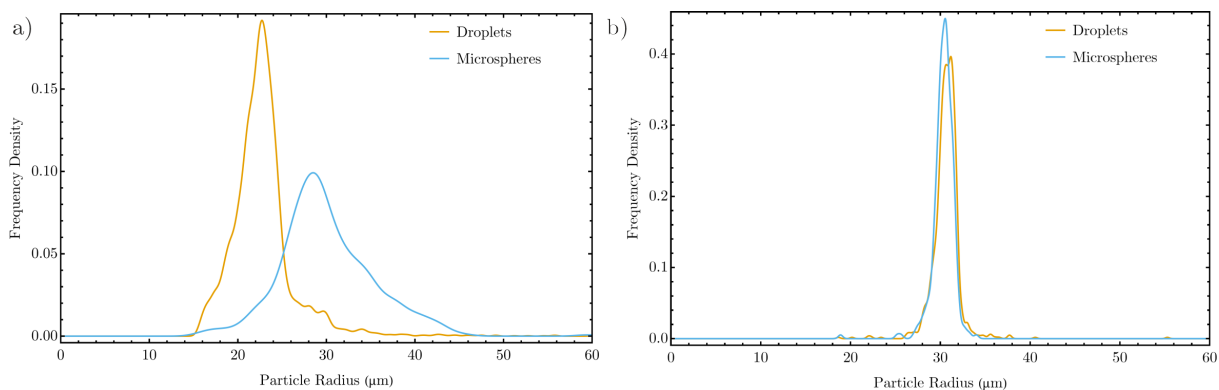


Figure 3.26: Histograms showing frequency of original droplet radii, and resulting microsphere radii in their respective emulsions and suspensions for a) 2.5 % w/w alginate microspheres, and b) 1 % agarose microspheres.

Table 3.5: A summary of the differences in agarose and alginate microsphere properties.

	Alginate microspheres	Agarose microspheres
Time stability	Stable stored as suspension	Stable stored as suspension
Heating stability	Stable up to 65 °C	Melt upon heating
Centrifugation Stability	Damaged upon centrifugation	Can be easily centrifuged
Swelling	Swell upon de-emulsification	Do not swell upon de-emulsification

3.3.5 Summary

In summary, both alginate and agarose microspheres are time stable, and should be stored as suspensions once cross-linking has occurred. Alginate microspheres can be heated, whereas agarose microspheres lose all structure upon heating, limiting their use as micro-reactors which involve heating steps. Although we discuss in Section 4.4.3 why dissolution upon heating may also be an advantage for PCR based applications. Conversely, agarose microspheres can be centrifuged, allowing for easy washing for dilution of reagents, whereas alginate washing will take time as sedimentation must occur by gravity alone. Finally we saw that alginate microspheres swell upon de-emulsification, and this should be taken into account when flowing them in subsequent microchannels for re-encapsulation in new droplets. These differences are summarised in Table 3.5.

3.4 Chapter Summary

The work in this Chapter intended to develop methods to reliably produce suspensions of agarose and alginate microspheres with varying compositions. We also aimed to evaluate their compatibility with common steps in biological assays, such as washing, PCR, re-encapsulation,

and other experimental requirements.

In Section 3.1 we developed a tubing and slide heater method to produce 1 % w/w agarose microspheres. We also used a CaEDTA and acetic acid ligand exchange method to produce 2.5 % alginate microspheres. These two methods are key to this thesis as they are used to produce all microspheres used in further work investigating a HIV latent reservoir assay in Chapter 4 and investigating the trapping ability of microspheres in Chapter 5.

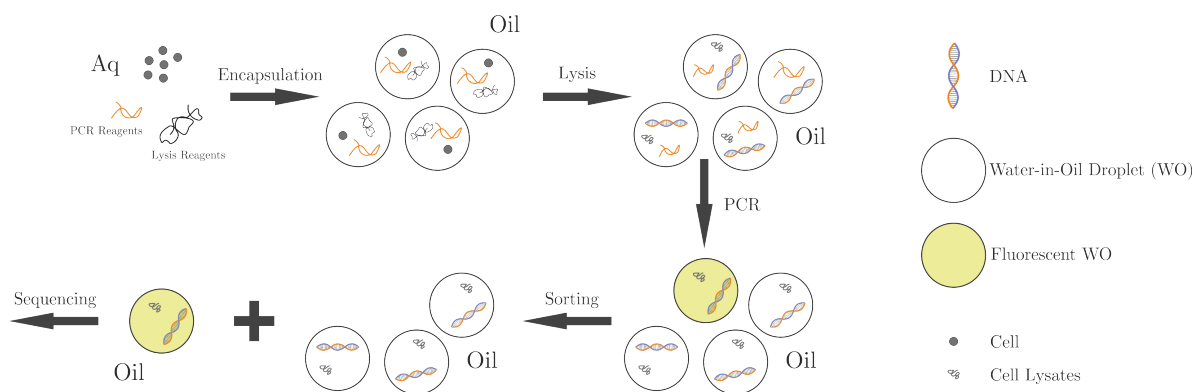
In Section 3.2 we used rheological techniques to investigate bulk properties of hydrogels of different compositions. We used oscillatory measurements to determine how viscoelastic the hydrogel samples were, and how their mechanical strength compared to each other. We theorised that mechanical strength relates to pore size of the hydrogels (providing the type of cross-linking bond is consistent), and then predicted that hydrogel compositions with a higher mechanical strength will trap DNA more efficiently, a topic of investigation we will return to in Chapter 5. Section 3.3 investigated whether the microspheres are compatible with the reaction steps required for the HIV latent reservoir assay proposed in Chapter 4. We showed they are time stable, allowing for use in a multi-day assay. Alginate microspheres were shown to be more stable with respect to heating than agarose microspheres. While this would be problematic for running PCR on a suspension of microspheres, instead re-encapsulating the microspheres into new droplets will allow PCR to run without losing single-cell resolution. PCR is discussed further in Section 4.4. We developed washing protocols for alginate and agarose microspheres which will be used in later chapters. Finally we showed how alginate microspheres swell upon de-emulsification while agarose microspheres do not, an important property to account for when re-encapsulating microspheres as part of the HIV latent reservoir assay.

Chapter 4

The HIV Latent Reservoir Assay

In Chapter 1 we discussed the limitations of current single cell sequencing technologies, and that there would be a large benefit to the development of an assay that can sort cells looking for a rare population - such as intact HIV provirus integrated in T cells. In this chapter we propose and test an assay to combat the current limitations. To efficiently sequence HIV provirus at a single cell level, a technology is required that can sort DNA originating within infected cells from DNA originating within uninfected cells, while maintaining single cell resolution.

This chapter begins by proposing a new assay using the hydrogels microspheres developed and tested in Chapter 3, showing the current limitations that have led to size controlled diffusion using hydrogels being a necessary adaptation of current technologies. We then focus on the three key steps in this assay, detailing the challenges raised in their development, comparing agarose and alginate hydrogels in their abilities to avoid these. Firstly, cell lysis methods are discussed, and the most promising are applied to cells trapped in hydrogel microspheres in order to find a reliable method that doesn't damage the microspheres (and lose the single cell resolution). Secondly, a new microfluidic device is used to re-encapsulate hydrogel microspheres into larger droplets, investigating how channel geometries affects successful re-encapsulation. Washing techniques to improve re-encapsulation ease and efficiency are shown. This section ends by showing successful re-encapsulation of DNA trapped within hydrogel microspheres. Finally in-droplet PCR of DNA and the subsequent bulk and in-droplet PCR of DNA trapped in microspheres is discussed, showing the current limitations of the hydrogel platform.



Aq = Aqueous Continuous Phase

Oil = Oil Continuous Phase

Figure 4.1: The initially proposed microfluidic HIV reservoir assay. Cells are encapsulated alongside lysis and PCR reagents. Nuclear DNA is released and proviral HIV DNA is detected by presence of a fluorescent signal.

4.1 A Proposed New Assay

4.1.1 Lysis and PCR in Droplet Micro-reactors

In theory, sorting nuclear DNA based on the presence of HIV provirus at a single cell resolution can be solved by a simple droplet microfluidic assay (Figure 4.1). If single cells were initially encapsulated in water-in-oil droplets alongside lysis reagents and PCR reagents, the Poisson distribution could ensure droplets contained at most one cell [38]. Cell lysis would then release the genomic DNA, and PCR could detect and amplify a target sequence. Using PCR with a fluorescent probe allows fluorescent droplets to be sorted on another microfluidic device, leading to a collection of droplets that only contain the nuclear material from T cells that contained provirus. These could then be sorted onto a plate and sequenced, with every well containing proviral DNA from separate infected T cells (Figure 4.1). It has been shown in unpublished work by Dr Lucia Parolini (Aarts and Frater groups, University of Oxford), that while this system can successfully lyse cells, subsequent amplification can only occur upon significant dilution of the droplets - it appears that excess lysis reagents or remaining cell lysates disrupt PCR [9]. While this dilution could be achieved by merging a droplet containing nuclear DNA and one containing PCR reagents, this would have to produce a significantly larger droplet, which then poses problems for successive sorting into specific wells on a plate for sequencing.

The solution to this problem would be to remove the excess lysis reagents and cell lysates from the micro-reaction vessel, while keeping long chain genomic DNA trapped at a single cell resolution.

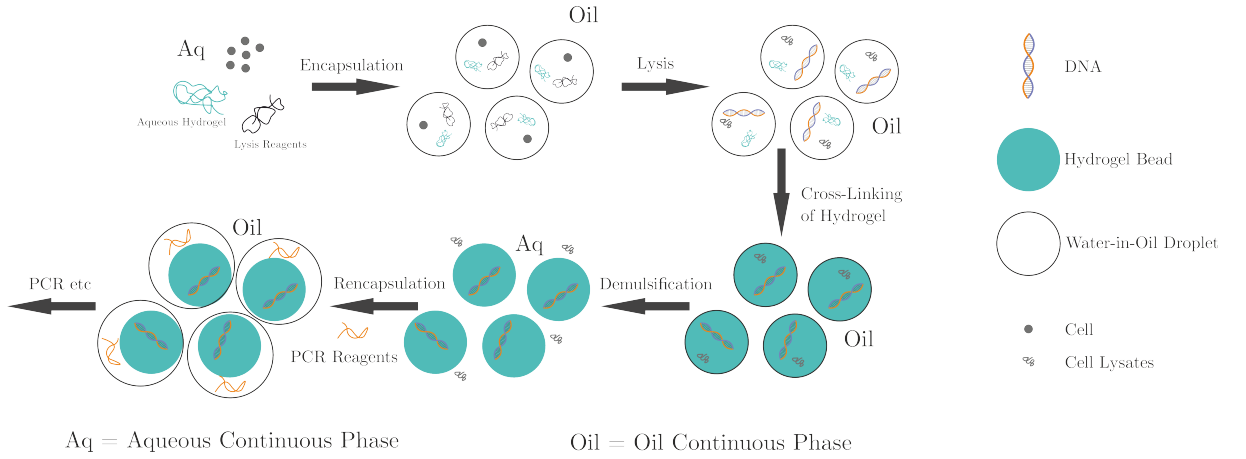


Figure 4.2: The new proposed combined hydrogel and microfluidic HIV reservoir assay. Nuclear DNA lysed from a single cell is trapped within a hydrogel microsphere and re-encapsulated alongside PCR reagents for subsequent detection of HIV via PCR.

4.1.2 A Hydrogel Solution

In Chapter 1 we theorised that hydrogel matrices can be used as a micro-sieve for droplet micro-reactors. In this case it would be useful to contain the large nuclear DNA within a droplet, while allowing the relatively small lysis reagents and cell lysates to be washed out.

In this chapter we therefore propose a combined hydrogel and microfluidic assay to achieve this. We encapsulate cells, lysis reagents and sol-phase-hydrogel into a water-in-oil droplet and allow lysis to occur (controlling cell lysis to occur post encapsulation to maintain single cell resolution). Next, the hydrogel will be induced to cross-link to form a gel-phase, and de-emulsification of the emulsion can produce a suspension of hydrogel microspheres in water, each containing nuclear DNA. These will then be washed to remove excess lysis reagents and cell lysates, and then re-encapsulated in new water-in-oil droplets alongside PCR reagents. This new emulsion will then be thermocycled to produce fluorescent droplets where provirus was present. These will be sorted and sequenced as described above. This is summarised in Figure 4.2.

Initial encapsulation of aqueous hydrogel chains, lysis reagents and cells, and subsequent de-emulsification of droplets is trivial, and the cross-linking of hydrogels was discussed in Chapter 3. Therefore this chapter focuses on the method development of the remaining steps of the assay. Section 4.2 discusses several cell lysis techniques, and then adapts chemical lysis methods to ensure microsphere survival alongside successful release of genomic DNA. Section 4.3 investigates how changing channel geometry and microsphere dimensions affects successful microsphere re-encapsulation, before showing that DNA remains trapped inside the microspheres during this re-encapsulation. Section 4.4 discusses the theory and methods used for PCR in this thesis. We

focus on the difficulties in adapting PCR to work within hydrogel matrices, using both qPCR and in-droplet PCR methodologies. We end in Section 4.5 by discussing the advantages and disadvantages of alginate and agarose microspheres, discussing where there is the potential for future work to improve these.

4.1.3 A Model Cell Line

While the intention of this assay is to detect HIV infected T cells, working with these brings an inherent risk of infection. To reduce risk of infection during this thesis, Jurkat cells were used instead. The Jurkat cell line consists of non-infectious T cells originating from a cancer patient. They have a mutation that allows them to overcome the Hayflick limit, a phenomenon that causes eventual death of a cell population after a certain number of divisions [211]. They are often therefore referred to as an "immortal" cell line. As these cells do not contain HIV DNA, the risk of infection is negligible. Here the ALB gene was used as a target for PCR rather than part of the HIV proviral DNA sequence. This codes for albumin proteins found in blood plasma, the most abundant protein found in human blood [212]. In this thesis, Alb-PCR was used as a detection method for generic genomic DNA lysed from T cells.

4.2 Cell Lysis

The aim of this section was to develop a method of producing hydrogel microspheres containing trapped genomic DNA at a single-cell resolution. This was achieved by encapsulating Jurkat cells inside droplet micro-reactors and the subsequent breakdown of the cell to release genomic material. Section 4.2.1 opens with an overview of various lysis techniques, comparing their suitability in droplet micro-reactors and choosing the techniques to test in this thesis. Section 4.2.2 then describes the investigation to determine the compatibility of these lysis methods with hydrogel microspheres. Section 4.2.4 concludes by showing the successful release of genomic DNA using these adapted techniques.

4.2.1 Lysis Techniques

Cell lysis refers to the general process of controlled damage to a cell membrane or the membrane of an internal component of the cell (such as the nucleus) in order to release biological material from confinement, often as a part of larger bioassays. This thesis works with human T cells, where a cell consists of a cell membrane surrounding the whole cell, and genomic material is

trapped within a nucleus (Section 1.3.2). Unless otherwise stated, for the rest of this thesis cell lysis refers to the lysis of mammalian cells. Different cell lysis techniques can cause different cell breakdown, so they are typically designed for the specific purpose required. Partial cell lysis typically refers to the breakdown of the cell wall to release the cytoplasmic components, whereas complete cell lysis is required to also breakdown the nucleus to release genomic DNA and RNA [213]. The assay proposed in Section 4.1 requires genomic DNA, so this Section focuses on lysis techniques that release genomic DNA from confinement. The many cell lysis techniques can broadly be summarised in two categories, mechanical and non-mechanical.

Mechanical lysis techniques consist of a force applied to break the cell membranes without using chemicals or enzymes that could disrupt further analysis of cellular material such as PCR [214]. These include methods such as shearing [215], sonication to form and rupture cavities [216, 217], microfluidic acoustic oscillation [218], or grinding techniques such as bead mills or rotor-stator homogenisation [214, 219]. These techniques are difficult to replicate in droplet micro-reactors, and also are known to denature proteins due to heating or shearing [214, 217]. This can be a problem if DNA is required to not be denatured or fragmented for further analysis. Therefore mechanical lysis is not used in this thesis.

Non-mechanical lysis can be chemical, enzymatic, physical, or a combination of these. Chemical lysis uses a buffer to disrupt the cell membrane. Mammalian lysis typically uses surfactants to disrupt lipid-lipid, lipid-protein, and protein-protein interactions in the lipid bilayer of the cell membrane [213]. These surfactants can be non-ionic (e.g. Triton X-100 or Tween 20), anionic (e.g. sodium dodecyl sulfate, SDS) or cationic (e.g. cetyltrimethylammonium bromide). These lysis buffers can also contain chaotropic agents such as EDTA or guanidinium thiocyanate, which disrupt hydrogen bonding between water molecules. This weakens the hydrophobic effect which stabilises protein structure and therefore denatures the protein structure of the cell membrane [220]. EDTA and other chelating ligands also bind to divalent metal ions. As these metal ions are also required by enzymes which degrade DNA and other proteins (DNases and proteinases), it is beneficial to trap divalent ions with chelating ligands to reduce this degradation. Chemical lysis is easily scalable to work in droplets, with the buffers easily encapsulated. Chemical lysis will be investigated in this thesis.

Enzymatic methods work similar to chemical methods, using enzymes as disruptors of protein structure. As enzymes often have optimum conditions required for efficient function, pH and temperature often have to be controlled for successful lysis [213]. Specific enzymes can be used

to lyse specific cells, or for specific extraction purposes; such as the use of proteinase K to isolate genomic DNA [221, 222]. Enzymes are often used in conjunction with chemical buffers for cell lysis.

Physical lysis methods include the heating of cells in order to denature proteins [223], freeze-thaw cycles [224] or osmotic shock [225, 226]. These techniques also have the potential to denature DNA, but for our purpose, dsDNA will need to be denatured to ssDNA during PCR anyway (Section 4.4.1). Some physical methods are investigated through the rest of this thesis as they are easily adapted for lysis in droplet micro-reactors.

To produce hydrogel microspheres containing trapped nuclear DNA, cell lysis can occur at several points in the assay. Firstly, cells could be trapped inside hydrogel microspheres, and then this suspension can be washed first with a lysis buffer, and then with water to dilute out this buffer and cell lysates. Alternatively, cells, sol-phase hydrogel, and a lysis buffer could be encapsulated in droplets. Then cell lysis could occur prior to the induced cross-linking of the hydrogel, allowing washes post emulsion-breaking to dilute out this buffer and cell lysates. This would produce a suspension of hydrogel microspheres trapping genomic DNA. For either method to be successful, the lysis method chosen must not also disrupt the cross-linking in hydrogels. The next few sections test microsphere compatibility and the effectiveness of chosen lysis techniques using a mixture of both of these methods. We started in Section 4.2.2 by investigating how hydrogel microspheres respond to some chosen chemical and physical lysis techniques.

4.2.2 Testing Lysis Methods - Microsphere Survival

Physical Lysis - The only physical lysis technique used in this thesis was heating. The ability of hydrogel microspheres to maintain structural integrity during heating was investigated in Section 3.3.2.

Chemical Lysis - This thesis works from two "base" lysis buffers. The first contained 50 mM TRIS-HCl, 0.1 % v/v SDS and 1 % proteinase K, and was developed by Dr Lucia Parolini. The second contained guanidinium thiocyanate, a known chaotropic agent [227]. The concern for compatibility with hydrogel microspheres here is that the ionic cross-links between alginate chains will be disrupted by some of these ionic buffers. These buffers are designed to disrupt the ionic interactions holding cell structure together, so it is expected that they will disrupt the ionic interactions holding hydrogel chains together. This section therefore focuses on testing a range of lysis buffers to determine whether they disrupt the structure of the hydrogel microspheres.

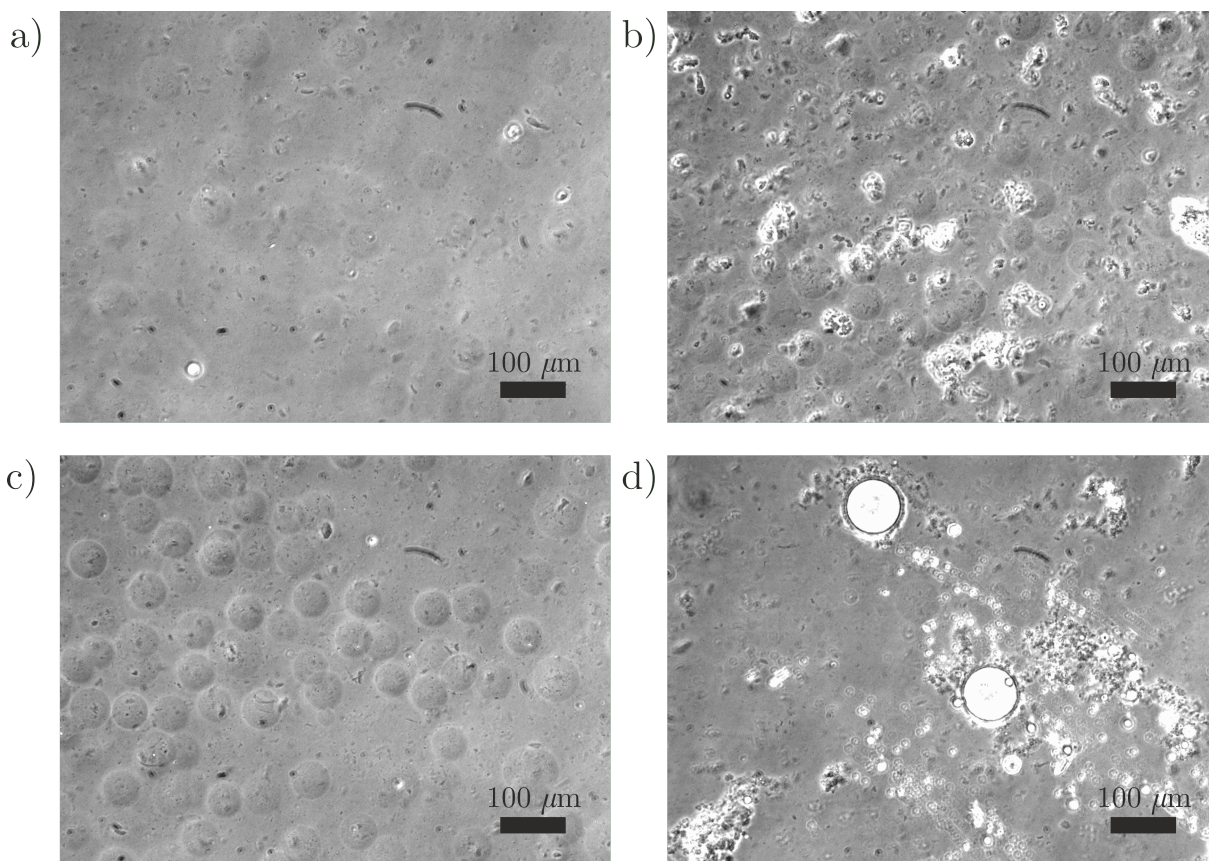


Figure 4.3: Bright-field images of 1 % w/w alginate microspheres in a lysis buffer of a) 5 % v/v TRIS-HCl 1M, b) 0.1 % v/v SDS, c) 1 % proteinase K or d) all the above.

Two methods were used, either addition of lysis buffer to existing microspheres, or the creation of microspheres with the presence of a lysis buffer.

To test the compatibility of the first lysis buffer with alginate microspheres, a suspension of 1 % alginate microspheres was added to different lysis buffers to produce a final concentration of 50 mM TRIS-HCl, 0.1 % v/v SDS or 1 % v/v proteinase K respectively, as well as a buffer containing all of these (Figure 4.3). Here we see that proteinase K causes no apparent damage to microsphere structure (comparable to images shown in Section 3.1.2). 50 mM TRIS-HCl reduced the frequency of microspheres, and 0.1 % v/v SDS resulted in a messy sample, where microspheres seemed deformed from their usual circular shape. The microspheres added to the full lysis buffer seemed to be completely destroyed as none could be found in the sample.

To test whether the ionic guanidinium thiocyanate interferes with the ionic cross-linking of alginate chains, guanidinium thiocyanate was added to 1 % w/w alginate droplets prior to cross-linking at 0.5 %, 5.0 %, 12.5 % and 50 % v/v final concentrations (Figure 4.4). Cross-linking and breaking of the emulsion were achieved using the procedures developed in Sections 3.1.2 and

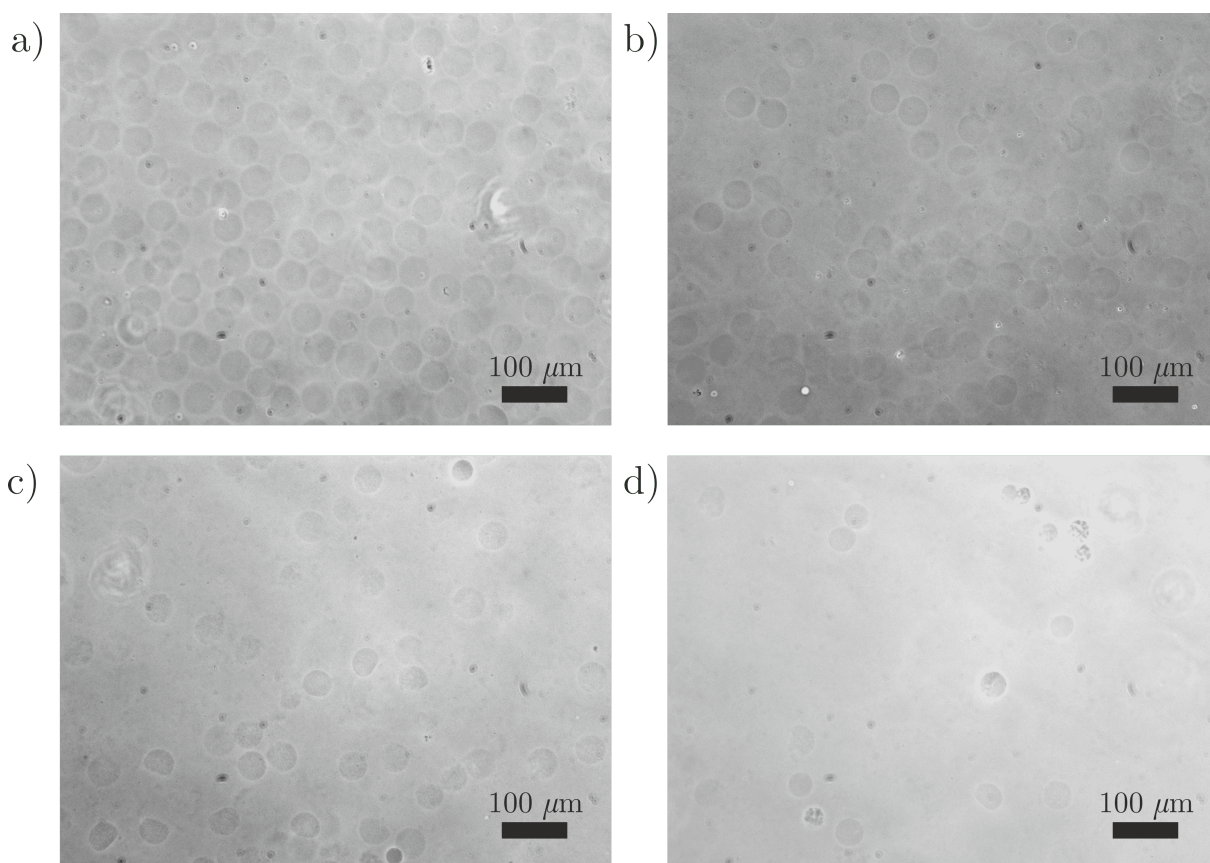


Figure 4.4: Bright-field images 1 % w/w alginate microspheres produced from droplets originally containing a) 0.5 %, b) 5.0 %, c) 12.5 % and d) 50 % v/v guanidinium thiocyanate.

2.2.4 respectively. As the concentration of guanidinium thiocyanate increased, the microspheres decreased in frequency and became more misshapen, suggesting that cross-linking is disrupted by ionic interactions. The 0.5 % and 5.0 % guanidinium lysis buffers produced a stable enough suspension for subsequent analysis, but any higher concentration than this proved too destructive for successful use.

4.2.3 Testing Lysis Methods - Lysing Jurkat Cells

While it seems trivial, a lysis buffer must successfully release genomic DNA as well as not damaging the hydrogel microspheres. This section will take the lysis buffers used in Section 4.2.2 and assess their effectiveness at cell lysis. No hydrogel component is present in the experiments discussed in this section.

Physical Lysis - Droplets containing Jurkat cells were heated to 100°C for 10 min in order to adapt work showing this would release DNA from cells in bulk [223]. This resulted in no apparent lysis in droplets, presumably due to poor heat transfer from the Eppendorf Thermomixer compact

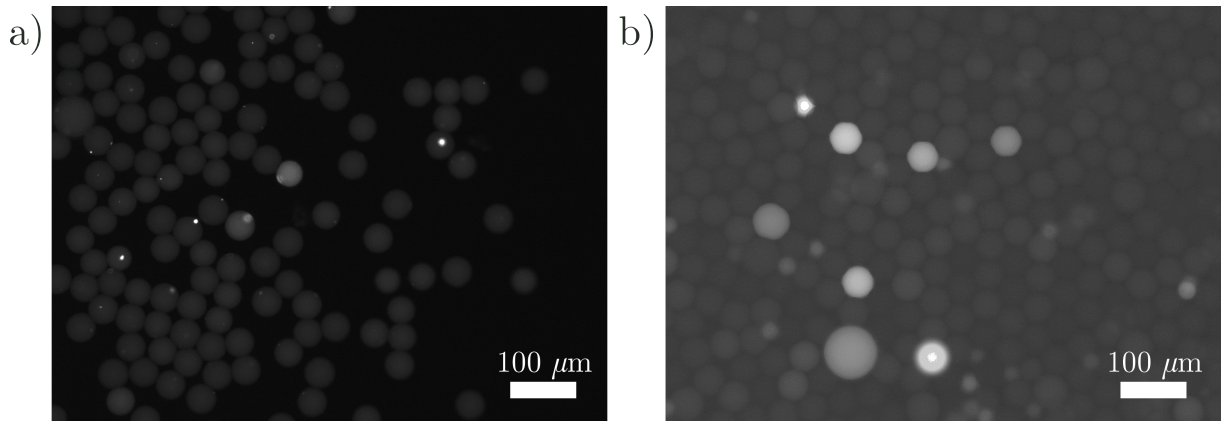


Figure 4.5: Droplets containing fluorescently stained Jurkat cells with a) no lysis buffer and b) a lysis buffer with final concentration 5 % v/v TRIS-HCl 1M, 0.05 % v/v SDS and 1 % proteinase K.

to the cells within an emulsion. This suggests use of heating as a cell lysis technique would require more extreme conditions, either a higher temperature or heating for longer.

For use with agarose microsphere, lysis would have to occur in emulsion form (compartmentalised water-in-oil droplets) and not suspension form (microspheres-in-water). This is due to the agarose samples used in this thesis having a melting temperature of around 50°C (Section 2.1), so heating a suspension of agarose microspheres above this would lose all single cell resolution. Heating droplets runs the risk of destabilising the emulsion and again losing single cell resolution (Section 1.1.2).

While there is potential to adapt droplet heating as a technique here, chemical lysis was first investigated in depth as it is theorised to not cause similar disruptions to droplet stability.

Chemical Lysis - To test the first lysis buffer, Jurkat cells stained with CellTracker™ CM-Dil fluorescent dye (Section 2.3.2) were encapsulated to produce droplets with final concentration 5 % v/v TRIS-HCl 1M, 0.05 % v/v SDS and 1 % proteinase K. The resulting emulsion was heated at 50°C for 1 h using the method described in 2.2.5. This heating was to achieve the optimal conditions for the proteinase K enzyme to operate. Note this contains half the concentration of SDS in the initial buffer. In Section 4.2.2 we saw 0.1 % v/v SDS caused damage to microsphere structure. We hypothesised that the SDS was the main cause of damage to microspheres, so tested whether reducing this still caused cell lysis. In Section 4.2.4, we see that this is a sufficient decrease to the SDS concentration to ensure microsphere survival. Figure 4.5 shows the resultant emulsion imaged using a DSred filter. The droplets are now homogeneously fluorescent, showing complete lysis of the cells as no fluorescent spherical structures are still visible. This lysis buffer is therefore shown to be successful at cell lysis.

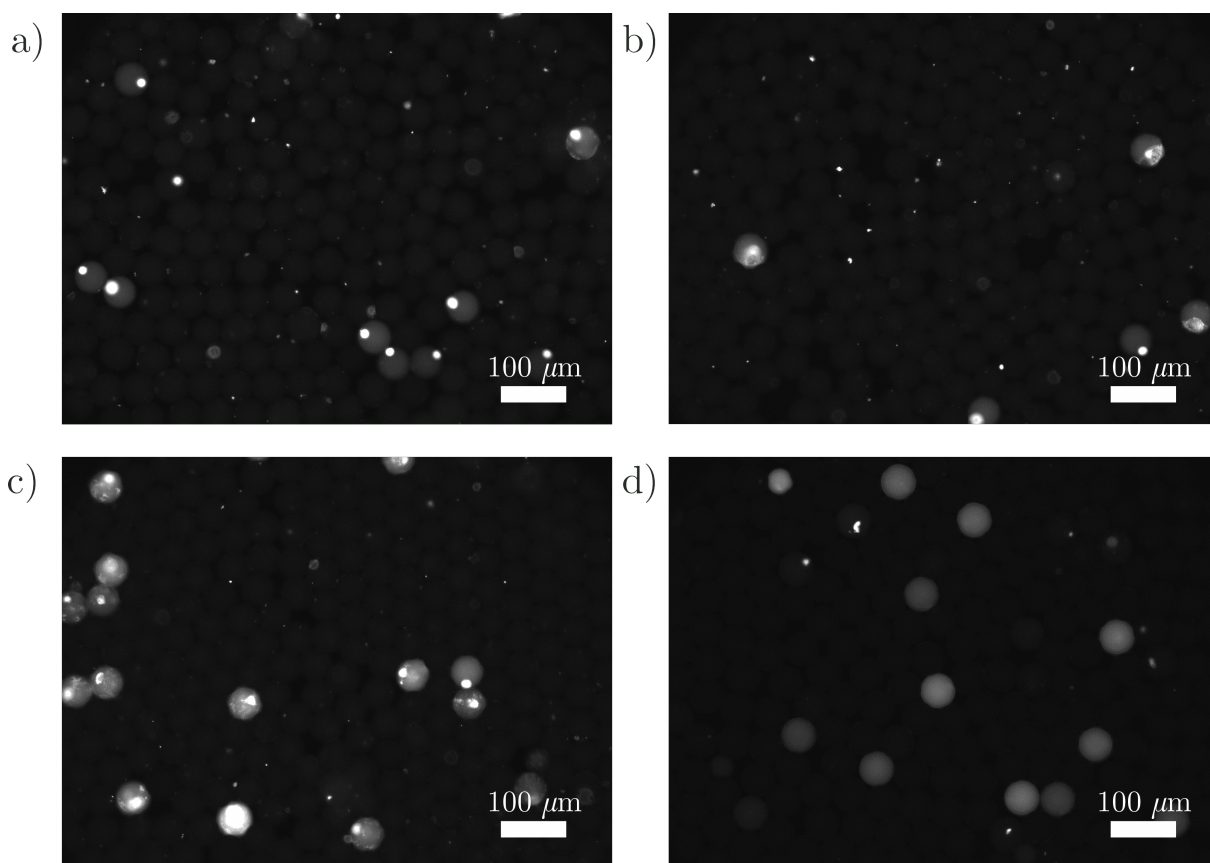


Figure 4.6: Droplets containing fluorescently stained Jurkat cells with final concentration a) 0.5 %, b) 5.0 %, c) 12.5 %, or d) 50 % v/v guanidinium thiocyanate.

To test the second lysis buffer, Jurkat cells stained with CellTracker™ CM-Dil fluorescent dye (Section 2.3.2) were encapsulated into droplets alongside four different final concentrations of guanidinium thiocyanate (0.5 %, 5.0 %, 12.5 % and 50 % v/v). The resulting emulsion was imaged using a DSred filter (Figure 4.6). Here Jurkat cells appear as bright spots with a diameter on the order of 10 μm . For the 0.5 % sample, the Jurkat cells appear intact, with some fluorescence seen in the rest of the droplet suggesting the contents of the cell have begun to no longer be confined within the cell membrane. As the concentration of guanidinium thiocyanate increases from 5.0 % to 12.5 %, the cell loses spherical structure, with more successful lysis occurring at the higher concentration. For the 50 % sample, no remaining cell structure can be seen, the droplets appear homogeneously fluorescent and complete cell lysis is believed to have occurred. However, complete cell lysis only happens at concentrations of guanidinium thiocyanate high enough to disrupt the cross-linking of alginate, so guanidinium thiocyanate was decided to be an unsuitable method of cell lysis within alginate microspheres.

4.2.4 Lysing Cells in Hydrogel Microspheres

We showed in Section 4.2.3 that a 5 % v/v TRIS-HCl 1M, 0.05 % v/v SDS and 1 % proteinase K chemical lysis buffer successfully lyses cells. The aims of this section were to show that reducing SDS concentration reduces damage to hydrogel microspheres and to use this buffer to produce suspensions of alginate and agarose microspheres which contain trapped genomic DNA. We chose not to further investigate lysis by heating, as chemical lysis techniques did not require further droplet stabilisation to counteract the lysis method.

Initially, Jurkat cells were fluorescently stained using the CellTracker™ CM-Dil dye and then encapsulated in droplets alongside a final concentration of 2.5 % alginate mix. These were cross-linked using acetic acid (as described in Section 3.1.2) and broken using perfluorooctanol (as described in Section 2.2.4). The resulting suspension was then mixed with a lysis buffer to give final concentrations of 5 % v/v TRIS-HCl 1M, 0.05 % v/v SDS. This suspension was heated at 50 °C for 1 h, and then imaged. Figure 4.7 shows images of the resultant suspension taken using phase-contrast imaging, and fluorescent microscopy using a DSred filter. Note these are not the same field of view. We see the spherical alginate microsphere structure is undamaged by this new lysis buffer, showing that decreasing the SDS concentration to 0.5 % is sufficient to ensure microsphere compatibility. However the Jurkat cells appear as small white circles in phase-contrast imaging, suggesting they remain intact in structure, despite these conditions lysing them in droplets. Imaging using a DSred filter confirms these are unlysed Jurkat cells. For comparison, Figure 4.7c shows a phase-contrast image of a suspension of 2.5 % alginate microspheres containing Jurkat cells that were not exposed to a lysis buffer. Here the Jurkat cells appear identical to those exposed to the lysis buffer, showing this lysis method is ineffective. The Jurkat cells also appear largely free from the alginate microspheres.

So far we have attempted cell lysis after cross-linking of the hydrogel. In Section 4.2.1, we proposed an alternative order of events, where the cells are lysed prior to cross-linking of the hydrogels. Jurkat cells were encapsulated in droplets with a final concentration of 5 % v/v TRIS-HCl 1M, 0.05 % v/v SDS and 1 % proteinase K chemical lysis buffer. The cells entered the device using a different inlet to the lysis buffer, so lysis should only occur post encapsulation, ensuring single cell resolution. This was done using the SE device (rather than the aaSE device), so no acetic acid was present at this stage, and no cross-linking occurred. After the droplets were heated at 50 °C for 1 h, the oil phase of the emulsion was replaced with 50 μ L 0.5 % AA in HFE and allowed to rest overnight for cross-linking. This emulsion was broken, and then stained with

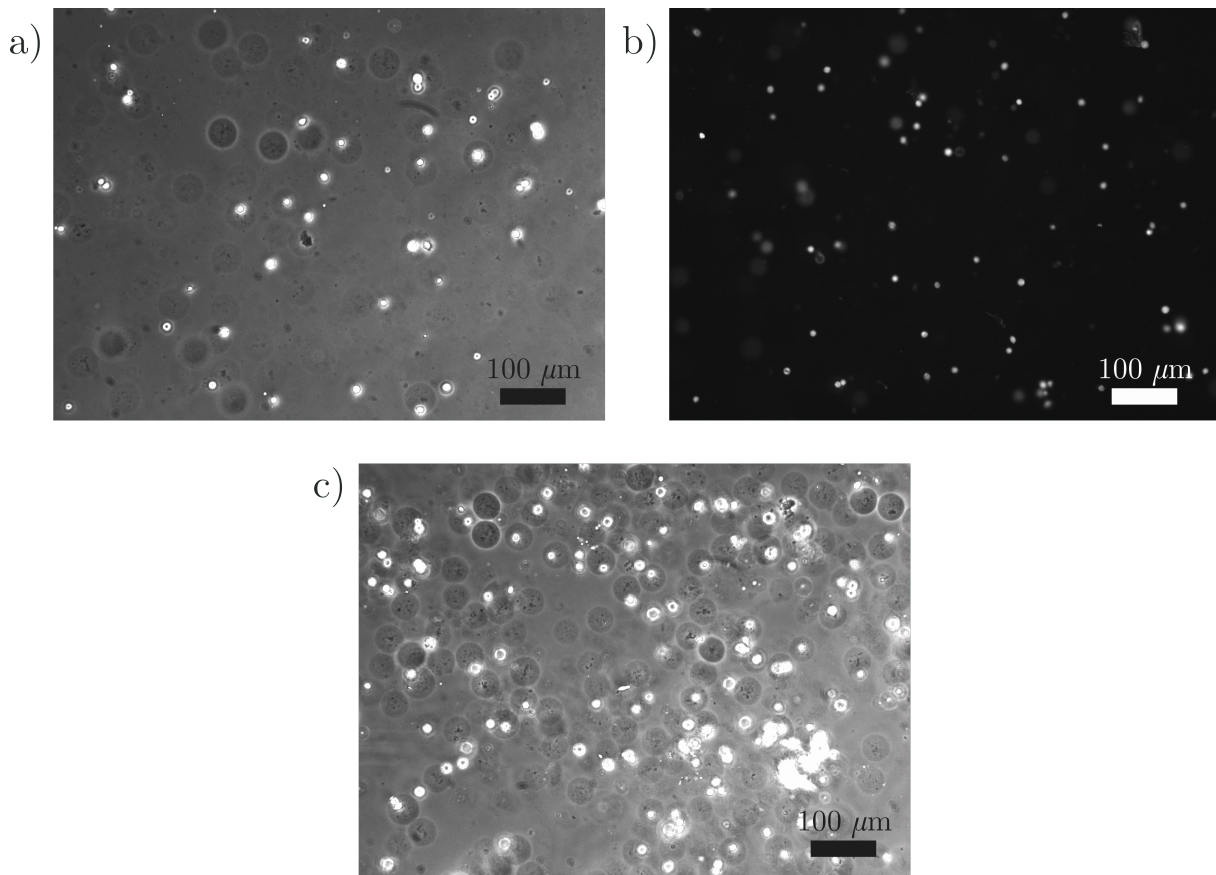


Figure 4.7: 2.5 % alginate microspheres containing Jurkat cells suspended in a 5 % v/v TRIS-HCl 1M, 0.05 % v/v SDS and 1 % proteinase K lysis buffer, imaged by a) phase-contrast microscopy and b) fluorescence microscopy. c) A phase-contrast image of 2.5 % alginate microspheres containing Jurkat cells with no lysis buffer present.

SYBR Gold to fluorescently label DNA (see Section 2.3.3). The resulting suspension was imaged with a FITC filter, and is shown in Figure 4.8. Here we see successful lysis of a Jurkat cell and the release of DNA, which is then trapped within a 2.5 % alginate microsphere. This is shown by localised fluorescence within a microsphere. The presence of dark microspheres show that this fluorescence is seen only for droplets which contained a cell, and that the stain does not bind to alginate chains and fluoresce. It also confirms that DNA does not diffuse into microspheres which did not initially contain a cell, therefore ensuring single-cell resolution. Upon decreasing the exposure of the left image to produce the rightmost, the DNA strands can be seen, potentially showing the pore structure of the hydrogel. This is investigated in more depth in Section 5.4.3. Finally, this order of lysis and subsequent cross-linking was repeated for agarose microspheres. Droplets were made using the SE device and tube heater (Section 3.1.1) containing a final concentration of 1 % w/w ULG agarose, and a lysis buffer of 5 % v/v TRIS-HCl 1M, 0.1 % v/v SDS and 1 % proteinase K. Note we have returned to a 0.1 % v/v SDS concentration as SDS does

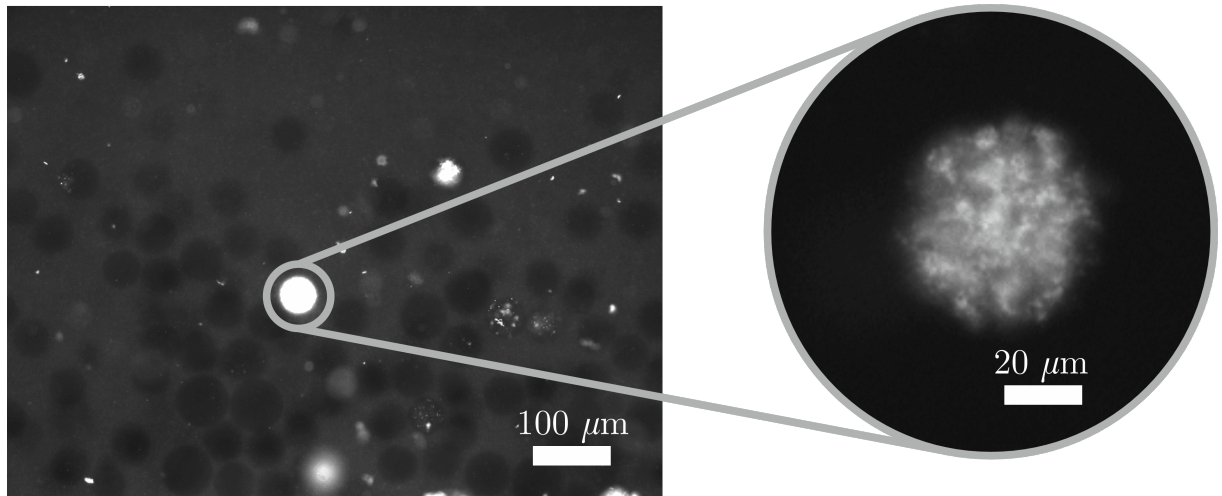


Figure 4.8: Successful cell lysis and subsequent cross-linking of alginate chains to produce fluorescently stained DNA trapped within 2.5 % alginate microspheres. The first image is over exposed to show the presence of "dark" microspheres. The second image is a zoomed in and unsaturated version of the first image.

not disrupt the physical cross-links in agarose in the same way SDS disrupts the chemical cross-links in alginate. This emulsion was heated at 50 °C for 2 h, allowed to cross-link overnight at 5 °C before being broken and stained with SYBR™ Gold. The resulting suspension was imaged using a FITC filter and is shown in Figure 4.9. As with the alginate sample, fluorescently stained DNA is seen localised within hydrogel microspheres, with the presence of dark microspheres showing this is not simply a staining of agarose. This suspension has significant differences in appearance compared to the alginate suspension however. While some agarose microspheres show the arrangement of DNA strands within the hydrogel matrix, others seem more homogeneously fluorescent. There are also long strands of DNA seen outside the microspheres, suggesting DNA is not completely trapped within the hydrogel matrix. The presence of dark microspheres again shows DNA is not diffusing back into agarose microspheres, showing single cell resolution is maintained.

Both hydrogels demonstrated the capacity to trap DNA originating from Jurkat cells, and we can therefore move onto developing the re-encapsulation techniques required to create an emulsion of these suitable for PCR. Comparing the efficiency of DNA trapping within agarose and alginate microspheres is investigated further in Chapter 5.

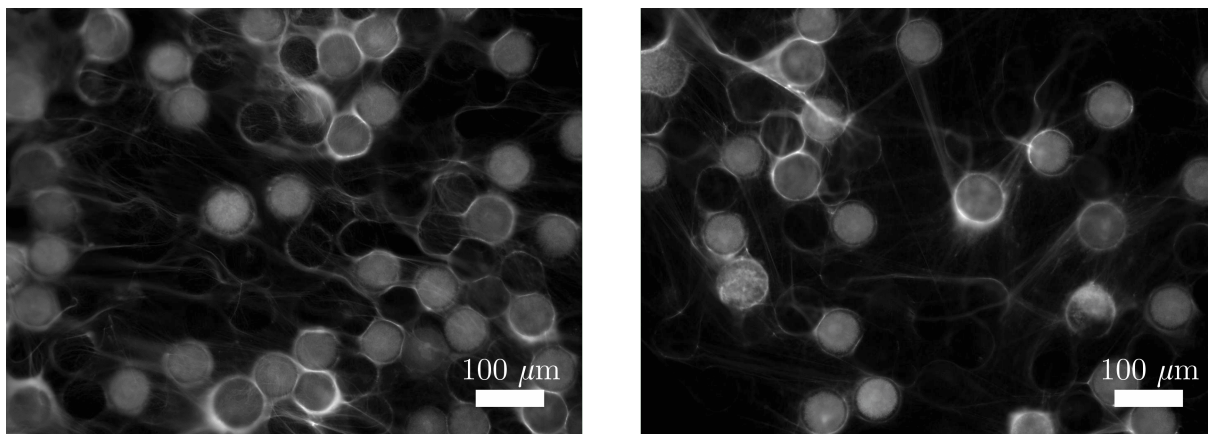


Figure 4.9: Successful cell lysis and subsequent cross-linking of agarose chains to produce fluorescently stained DNA trapped within 1 % w/w agarose microspheres. Two different fields of view are shown.

4.3 Re-encapsulation

Now that genomic DNA has been trapped within hydrogel microspheres, the next step of the proposed assay is to re-encapsulate these within new droplets for subsequent PCR. Using droplets for PCR allows the use of agarose, which would otherwise melt during thermal cycling, and ensures single-cell resolution over the timescale required for PCR. This section started by investigating the ability to trap fluorescent alginate microspheres inside new droplets using a re-encapsulation device. Then we combined this with the work shown in Section 4.2.4 to achieve successful re-encapsulation of microspheres containing trapped genomic DNA.

4.3.1 Re-encapsulation of Microspheres

The devices used in this thesis to re-encapsulate microspheres were designed by Dr Lucia Parolini. Three sizes of devices were then made, with varying channel widths and heights (heights shown in Table 2.1 in Chapter 2). They consisted of an entry channel for hydrogel microspheres surrounded by aqueous streams for PCR reagents. There is then the typical oil channel and a flow focusing junction for droplet production, and an outlet for the produced emulsion (Figure 4.10). The channel dimensions varied were the width of the microsphere input channel and the droplet output channel. A 35-50 device has a microsphere input channel of width 35 μm and a droplet output channel of width 50 μm . Three devices were made, with dimensions of 25-45, 35-50, and 40-55.

We first investigated whether microspheres can be re-encapsulated without causing any structural damage. We varied size of microspheres and the dimensions of the re-encapsulation device. The

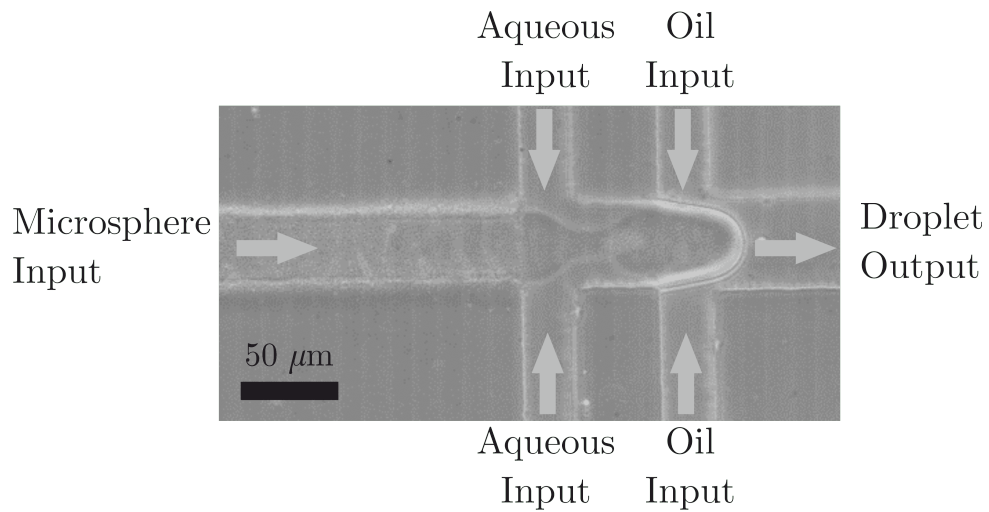


Figure 4.10: A 35-50 re-encapsulation device used to re-encapsulate hydrogel microspheres within droplets. Direction of flow, and contents of input and output channels are labelled.

size of microspheres was controlled using by varying the output channel width of the aaSE device used to initially produce microspheres. This output channel width is comparable to the diameter of the microspheres produced.

Emulsions of fluorescein tagged 1 % alginate microspheres were made as described in Chapter 3 using aaSE devices. Two different devices were used, with output channel widths of 20 μm and 30 μm . These were broken using PFO as described in Section 2.2.4. The subsequent suspensions of microspheres were then backfilled into tubing as described in 2.2.3, and flowed into the microsphere input on the re-encapsulation devices. The other aqueous input was used to flow deionised water. These were both pushed at $1 \mu\text{L min}^{-1}$. The continuous phase was made of 1 % EA in HFE, and flowed at around $7 \mu\text{L min}^{-1}$. Droplets were then collected and imaged using fluorescence microscopy using a FITC filter. This was repeated for the three different channel widths, 25-45, 35-50, and 40-55. The droplets imaged are shown in Figure 4.11.

The 20 μm microspheres re-encapsulated with little structural damage in all devices tested, appearing spherical as shown for un-re-encapsulated microspheres in Section 3.1.2. At the undiluted concentration of microspheres used there were multiple microspheres encapsulated per droplet. In the final proposed HIV reservoir assay, roughly one-in-ten microspheres will contain a cell, so the chance of a droplet having genetic material from more than one cell is low. This could be further reduced by dilution of the initial suspension. The frequency of a multiply occupied droplet increases with increased channel width. This would make sense as in the 35-50 device, two 20 μm microspheres can pack closely (they would only need to deform to reduce their dimensions in the direction of the channel width to 87.5 % to completely pack side

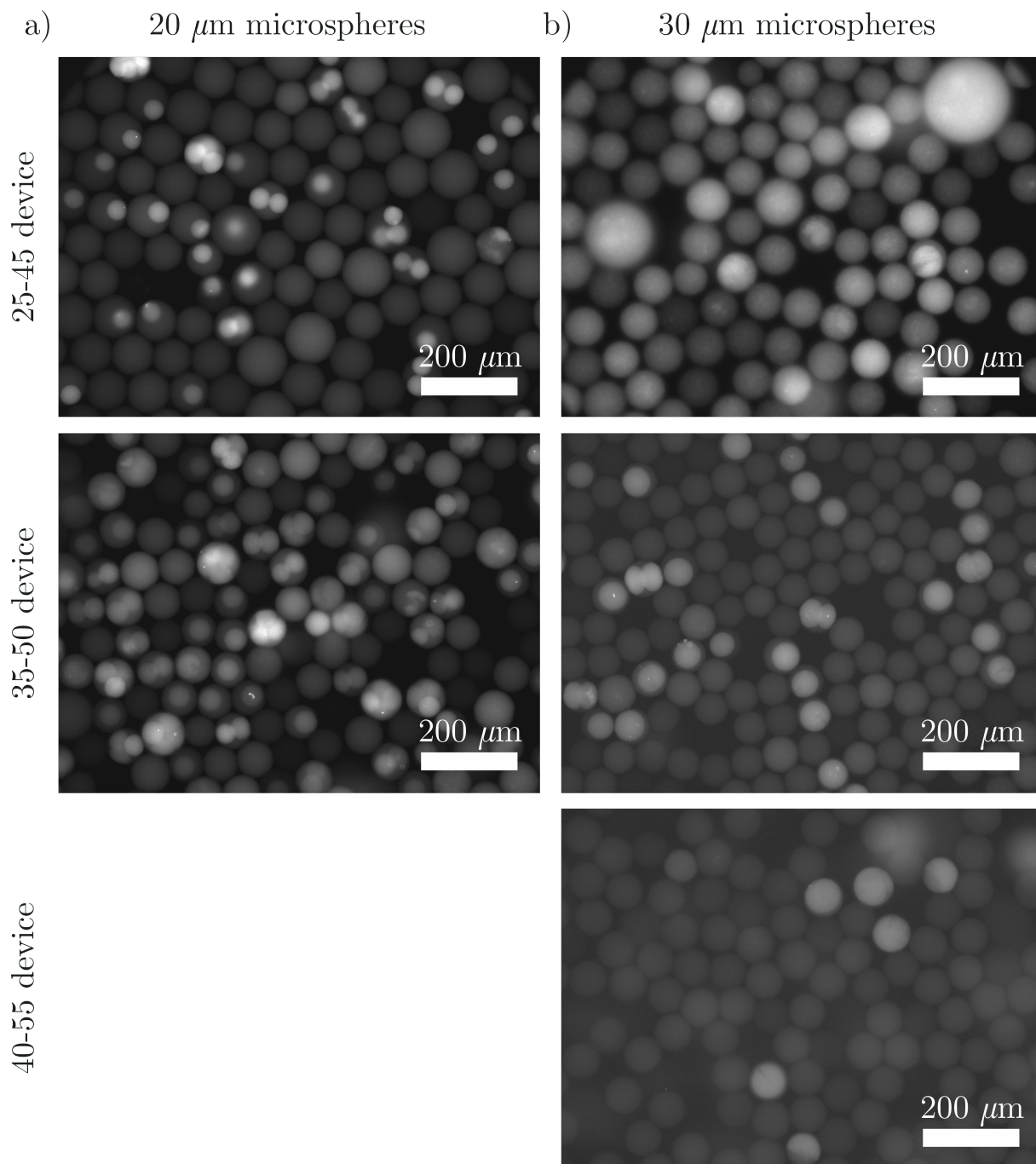


Figure 4.11: Fluorescent 1 % alginate microspheres re-encapsulated within droplets. Two aaSE devices of different channel widths were used to produce microspheres of 20 μm or 30 μm diameter, and these were encapsulated in new droplets using 25-45, 35-50 or 40-55 re-encapsulation devices.

by side) and enter the same droplet. As the channel width increases, more microspheres are able to pack perpendicular to the direction of flow. Now the chance of multiple microspheres passing the flow focusing junction within the time frame of a single droplet creation increases, increasing the chance of multiple occupancy within a droplet.

The 30 μm microspheres however sustained structural damage depending on the device used. As expected, forcing microspheres of 30 μm diameter through a channel of 25 μm in width resulted in the microspheres often partially or completely rupturing into two half spheres. Using the 35-50 device reduced this, but resulted in occasional multiple occupancy of droplets (though not at as high a frequency as the 25-45 device). Interestingly, the rupturing of spherical microspheres returned upon use of the 40-55 channel. This is consistent with experiments in which centrifuging alginate microspheres causes structural damage when diluted, but not when centrifuged undiluted (i.e. when close packed). We speculate that when the microspheres have an "excess volume" in which they can move and bump into each other they sustain damage, but when confined in a smaller volume and are close-packed they do not. Note that some droplets seem homogeneously bright, these could result from complete destruction of microsphere structure, or from the droplets forming being of the same size as the microspheres.

Complete destruction of microspheres was observed to result from the channel being blocked by debris. The constant flow syringe pumps would continue to push volume through the blockage, rupturing microspheres and causing the loss of spherical structure. To prevent this, beads were washed as described in Section 3.3.3, and the droplet size was controlled to be larger than that of the original beads by increasing the flow rate of the aqueous phases. This produced the emulsion imaged in Figure 4.12. Note the microspheres were overexposed in order to be able to see the droplets, which were otherwise too poorly contrasted with the background. This large contrast between microspheres and droplets allows us to clearly show spherical microspheres encapsulated in larger droplets. This assures us that the fluorescence signal is not simply a result of damaged microspheres producing fluorescently stained non-cross-linked alginate chains, which would produce a homogeneously fluorescent droplet.

To reduce structural damage to the microspheres and to reduce multiple occupancy of droplets, this thesis proceeded by encapsulating 30 μm hydrogel microspheres using the 35-50 device.

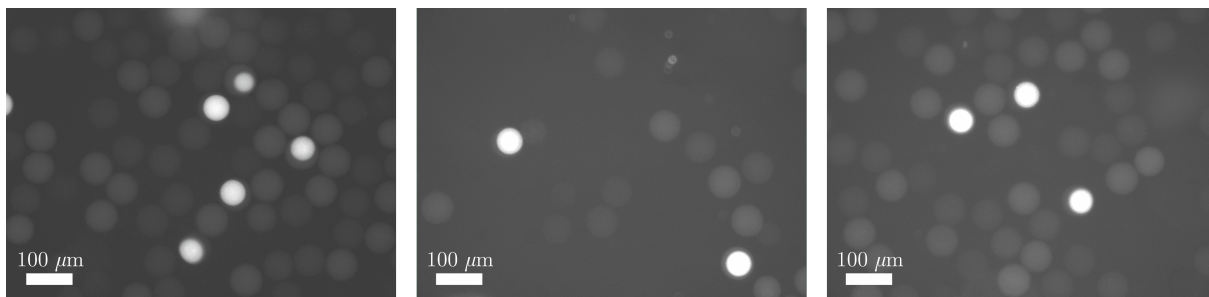


Figure 4.12: Washed fluorescent 2.5 % alginate hydrogel microspheres were re-encapsulated within droplets. Three different fields of view are shown.

4.3.2 Re-encapsulation of Trapped Nuclear DNA

We have shown that hydrogel microspheres can be successfully re-encapsulated within droplets. Now it is important to test whether genomic DNA remains trapped in the microspheres during this process.

Jurkat cells were encapsulated into droplets containing 1 % w/w agarose or 2.5 % alginate and their relevant lysis buffers developed in Section 4.2.4. These were left at room temperature for either 2 h (agarose) or 1 h (alginate) respectively to allow lysis to occur. Cross-linking was then induced by either leaving the emulsion at 5 °C overnight (agarose) or replacing the continuous phase of the emulsion with 0.5 % AA in HFE and leaving overnight (alginate). The emulsions were then broken as in Section 2.2.4. The resulting suspensions were washed as described in Section 3.3.3. Note that the washing of agarose microspheres took 5 min whereas the washing of alginate microspheres took 2 h as described in Section 3.3.3. The washed suspensions were then stained with fluorescent intercalating SYBRTMGold dye (Section 2.3.3) and re-encapsulated using the 35-50 device as shown in Section 4.3.1. The resulting emulsions were imaged and are shown in Figure 4.13. Here we display three fields of view of each sample to show a representative frequency of fluorescent droplets. We see approximately one fluorescent droplet per view of view which roughly corresponds with the frequency of positive microspheres shown in Figure 4.9. There is a slight decrease, which would be expected as not all droplets contain a microsphere. Both agarose and alginate re-encapsulated within droplets appear as they did prior to re-encapsulation (as shown in Figures 4.8 and 4.9). As before, the alginate microspheres show retention of fluorescently stained DNA, highlighting the arrangement of DNA strands within a microsphere. The agarose microspheres again show some retention of DNA when compared to the dark population of droplets (which represent the encapsulation of the continuous phase). If all the DNA was not trapped within a microsphere then all the droplets would be expected to be homogeneously

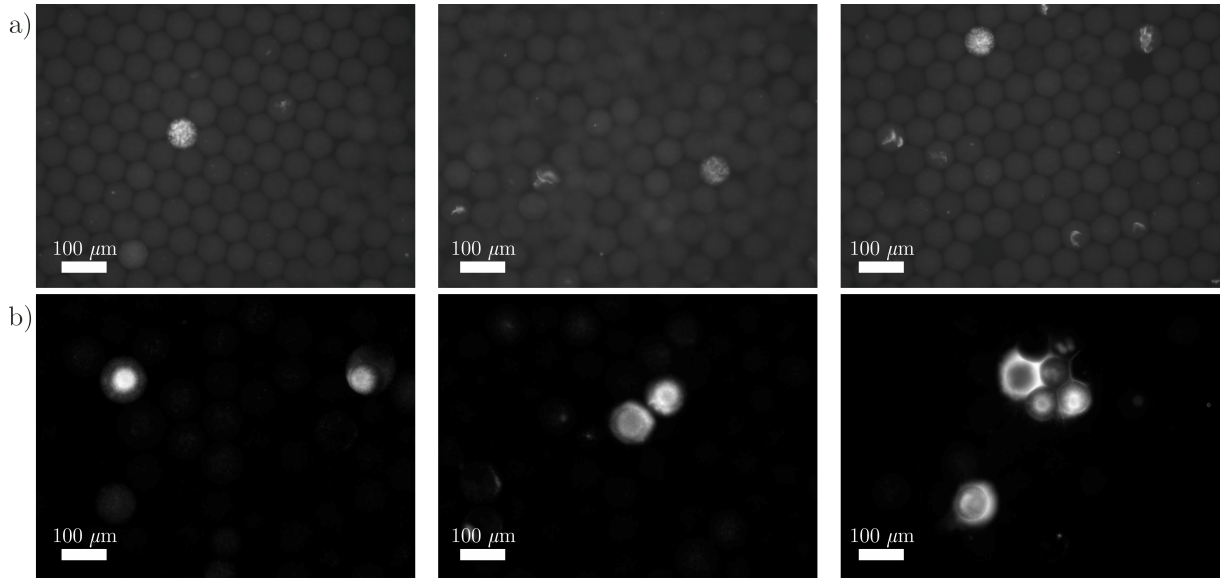


Figure 4.13: Re-encapsulation of DNA trapped in a) 2.5 % alginate and b) 1% w/w agarose microspheres. Three different fields of view are shown for each type of microsphere.

fluorescent. Instead we see encapsulation of microspheres containing some DNA, though not as clearly trapped as in the alginate case. By eye, it appears that alginate microspheres trap a higher proportion of genomic DNA than agarose. We use quantifiable methods to investigate the efficiency of alginate and agarose microspheres to trap DNA in Chapter 5.

4.4 PCR

DNA has been trapped within hydrogel microspheres, and a method has been shown to re-encapsulate these within new droplets. This section details the method development to use PCR to detect droplets containing a particular target sequence of DNA. Section 4.4.1 describes the general theory behind PCR, and Section 4.4.2 details the specific reagents and thermal cycling methods used in this thesis. We then investigated the PCR efficiency within agarose and alginate networks in Sections 4.4.3 and 4.4.4 respectively.

4.4.1 Theory

Polymerase chain reaction (PCR) is a DNA analysis and replication technique. In general terms, PCR detects a particular target sequence of DNA and replicates it, amplifying or concentrating this DNA sequence. This method can be coupled with a probe which fluoresces upon successful replication, resulting in a quantitative measure of reaction progress, or DNA concentration (based on fluorescence intensity as a function of time) [149]. This is referred to as qPCR (quantitative

PCR).

There are several key components in a PCR reaction [149]:

Template DNA - This is the double-stranded DNA sample containing the target sequence.

Primers - Two or more single-stranded oligonucleotide sequences complementary to sequences either side of the DNA target. One binds to the "top" DNA strand and the other to the "bottom" strand, in order to copy both strands of the DNA. The two primers are often referred to as forwards (F) and reverse (R) primers.

dNTPs - Deoxynucleotide triphosphates (the structural unit of base, sugar and the tri-phosphate group) are the molecular precursors to DNA and RNA. There are 4 types of dNTPs required for PCR, corresponding to the 4 bases in DNA.

DNA Polymerase - An enzyme which is responsible for making a DNA copy of a sequence. PCR often uses the thermostable Taq polymerase, with a optimal activity at around 72 °C [228].

Reporter Molecules - Fluorescent materials used to quantify how successful PCR is. There are many kinds of probes able to be used for this. Non-specific DNA dyes such as SYBR [®]Green fluoresce upon binding to any dsDNA. By dyeing DNA samples of known concentration, qPCR then shows the exponential increase of DNA over time [229]. There are also many fluorescent primer- or probe-based detection systems. These typically are sensitive to a particular target sequence, so only fluoresce on presence of amplified target sequence (often called an amplicon - a DNA strand or sequence that is the source or product of PCR).

PCR consists of three main steps [149]:

Denaturing - The template DNA is heated (95 °C) to break the hydrogen bonds holding the two strands together. This allows the primers to then conjugate to the now single-stranded DNA by forming new hydrogen bonds.

Annealing - The temperature is reduced (to 40 - 60 °C) so the primers can form new hydrogen bonds to complementary DNA sequences.

Polymerisation - The temperature is again raised, this time to the optimal activity point of the DNA polymerase used, but not so high as to cause the primers to detach from the DNA target sequence (usually around 70 °C). The polymerase synthesises new DNA strands complementary to the target.

Repetition - These three temperature stages are then repeated 30-40 times, a process known as thermocycling. This generates an exponential growth in concentration of the target sequence as each new strand produced can act as a subsequent source for successive rounds of PCR.

4.4.2 PCR Reagents and Methods

This thesis uses hydrolysis probes. These take advantage of the directional 5' to 3' activity of specific polymerases. They consist of a complementary sequence to a part of the DNA target between the position of the two primers. The 5' end has a fluorescent source and the 3' end a quencher. During annealing, the probe binds to the target ssDNA. As the fluorescent source and quencher are close in proximity, fluorescence is not seen. During polymerisation, the polymerase enzyme cleaves the source from the quencher, separating them and allowing the fluorescent signal to be detected [230].

The albumin primers used in this thesis were F: 5'-GCT GTCATC TCT TGT GGG CTG T-3' and R: 5'-AAA CTC ATG GGA GCT GCT GGT T-3'. The primers were obtained from Eurofins Genomics. A double-quenched probe was used to increase the signal to noise ratio of fluorescence [231]. The probe was obtained from Integrated DNA Technologies TM and designed by Dr Mathew Jones (previous member of the Frater group) with a structure of 5'/56-FAM/CCT GTC ATG/ZEN/CCC ACA CAA ATC TCT CC/3IABkFQ/-3'. Here FAM is the fluorescein based fluorescence reporter, and ZEN and 3IABkFQ represent the ZEN and 3' Iowa Black® FQ quenchers respectively. As this probe is based on fluorescein, images taken with the intention of showing successful PCR amplification use a FITC filter. These were made to a 10 μ M working stock using DNase and RNase-free water from initial 100 μ M solutions. Final concentrations of the PCR reaction mixture were 0.4 μ M for the primers and 0.2 μ M for the double-quenched probe.

All other reagents required for PCR are contained within a LightCycler® 480 Probes Master, including FastStart Taq DNA Polymerase, which is designed for use with hydrolysis probes. This was diluted from an initial 2x concentration to a 0.8x concentration in the PCR mix.

For comparison of PCR efficiency without lysing new cells for every experiment, 8e5 derived DNA extracted by Daniel Coneyworth (Frater group, University of Oxford) was used as a positive control. 8e5 is a human cell line of HIV-1 infected CEM cells [232]. Where this DNA was used in qPCR experiments, the final concentration was 2.6 ng μ L⁻¹. In all qPCR experiments, for comparison purposes, a positive control containing this DNA and PCR reaction mix, and a negative control of PCR reaction mixture only were tested.

Unless otherwise stated, the thermocycling procedure for all future PCR experiments was 10 min at 95 °C for denaturing, then 45 cycles of 15 s at 95 °C and 1 min at 60 °C. When run on bulk samples for qPCR, the heated lid was set at 105 °C. qPCR experiments were run on a

QuantStudio™3 Real-Time PCR System from Applied Biosystems, Thermo Fisher Scientific. Here the measurements of fluorescence signal were taken during every 60°C annealing step. qPCR data was collected using Thermo Fisher Data Connect and analysed in Mathematica. Thermocycling of droplets used Igepal and mineral oil as described in Section 2.2.5 to reduce droplet coalescence upon heating. A MJ Research PTC-200 Thermal Cycler was used for droplet thermocycling.

4.4.3 PCR in Agarose Hydrogel Matrices

This section first shows attempted bulk qPCR of a suspension of many agarose microspheres which contain trapped DNA from lysed Jurkat cells. This is required to determine whether the agarose matrix inhibits PCR. We then adapted this method for in droplet PCR, detecting target DNA presence within a single microsphere. The aim here is to develop a method showing a clear difference in fluorescence intensity between droplets which contain the target DNA and those which do not, allowing subsequent fluorescent activated droplet sorting.

The ultra low gelling agarose used in this thesis was shown to transition to a sol-phase at 60°C (see the preparation of agarose microspheres in Section 3.1.1). As the PCR method used in this thesis heats the sample to 95°C, the structure of the hydrogel network should not inhibit the ability of PCR reagents to arrange themselves in the correct orientation for PCR.

Initially, qPCR was attempted on a suspension of 1 % w/w agarose microspheres containing trapped DNA. These were produced as in Section 4.2.4, via the encapsulation of Jurkat cells in sol-phase agarose, followed by cell lysis and subsequent cross-linking of the agarose chains. These were washed by a factor of ten dilution in water, and centrifugation as described in Section 3.3.3. Then PCR reaction mixtures were made to a final concentration of 6.4 % v/v of the sedimented microspheres per 25 µL reaction mixture. This produces an DNA concentration of sample estimated to be the same as the positive control. The qPCR data showing fluorescence signal measured every cycle is shown in Figure 4.14. Successful amplification for the positive control is shown by an increase of fluorescence signal over time, and no amplification is seen in the negative control as fluorescence signal remains constant throughout. The suspension of agarose microspheres does show an increase in fluorescence signal, indicating successful PCR. As the signal rises later than the positive control, this suggests PCR efficiency is reduced by the presence of agarose.

To then test this in droplets, 1 % w/w agarose microspheres trapping DNA lysed from Jurkat

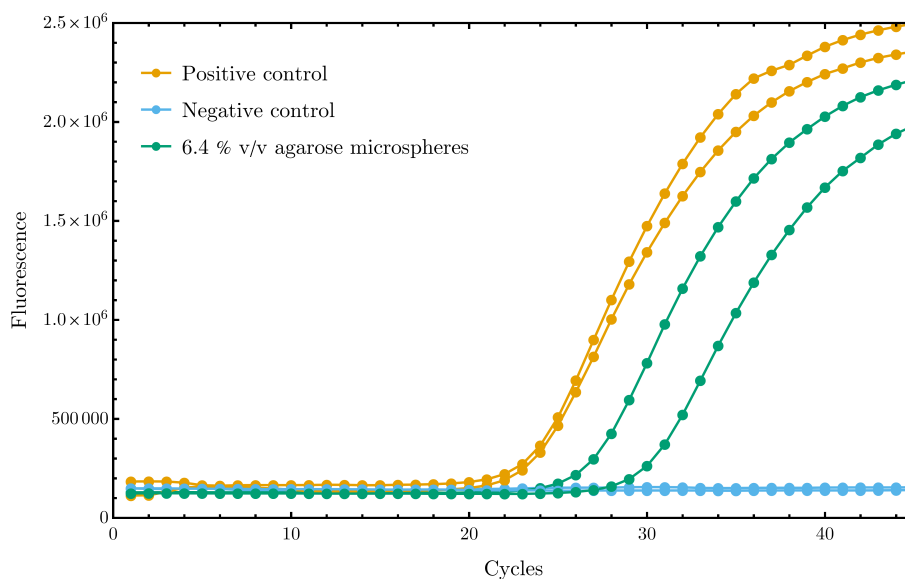


Figure 4.14: qPCR data showing the fluorescence signal as a function of time (number of PCR thermal cycles). A suspension of 1 % w/w agarose microspheres was produced containing DNA lysed from within a Jurkat cell. These were washed, and added to PCR mixtures at a final concentration of 6.4 % v/v. Controls included a positive of DNA of known concentration, and a negative containing no microspheres or added DNA.

cells were washed and encapsulated in new droplets also containing the above described PCR reaction mix and Igepal, as described in Section 4.3.2. The droplets were thermocycled using the droplet heating stabilisation methods described in Section 2.2.5, and the thermal cycling method described in 4.4.2. The resulting emulsion was imaged using a FITC filter, and these images are shown in Figure 4.15. Again we show three different fields of view of the same sample, here to illustrate the observation of multiple successful compartmentalised PCR reactions. A clear separation of two populations is shown with successful PCR is shown in bright droplets, and no PCR in dark droplets. Note that droplet coalescence has once again become a problem here. Repeated attempts of re-encapsulating microspheres with PCR reagents resulted in a high polydispersity in droplet size. Using mineral oil and Igepal to improve the thermal stabilisation of droplets resulted in a significant increase in monodispersity when only encapsulating lambda DNA. However, any encapsulation of hydrogel microspheres alongside PCR reagents resulted in a large polydispersity of droplet size, even without any DNA present. This is certainly an issue that will need further work before a droplet sorting method can be developed, but we have shown that PCR of DNA trapped within agarose microspheres is possible.

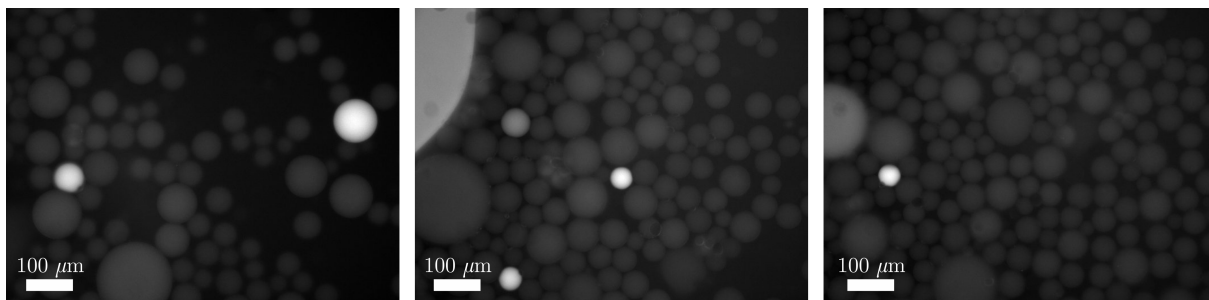


Figure 4.15: Fluorescent images using a FITC filter showing an emulsion of water-in-oil droplets encapsulating 1 % w/w agarose microspheres with trapped genomic DNA, and the PCR reaction mixture. The droplets underwent thermal cycling and then were imaged. Droplets in which successful PCR occurred are significantly more fluorescent than the others. Three fields of view are shown.

4.4.4 PCR in Alginate Hydrogel Matrices

As in Section 4.4.3, this section first shows qPCR results for suspensions of alginate microspheres containing trapped DNA to determine whether alginate matrices inhibit PCR and whether this can be overcome. This work is then replicated within droplets at a single cell resolution.

PCR in alginate networks is a more complicated system. PCR relies on hydrogen bonding and other charge based interactions, so using a hydrogel that also uses charge-based interactions to cross-link could interfere with PCR. While some literature shows successful PCR in alginate networks with no modifications [209], others required the use of potassium chloride or sodium chloride salts as charge screening agents [233]. Li et al used a 7 mmol dm^{-3} potassium chloride and 75 mmol dm^{-3} sodium chloride concentration to overcome alginate inhibition of PCR [233].

Initially qPCR of 2.5 % alginate microspheres containing genomic DNA lysed from Jurkat cells was attempted. A suspension of these was produced as described in Section 4.2.4, then diluted by a factor of ten. After 2 hours, the sediment was removed and added to the PCR reaction mix, and underwent thermal cycling. Two concentrations of microsphere sediment were tested, 50 % v/v and 6.4 % v/v. Figure 4.16a shows successful amplification only occurred for the positive control, and not for any sample containing alginate. To remedy this, the salt buffer proposed by Li et al [233] was tested. A 1x salt concentration here refers to the concentrations used by Li et al, 7 mmol dm^{-3} potassium chloride and 75 mmol dm^{-3} sodium chloride. Three mixtures containing final concentrations of 2.0 % AlgEDTA and 1x salt, 1.6 % AlgEDTA and 2x salt, and 0.2 % AlgEDTA and 1x salt were trialled. All used the same DNA concentration as the standard positive control, $2.6 \text{ ng } \mu\text{L}^{-1}$ of 8e5 extracted DNA. Figure 4.16b shows the fluorescence

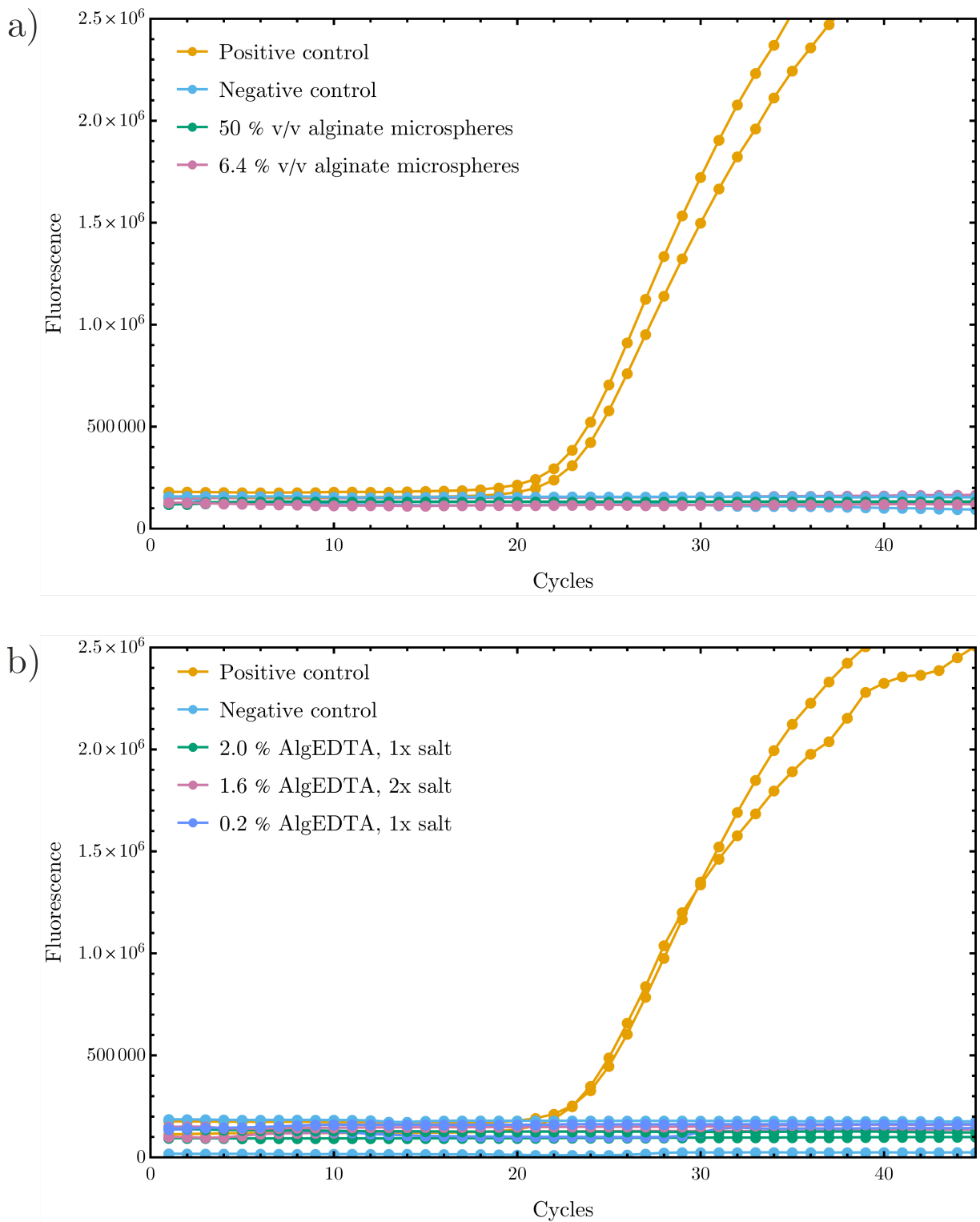


Figure 4.16: qPCR data showing the fluorescence signal as a function of time (number of PCR thermal cycles). a) A suspension of 2.5 % alginate microspheres was produced containing DNA lysed from within a Jurkat cell. These were washed, and added to PCR mixtures at a final concentration of 6.4 % v/v. Controls included a positive of DNA of known concentration and a negative containing no microspheres or added DNA. b) Bulk AlgEDTA mixtures of varying final concentrations were added to salt buffers. A 1x salt concentration represents 7 mmol dm^{-3} potassium chloride and 75 mmol dm^{-3} sodium chloride. All concentrations listed are final concentration of reaction mixture.

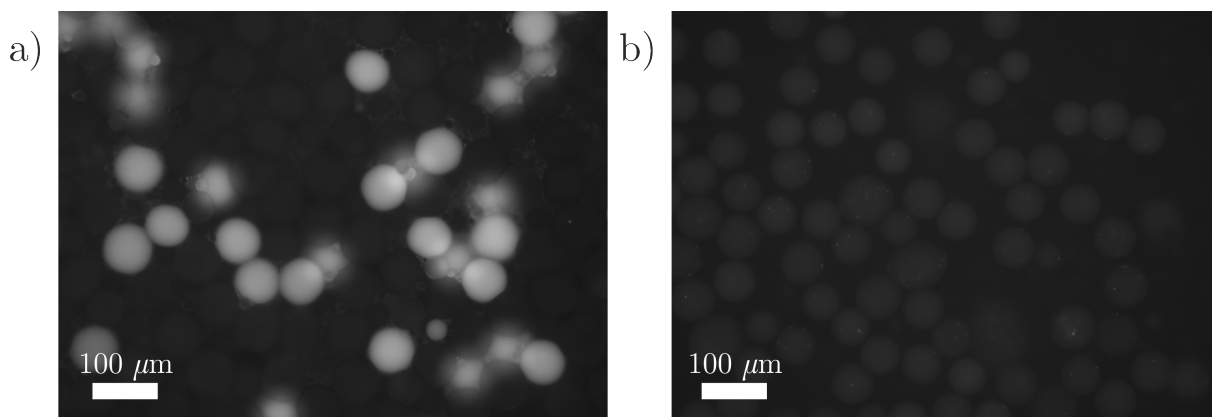


Figure 4.17: Fluorescence images using a FITC filter of W/O droplets containing $2.6 \text{ ng } \mu\text{L}^{-1}$ of 8e5 extracted DNA and PCR reaction mix, where the continuous phase was replaced with 0.5 % AA in HFE after droplet formation and allowed to stand overnight. The droplets shown contained either a) no AlgEDTA mix or b) final concentration of 2.5 % AlgEDTA mix.

signals as function of time. Again only the positive control showed successful amplification. Here as the alginate has not been cross-linked, we hypothesise it is not the hydrogel structure that is inhibiting PCR, but rather the alginate chains themselves. While calcium ions are known to inhibit PCR, this is reversed by addition of chelating ligands such as EGTA (ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid) used at the same concentration as the calcium [234]. As our system uses a chelated calcium EDTA ion (as evidenced by the lack of cross-linking without acetic acid presence), this inhibition is not the cause here.

To check whether the acetic acid was the cause of PCR inhibition, an emulsion was made of droplets encapsulating PCR reaction mix and $2.6 \text{ ng } \mu\text{L}^{-1}$ of 8e5 extracted DNA. Another was made encapsulating PCR reaction mix and $2.6 \text{ ng } \mu\text{L}^{-1}$ of 8e5 extracted DNA and a final concentration of 2.5 % AlgEDTA. The continuous phase of both emulsions was replaced with 0.5 % AA in HFE after droplet formation had finished, and allowed to stand overnight. Both emulsions were thermocycled (using droplet heating techniques described in Section 2.2.5) and imaged with fluorescence microscopy (Figure 4.17). Fluorescent droplets showing successful PCR amplification are only present when the AlgEDTA mix is not, showing inhibition is caused by the alginate hydrogel, and not the acetic acid presence.

Ultimately we did not achieve PCR within alginate microspheres. However, literature suggests it may be possible with fine tuning of salt concentrations within the reaction buffer. While this currently rules out alginate microspheres for use with this specific assay, there are still other applications for their use as micro-sieves.

4.5 Chapter Summary

In Section 4.2, we successfully adapted a chemical lysis buffer to release genomic DNA without disrupting the structure of the hydrogel microspheres. We used this to produce both alginate and agarose microspheres which trap genomic DNA. In Section 4.3, we showed a method for the re-encapsulation of microspheres which minimises structural damage. We then used this method to show the re-encapsulation of microspheres that trap genomic DNA, showing the retention of DNA trapping during this process.

Finally, in Section 4.4, we successfully achieved PCR amplification and detection of genomic DNA within agarose microspheres. We also showed the difficulties in achieving successful PCR within alginate microspheres, despite literature showing this is possible [233]. It is possible that EDTA acts a chelating ligand of the divalent magnesium ions essential for PCR, causing PCR inhibition. Alternatively, competition between magnesium ions and un-crosslinked calcium ions for interaction with polymerases could be a source of inhibition. We hypothesis that fine tuning of the competitive binding between chelating ligands and cations could optimise this system for successful PCR as in the literature [233], and suggest future work should be directed around this. Adding to the difficulty of this fine tuning is the swelling of the microspheres shown in Section 3.3.4. This swelling could cause dilution of the buffer within the microspheres, making the precise buffer compositions unclear.

Some recommendations for future work include titrations to determine buffer composition or the magnesium concentration after dilution effects. We could mix the PCR positive controls with bulk lysates to check for inhibition. Alternative cross-linking agents other than calcium could be investigated to see if any exist which don't disrupt polymerase activity.

Despite the difficulties in achieving PCR within alginate microspheres, this Chapter shows a robust system for the trapping of DNA from individual cells, in combination with techniques for cell lysis and re-encapsulation of DNA-containing-microspheres. There are two key next steps for future research to be successful. Firstly, the droplets post-PCR need to be successfully sorted based on fluorescence (using a cell sorter) into a fluorescent population to then be sequenced, and a non-fluorescent population to be discarded. Secondly, this needs to be replicated using HIV infected T cells, to show the successful collection of the rare infected population of T cells. Chapter 5, explores quantifying this discovered ability of agarose and alginate microspheres to trap genomic DNA.

Chapter 5

The Trapping Ability of Microspheres

5.1 Introduction

In order for hydrogel microspheres to act as size based filters for compartmentalised bioassays, it is important to understand how well these microspheres can trap different biological material. The HIV latent reservoir assay developed in Chapter 4 relies on DNA being retained within the microspheres while cell lysates and excess lysis reagents can be washed out. It is also necessary for the DNA to remain trapped for a long enough timescale to complete this washing and then re-encapsulate the microspheres within new water-in-oil droplets alongside PCR reagents. This highlights the two main considerations that characterise the trapping ability of microspheres: the size of the material to be trapped, and the timescale for which the material is trapped.

This Chapter aims to quantify the ability of agarose and alginate microspheres to trap colloidal particles, cells and genomic material. We start in Section 5.2 by investigating the effect of alginate concentration, microsphere size and colloidal particle size on the trapping ability of alginate microspheres. We use polystyrene particles as a model for cells, and compare these with the trapping of Jurkat cells. In Section 5.3 we stain DNA and RNA with fluorescent dyes to study the kinetics of diffusion from within a microsphere. We vary the compositions of hydrogel microspheres and DNA length to determine trapping ability, quantifying this using fluorescent intensity measurements. Finally, in Section 5.4 we use fluorescence and confocal imaging of DNA trapped within a hydrogel microsphere to investigate the pore structure of hydrogel matrices.

5.2 Trapping Particles and Cells

One proposed assay discussed in Chapter 4 involved the trapping of T cells within hydrogel microspheres, which could then be washed with a lysis buffer to release the nuclear DNA. In order for this assay to be successful, cells would need to be trapped within hydrogel microspheres, and not diffuse out. This section uses polystyrene colloidal particles and Jurkat cells (Section 4.1.3) as models for T cells. We test how alginate concentration, particle size and microsphere size affect the trapping of microparticles.

5.2.1 Trapping Colloidal Microparticles

Jurkat cells were observed during experiments detailed in Chapter 4 to be polydisperse in size, having diameters of around 8 - 15 μm (consistent with that observed in literature [235, 236, 237]). Therefore, in order to investigate the effect of particle size on trapping within microspheres, monodisperse polystyrene particles were used as a model system. Three suspensions of differently sized polystyrene microparticles were obtained from Sigma, with mean diameters of 1.1 μm , 5 μm and 10 μm . They had a density of 1.05 g cm^{-3} and a concentration of 10 % w/w. Initial samples were stored at 5 °C and thoroughly mixed before use.

Polystyrene particles of a final 0.5 % v/v concentration were encapsulated into water-in-oil droplets alongside a final 1.0 % AlgEDTA mix using the protocol described in Section 2.2.3. Cross-linking of alginate chains was then induced using acetic acid as in Section 3.1.2, and the emulsion was left to rest overnight. The emulsion was then broken as described in Section 2.2.4. When these were imaged using bright-field microscopy (Figure 5.1), it was difficult to tell whether the microparticles were trapped within the microsphere due to the microspheres having poor refractive index contrast with the continuous phase. Instead, in order to observe relative motion between microparticles and microspheres, the resulting suspensions were flowed through a 35-45 re-encapsulation device. By recording the motion of microsphere, and slowing down the frame rate, it became possible to observe successful trapping. Section 4.3 described the 35-50 re-encapsulation device, and the method used to flow microspheres within it. Successful trapping here was defined as observing more polystyrene particles moving with an alginate microsphere rather than passing through the device separate from an alginate microsphere. Note that even if the polystyrene microparticles were well trapped, there was still the potential for free microparticles to appear. If the droplet initially created to form the alginate microspheres was the subject of local fluctuations in flow rates, it is possible a droplet would form containing

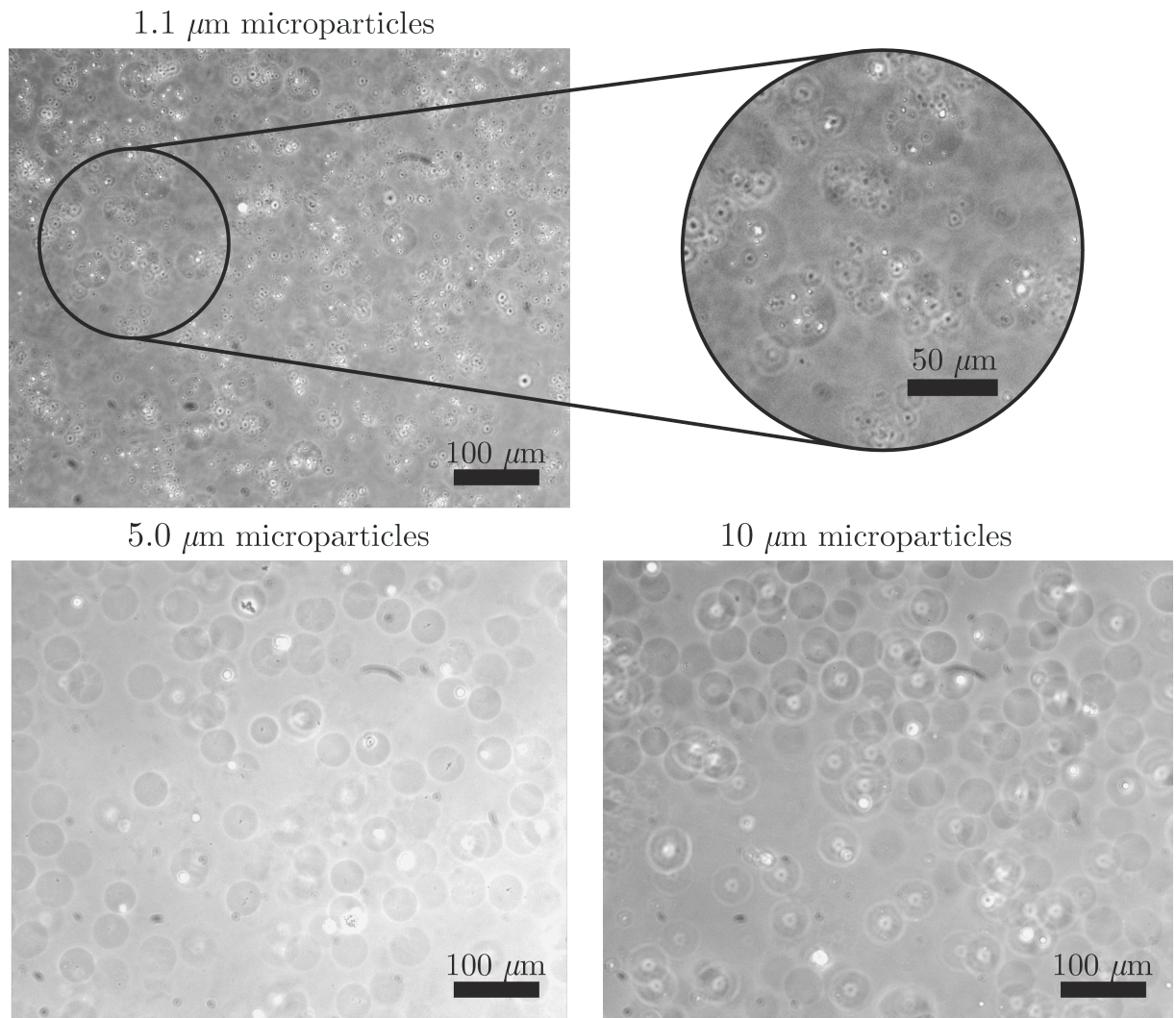


Figure 5.1: Phase-contrast images of 1.0 % alginate microspheres containing polystyrene microparticles with diameters of 1.1 μm , 5 μm and 10 μm . For the suspension containing 1.1 μm microparticles a zoomed in section of the suspension is shown.

only the aqueous inlet containing the microparticles, and not the sol-phase alginate. When the emulsion was broken, these microparticles would then appear not trapped, even though they were never trapped to begin with.

Figure 5.2 shows 1.0 % alginate microspheres originally containing polystyrene microparticles with diameters of 1.1 μm , 5 μm and 10 μm flowing through a 35-50 re-encapsulation microfluidic device. For each sample, three images taken successively with respect to time show the movement of a microsphere through the device. To count a set of conditions as successful trapping, 15-20 microspheres containing microparticles were observed. The presence of a microsphere is most obviously shown where the interface between the two aqueous inputs deforms to allow the microsphere through. The polystyrene microparticles are clearly seen due to a larger refractive

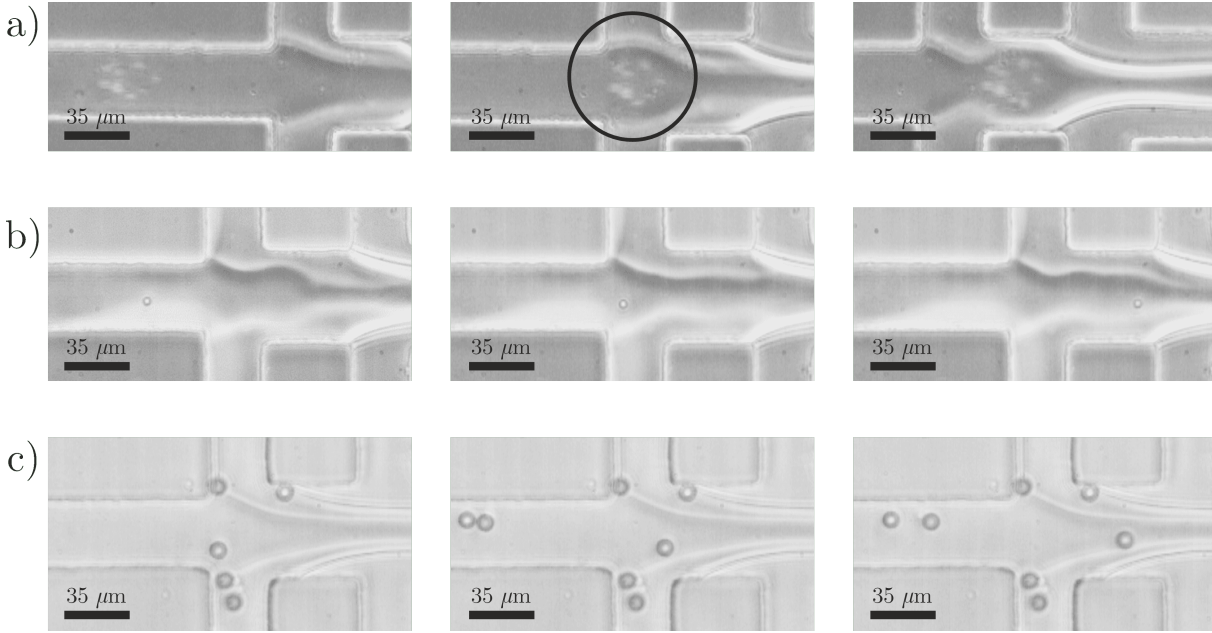


Figure 5.2: Bright-field images of 1.0 % alginate microspheres containing polystyrene microparticles with diameters of a) 1.1 μm , b) 5 μm and c) 10 μm flowing through a 35-50 re-encapsulation microfluidic device. For each sample, three images taken successively with respect to time are shown. A microsphere is circled for clarity.

index mismatch with the surrounding fluid. In Figure 5.2a we see an alginate microsphere approaching the junction in the first image, emerging from the junction in the second image, and having fully left the junction in the third image. Here we see that the 1.1 μm microparticles move within the microsphere, so are trapped. However, 5 μm and 10 μm microparticles move throughout the device with no accompanying alginate microsphere. This initially seems counter-intuitive; as particles get smaller, we would initially expect that for a given concentration of alginate (and therefore given pore size) the easier the particles would diffuse through the network. Simply, the smaller the particle the less likely it is to be trapped. However, we see the opposite. Literature reports pore sizes of alginate hydrogels in the 10 - 100 nm scale [203, 204, 205] and therefore the pore size is much smaller than the size of the microparticles. These pore sizes are also consistent with the mesh sizes estimated from plateau moduli in Chapter 3. This means changes in pore size should not dominate the different trapping behaviour observed here. This can be rationalised by looking at the formation of a microsphere. Assuming the polystyrene particles occupy a random position in the water-in-oil droplet, as the particles get bigger, they are more likely to exist at the edge of the droplet. If the particle is at the edge of the droplet when cross-linking occurs, then the particle will not be fully enclosed by the hydrogel, and will be released from the microsphere when the droplet is broken.

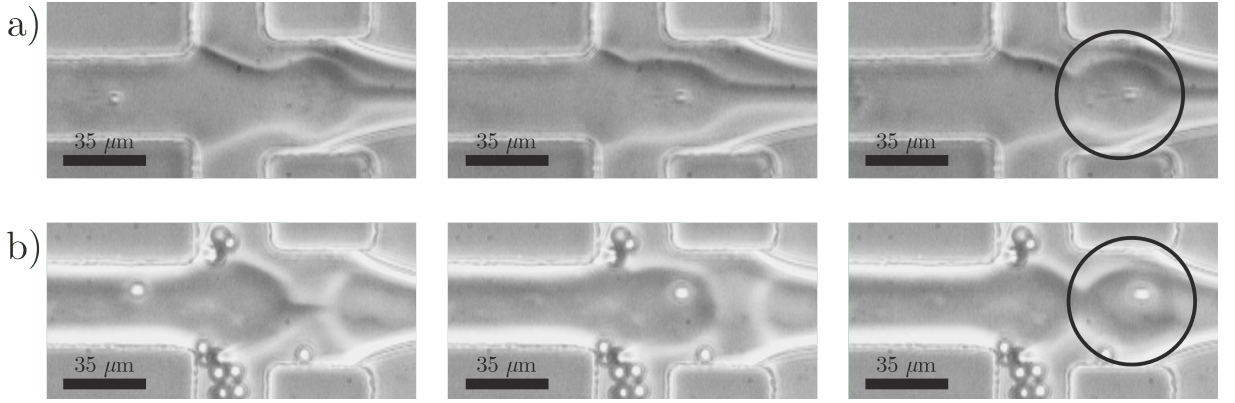


Figure 5.3: Bright-field images of 1.33 % alginate microspheres containing polystyrene microparticles with diameters of a) 5 μm and b) 10 μm flowing through a 35-50 re-encapsulation microfluidic device. For each sample, three images taken successively with respect to time are shown. A microsphere is circled for clarity.

Upon increasing the AlgEDTA mix concentration to 1.33 %, 5 μm and 10 μm microparticles became trapped within the hydrogel microspheres. Three images taken successively with respect to time of 5 μm and 10 μm microparticles moving with a 1.33 % alginate microsphere are shown in Figure 5.3. In Figure 5.3a, the first image shows a microsphere not containing microparticles emerging from the junction as a microsphere containing a 5 μm microparticle approaches from the left. This microsphere is more apparent as it emerges from the junction in the second two images, moving to the right. Figure 5.3b shows a similar migration of a 30 μm alginate microsphere containing a 10 μm microparticle. Note that while some free microparticles are seen at the junction, these could have originated from damaged microspheres, or from droplets that never contained sol-phase alginate. This suggests that concentration of the hydrogel matrix does have some contribution to how well polystyrene microparticles trap within hydrogel microspheres.

5.2.2 Trapping Jurkat Cells

As Section 5.2.1 has shown successful trapping of colloidal particles with diameter similar to that of Jurkat cells, we can now investigate whether Jurkat cells are trapped within alginate microspheres. Determining whether particles were trapped was shown to be difficult to determine with bright-field and phase-contrast imaging in Section 5.2.1. There we resolved this by observing relative motion between microparticles and microspheres, here we will use fluorescence microscopy of static images. By staining Jurkat cells with a dye that emits in a different wavelength to the alginate fluorescein, both can be observed clearly. In this section, images taken with a FITC filter show the alginate fluorescein signal, and images taken using the DSred filter show signal

originating from stained Jurkat cells. Note that here we cannot be completely sure the cells are trapped rather than existing above or below the plane of the microspheres.

All Jurkat cells used in this section were obtained as in Section 2.3.1, and stained with the CellTracker™ CM-Dil dye as described in Section 2.3.2. They were encapsulated into droplets alongside sol-phase alginate as described in Section 4.2.3, using the aaSE (acetic acid single emulsion) device described in Section 3.1.2 with no co-encapsulation or washing with lysis reagents. All alginate samples used were mixed with alginate fluorescein to provide a fluorescent label when imaged with a FITC filter (as described in Section 3.1.2), conserving the % AlgEDTA mix used.

Figure 5.4 shows a suspension of 1.0 % alginate microspheres-in-water containing Jurkat cells. By overlaying fluorescence images of the same field of view taken using DSred and FITC filters (Figure 5.4c), we see Jurkat cells trapped within the alginate microspheres. These are highlighted with grey circles in Figure 5.4c. When this was repeated using the 20 μm aaSE device in place of the standard 30 μm aaSE device, Jurkat cells were not trapped within 20 μm 1.0 % alginate microspheres. This is consistent with two hypotheses. To produce smaller hydrogel microspheres, initial encapsulation formed droplets with diameter 20 μm rather than 30 μm . In smaller droplets, with a conserved cell size, the cell is more likely to exist at the edge of the water-in-oil droplet, where the hydrogel network will not fully enclose the cell once cross-linked. If the cell is enclosed, as the microsphere is smaller, the distance required for diffusion out of the microsphere is less. Note that while we show here that 1.0 % alginate successfully traps Jurkat cells, polystyrene microparticles of size comparable to Jurkat cells were not trapped. This suggests that the surface chemistry or deformability of the trapped particles affects their trapping ability, and that polystyrene microparticles are a poor model for cells in this context.

5.2.3 Trapping Nanoparticles

The colloids described above were all much larger than the pore sizes reported for alginate gel networks, and those estimated in Chapter 3 for these hydrogel systems. Before investigating the trapping ability of DNA and RNA, we first attempted to trap fluorescent nanoparticles within the alginate microspheres. Polystyrene nanoparticles with a -COOH surface modification were obtained from Abvigen, with a diameter of 25 nm and concentration of 10 mg mL⁻¹. They had an excitation maximum at 488 nm and an emission maxima at 518 nm.

A 2.5 % AlgEDTA mix was encapsulated within water-in-oil droplets (as described in Section

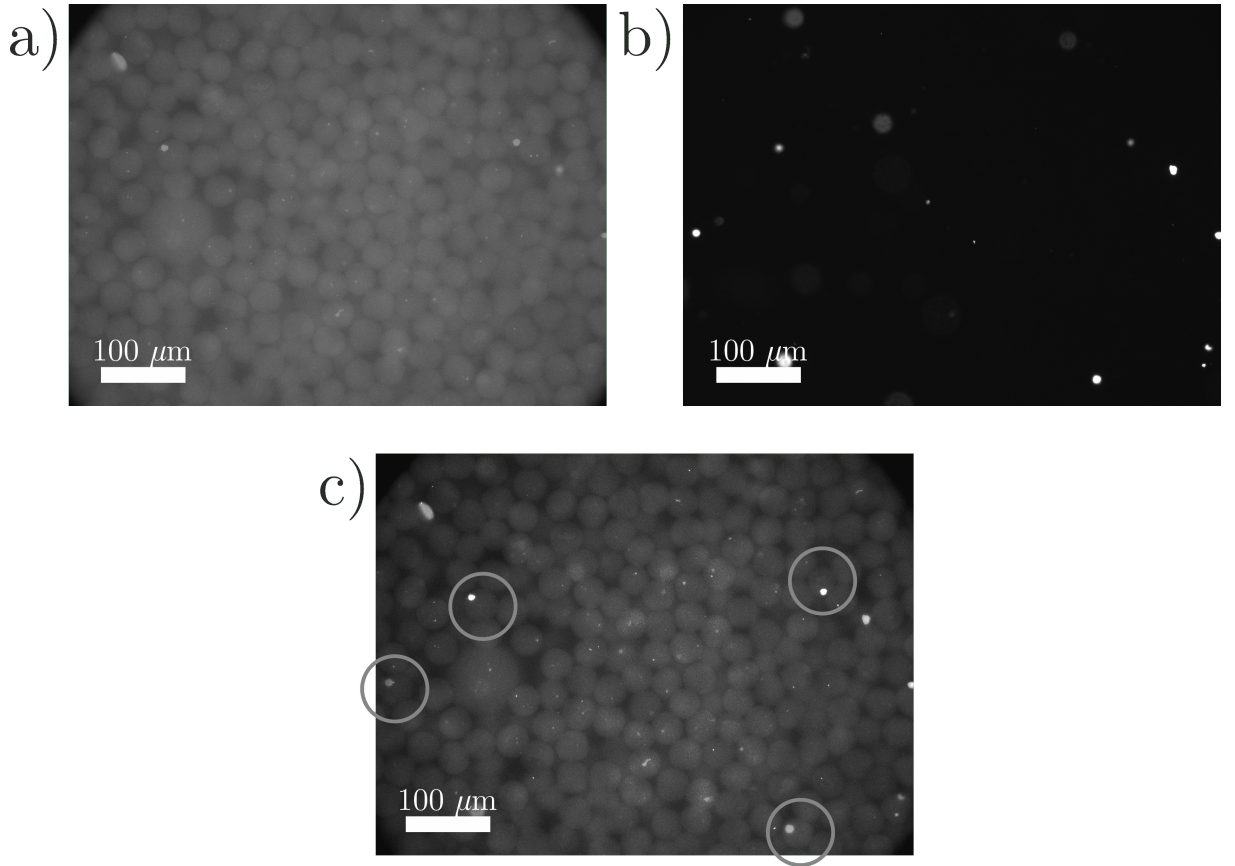


Figure 5.4: The same field of view of a suspension of 1.0 % alginate microspheres containing Jurkat cells imaged via a) FITC filter and b) DSred filter or c) with image a) and b) overlaid each other. Microspheres of interest, showing trapped Jurkat cells, are highlighted with grey circles. The field of view shown here was chosen to show a frequency of successful trapping representative of the entire sample.

3.1) alongside 1 % v/v of the polystyrene nanoparticle suspension obtained from Abvigen. The alginate was induced to cross-link to form microspheres within droplets and then the emulsion was broken with PFO (as in Section 2.2.4) to produce a suspension of 2.5 % alginate microspheres. These were imaged using a slide and cover slip. An example field of view is shown in Figure 5.5. As the particles would have been initially contained within the water-in-oil droplets, upon de-emulsification the location of the fluorescent signal shows whether the nanoparticles are trapped or not. As a bright homogeneous background is seen in Figure 5.5, while the microspheres seen in phase-contrast imaging are shown to be darker than the background, we can conclude the nanoparticles are not trapped within the alginate network. This is consistent as these particles are now similar in size to the pore sizes for alginate networks seen in the literature (and estimates in Chapter 3) [203, 204, 205], so diffusion through the networks would be expected.

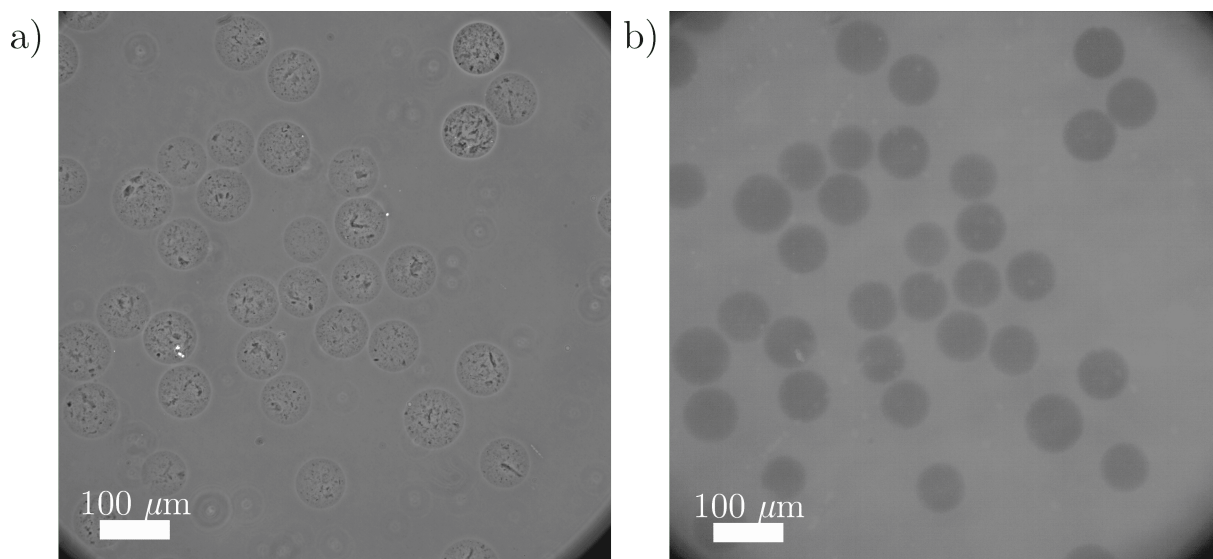


Figure 5.5: 2.5 % alginate microspheres made originally containing 25 nm fluorescent nanoparticles. The same field of view of the resulting suspension was imaged using a) phase-contrast microscopy and b) fluorescence microscopy using a FITC filter.

5.3 Trapping of DNA and RNA

5.3.1 Introduction

DNA and RNA are expected to diffuse out of hydrogel microspheres over time [238]. This is driven by three phenomena.

Firstly, there will be diffusion down the concentration gradient as the DNA originated within the microsphere, and the liquid phase of the suspension is diluted during washing, reducing the DNA concentration outside the microspheres.

Secondly, DNA confined within a hydrogel matrix will have a lower entropy than DNA unconfined in the liquid phase of the suspension. Therefore there is an entropic driving force for DNA to escape the hydrogel matrix.

Thirdly, alginate chains are negatively charged in solution, and so is DNA [149]. Electrostatic repulsion could therefore also be a driving force for the diffusion of DNA out of an alginate microsphere.

Diffusion of DNA through a hydrogel matrix was shown to be determined by pore size and DNA length in Section 1.4.1. Therefore we investigated the effect of hydrogel concentration, and DNA length on DNA diffusion timescales. We also investigated whether salt can be used to screen the electrostatic repulsion between alginate chains and DNA.

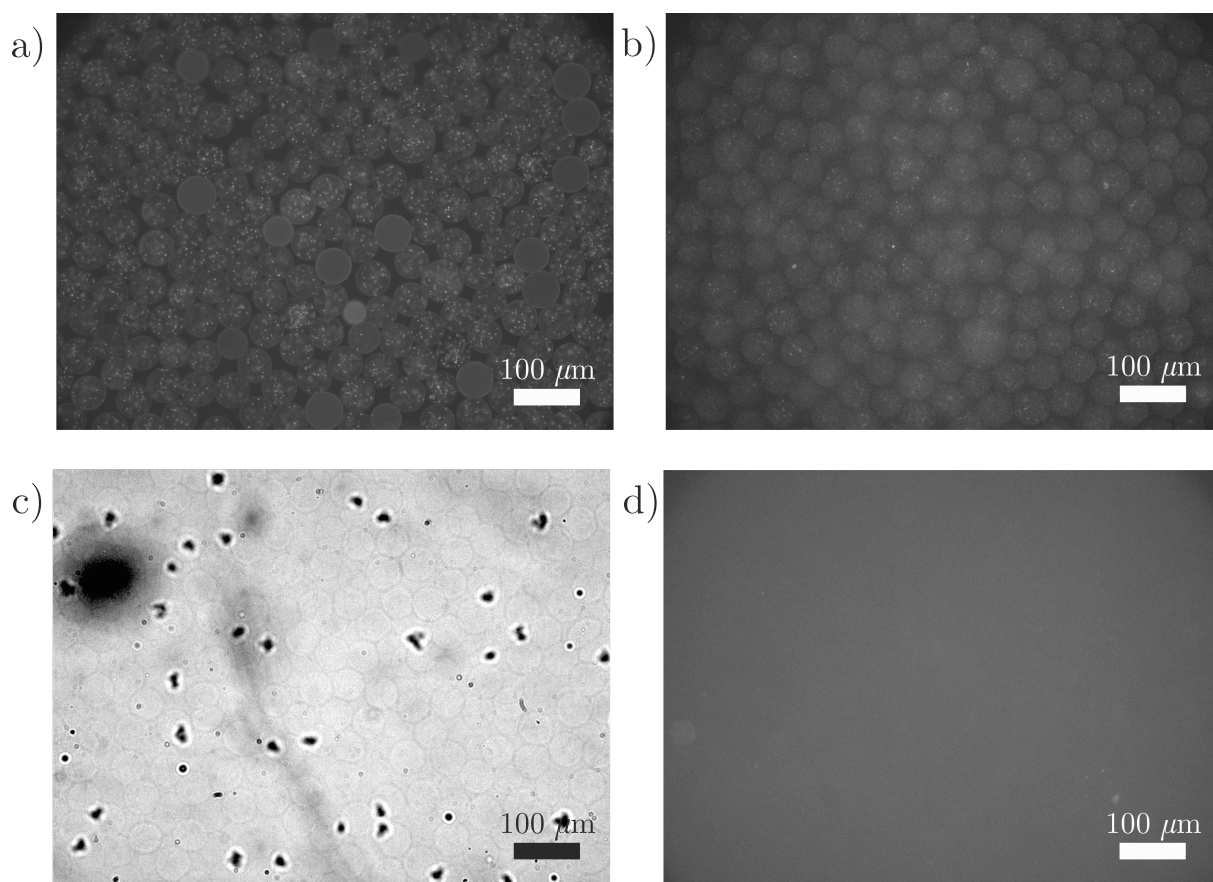


Figure 5.6: Fluorescence images taken using a FITC filter of a) droplets containing fluorescently labelled lambda DNA, b) droplets containing fluorescently labelled lambda DNA and cross-linked 2.5 % alginate. The emulsion from b) was broken and imaged by c) bright-field microscopy and d) fluorescence microscopy using a FITC filter. c) and d) show the same field of view.

5.3.2 Methods

Initially lambda DNA with molecular weight 32,300 kDa (Merck, obtained at $250 \mu\text{g mL}^{-1}$, 50,000 base pairs) was encapsulated within droplets to a final concentration of $12.5 \mu\text{g mL}^{-1}$. The droplets formed also contained a final concentration of 2.5 % AlgEDTA and SYBRTMGold (Section 2.3.3) to fluorescently label the DNA. Cross-linking was induced using acetic acid (Section 3.1.2). Another emulsion was made including $12.5 \mu\text{g mL}^{-1}$ lambda DNA and SYBRTMGold, but with no alginate present. These droplets were imaged using a FITC filter and shown in Figure 5.6a, while those also containing cross-linked alginate are shown in Figure 5.6b. The emulsion containing alginate microspheres was then broken (Section 2.2.4), and also imaged with a FITC filter (Figure 5.6d). In both emulsions, the DNA can be seen as bright spots within the droplets. When the suspension of microspheres was imaged, all fields of view were completely dark, suggesting this length of DNA was too small to be trapped within the 2.5 % alginate microspheres, and instead

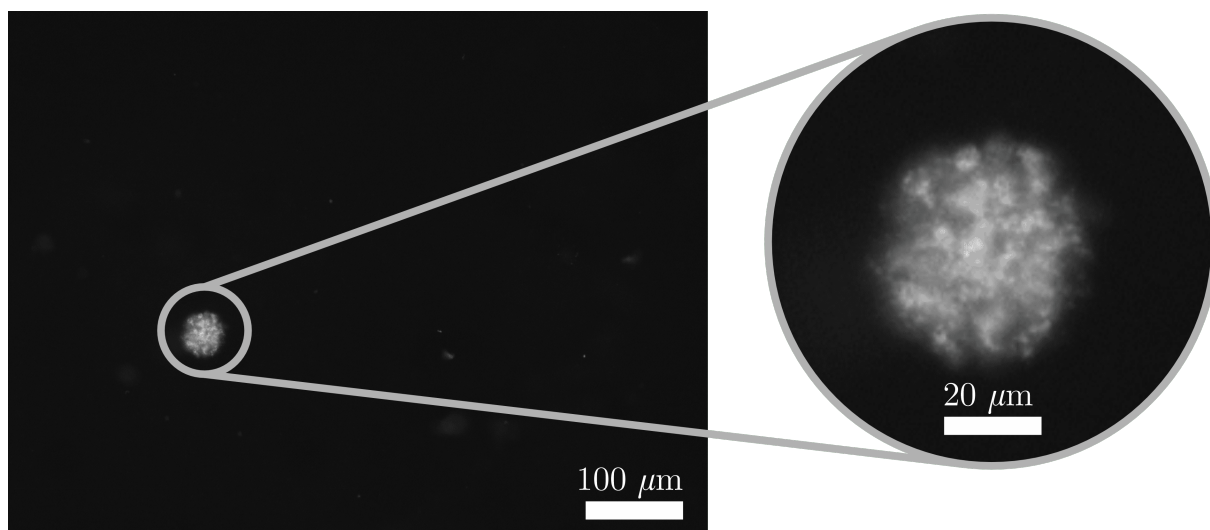


Figure 5.7: A fluorescence image taken using a FITC filter of a suspension of 2.5 % w/w alginate microspheres containing lysed Jurkat cells. One microsphere is enlarged to show SYBR Gold stained DNA trapped within the microsphere.

diffused out to produce a low level background fluorescence. Bright-field imaging of the same field of view (Figure 5.6c) showed successful microsphere formation, so unsuccessful microsphere formation was not the cause of the lack of DNA trapping. Instead, we trapped much longer nuclear DNA lysed from Jurkat cells within hydrogel microspheres. The male human genome contains 6.27 gigabase pairs [239]. As each base pair has a molecular weight of 660 kDa [240], unfragmented genomic DNA could have a molecular weight of up to 4.1×10^9 kDa.

Suspensions of microspheres containing trapped genomic DNA were produced as in Section 4.2.4. Jurkat cells were encapsulated in droplets containing sol-phase hydrogel and lysis buffers. The cells were lysed post encapsulation, and then cross linking of hydrogels was induced (by acetic acid for the alginate samples as in Section 3.1.2, and cooling for the agarose as in Section 3.1.1). Subsets of resulting emulsions were then broken every day for four or five days via the following process. 10 μ L of droplets were added to 10 μ L PFO and 10 μ L DI water. The oil layer was then removed. Figure 5.7 shows a suspension of 2.5 % w/w alginate microspheres containing lysed Jurkat cells after being stained with the DNA specific SYBR Gold dye as above. Trapped genomic DNA is clearly shown, illustrating that while the above lambda DNA was too short to be trapped, the much longer genomic DNA was trapped. 5 μ L of the resulting suspension was then added to 45 μ L. This was mixed and sedimented, either by gravity over 2 hours (alginate) or centrifugation for 3 min (agarose), as in Section 3.3.3. The sediment was then analysed for DNA concentration using the Qubit® fluorometer.

DNA concentration of a sample was determined using a Qubit®4.0 fluorometer. Samples

were stained using a dsDNA high specificity qubit assay kit (Invitrogen™). The machine was calibrated using two DNA standards of known concentrations kit prior to measurements taken (10 μL Qubit™1x dsDNA HS standards to 190 μL Qubit™1x dsDNA HS working solution). DNA concentration of a sample was determined by adding 1 μL sample to 199 μL Qubit™1x dsDNA HS working solution. This was then vortexed before measurement in the fluorometer. Three samples of each set of conditions were produced and measured using the fluorometer. Each of these were measured three times in the fluorometer, with the average DNA concentration measured noted. DNA concentrations were calculated by the fluorometer from fluorescence intensity and displayed in units of $\text{ng } \mu\text{L}^{-1}$. The mean and standard deviation of the three repeats of each sample are shown in all following figures.

Three concentrations of alginate microspheres were tested; 1 %, 2.5 % and 4.8 % AlgEDTA mix. These were compared with a 1 % w/w agarose sample. Finally a suspension of 2.5 % AlgEDTA microspheres were produced with an initial droplet concentration of 0.1 M sodium chloride.

5.3.3 Results and Discussion

We can estimate the persistence length of unlooped single stranded DNA to be 3 bp (1 nm) with a statistical segment (Kuhn) length then of double this, 6 bp [241]. This estimates the radius of gyration of the lambda DNA as on the order of 74 nm. Similar estimates give genomic DNA as having a radius of gyration of 25 μm . Therefore the lambda DNA can be thought of as being in-between the nanoparticles and the microparticles in terms of size. The lambda DNA is also on the size order of the reported pore size of alginate networks. It is therefore not surprising that the lambda DNA does not remain trapped within the alginate microspheres. The genomic DNA however is clearly much larger than the microparticles investigated, and therefore the trapping behaviour observed is expected. It is important to note these radii of gyration are estimates, as they do not account for hairpin loops, or other possible folding of the ssDNA strands.

Figure 5.8 shows the ratio of DNA concentration in the sediment to supernatant of the washed suspension of alginate microspheres. We assume the bottom 10 % of the sample is homogeneous and the top 90 % of the sample is also homogeneous. Here we compare the effect of alginate concentration on diffusion timescales. As time passes, all samples tend towards a ratio of 1 (shown by the black dashed line), showing equivalent DNA concentration within and outside hydrogel microspheres. This data is better presented as DNA concentration localised in the bottom 10 % of the sample, representing the DNA concentration still inside the microspheres.

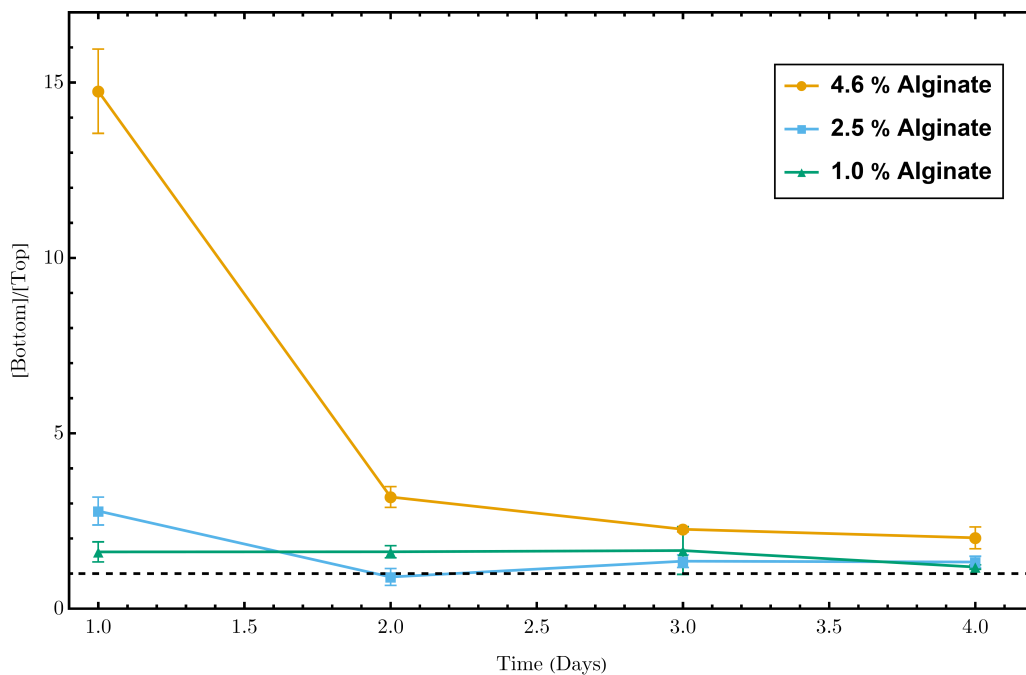


Figure 5.8: The ratio of DNA concentration localised in the bottom 10 % of the sample to the top 90 % of the sample, as a function of time after initial droplet formation. Error bars show standard deviation of the three repeats.

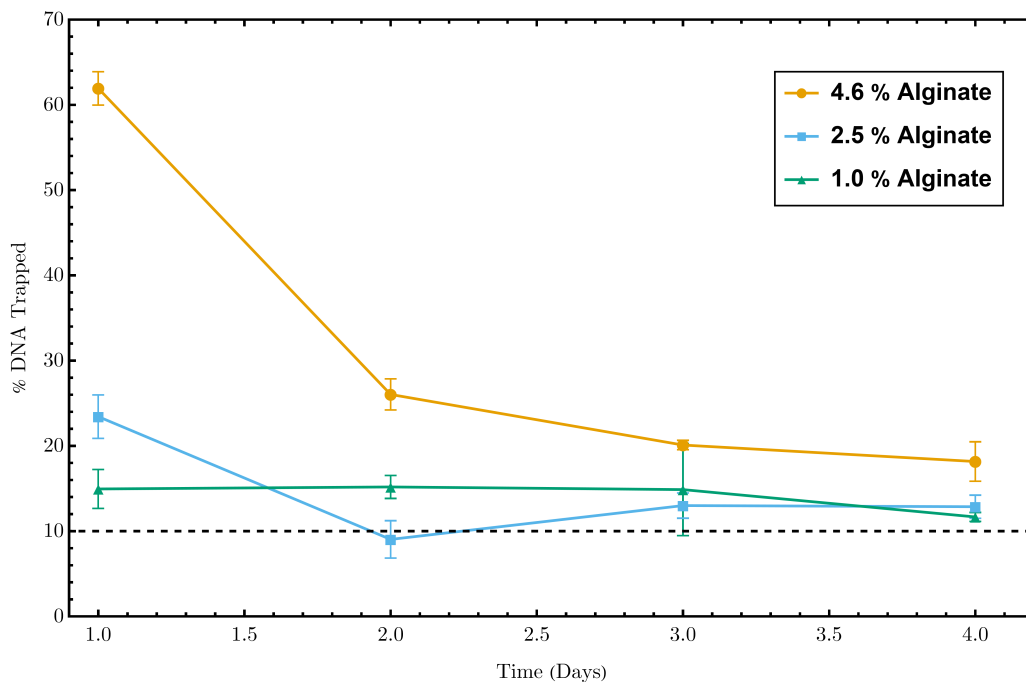


Figure 5.9: The percentage of DNA concentration localised in the bottom 10 % of the sample is displayed as a function of time after initial droplet formation. A comparison of 1 %, 2.5 % and 4.8 % alginate microspheres are shown. Error bars show standard deviation of the three repeats.

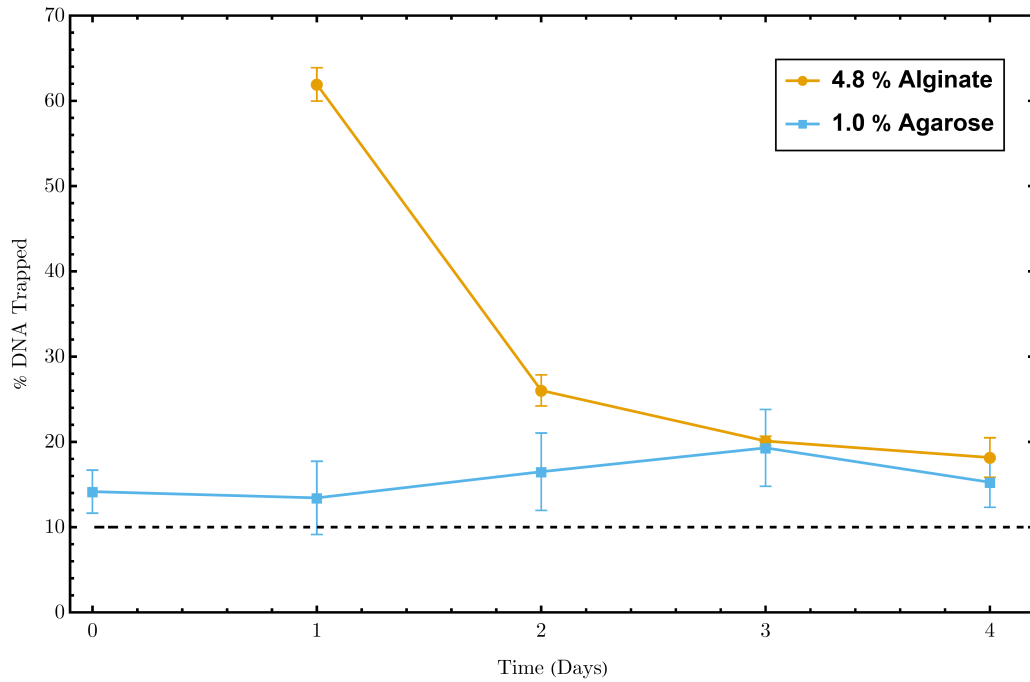


Figure 5.10: The percentage of DNA concentration localised in the bottom 10 % of the sample is displayed as a function of time after initial droplet formation. A comparison of 1 % agarose and 2.5 % alginate microspheres are shown. Error bars show standard deviation of the three repeats.

This is shown for the alginate samples in Figure 5.9. Note this value is still an estimate of the DNA concentration within the microspheres, as it does not take into account that the sediment of the sample is ≤ 74 % v/v microspheres (close packing volume fraction). Here the black dashed line represents 10 % DNA trapped, the value obtained from a sediment to supernatant ratio of 1. Here we see that the 1 % alginate microspheres did not effectively trap DNA, as the % DNA trapped remains on the order of 10 % throughout. As alginate concentration was increased, the % DNA trapped one day after microsphere formation increases, although both tend towards 10 % after 4 days. This is consistent with a higher % w/w alginate concentration reducing the pore size (as shown by pore size estimates in Section 3.2.4), resulting in a higher concentration of DNA remaining trapped after a day.

Figure 5.10 compares the two microsphere compositions used in Chapter 4: 1 % agarose and 2.5 % alginate microspheres. The agarose microspheres appear to not trap a significant proportion of the DNA present in the sample. This is consistent with data presented in Section 4.2.4, where microscopy techniques show a large amount of fluorescently stained DNA localised outside of the agarose microspheres, but not in the alginate microspheres. However it is inconsistent with our pore size estimates in Section 3.2.4, where agarose pore sizes were estimated to be smaller than those of alginate. One explanation for this is the large range of pore sizes reported in literature

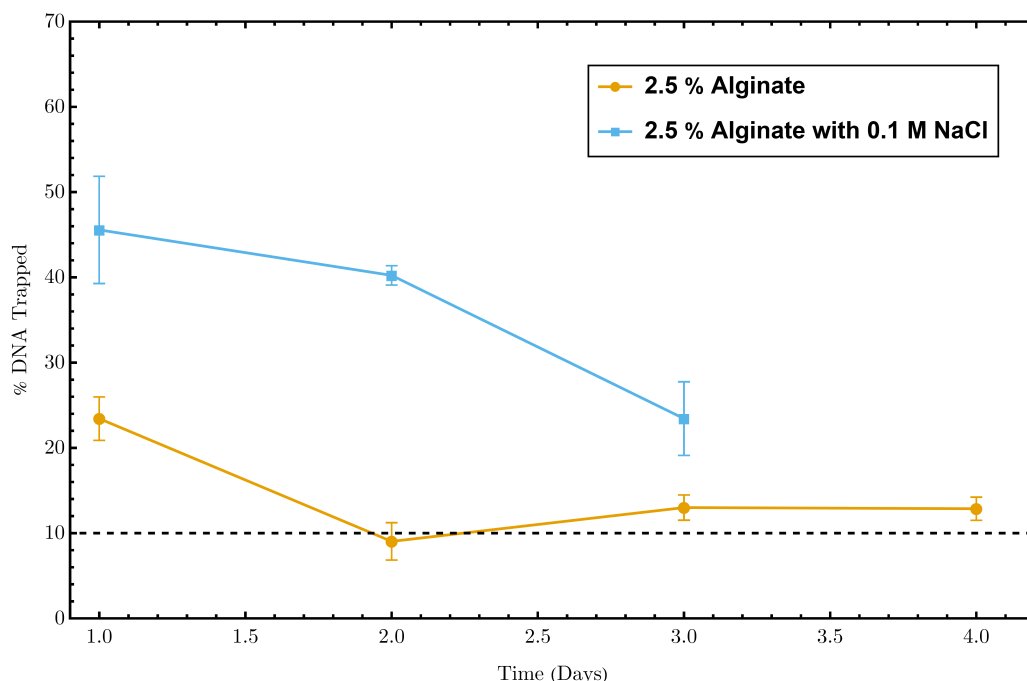


Figure 5.11: The percentage of DNA concentration localised in the bottom 10 % of the sample is displayed as a function of time after initial droplet formation. A 2.5 % alginate sample using no added salt concentration is compared with a final 0.1 M NaCl solution.

for agarose hydrogels, showing large variation [202].

Finally, Figure 5.11 shows that adding sodium chloride to a final concentration of 0.1 M to the dispersed phase of initial droplet formation (prior to alginate cross-linking) increases the initial DNA concentration trapped 1 day after microsphere formation. This salt also decreases the rate at which DNA diffuses out of the hydrogel matrix, showing a large benefit to DNA trapping ability caused by charge screening.

The 2.5 % alginate sample was also stained using a RNA high sensitivity assay kit. Any RNA stained by this technique was of a concentration below the detection threshold of the Qubit 4 fluorometer. This is consistent with published data suggesting RNA degrades over a short time period such as the 24 hours between cell lysis and the measurement of DNA concentration [242]. It is also consistent with the initial finding that lambda DNA is too short to be trapped within the same microspheres. As RNA is much shorter than DNA, it would be expected that RNA is also not trapped within the microspheres at this time interval.

5.4 Determining Pore Size using Imaging Techniques

5.4.1 Introduction

In Section 4.2.4, we lysed Jurkat cells encapsulated in droplets also containing sol-phase hydrogel. We then induced cross-linking, and broke the emulsion to produce a suspension of hydrogel microspheres which trap genomic DNA. Staining any DNA present with the SYBRTMGold fluorescent dye was used to show the successful trapping of genomic DNA. The resulting images (Figures 4.8 and 4.9) showed a network of fluorescent signal within a hydrogel microsphere, showing the location of trapped DNA. In this section we postulate that this fluorescent DNA structure represents the pore structure of the hydrogel matrix, and suggest the possibility of determining a characteristic length scale to describe this pore structure. In this section, we develop a methodology involving a combination of confocal and fluorescent microscopy techniques to produce high resolution images of hydrogel microspheres with varying compositions, and attempt to use Fourier transforms to determine a characteristic length scale of the hydrogels.

5.4.2 Fluorescent Imaging

Jurkat cells were prepared as discussed in Section 2.3.1. These were encapsulated into water-in-oil droplets using the SE device (Section 2.2.3) alongside sol-phase agarose or alginate hydrogels, as well as the relevant lysis buffers (Section 4.2.4). These were lysed before cross-linking took place overnight. Then the emulsions were broken, and the trapped DNA within the suspension of microspheres was stained using SYBRTMgold fluorescent dye (Section 2.3.3). These suspensions were then imaged using a cover slip and glass slide with double-sided sticky tape used as a spacer. These were imaged using a FITC filter on the Nikon microscope described in Section 2.2.6 with the 60x objective. Figure 5.12 shows nuclear DNA trapped within 1.0 % w/w agarose and 1.0 %, 2.5 % and 4.8 % alginate microspheres. The 1.0 % alginate microspheres show discrete fluorescent spots apart from each other, not showing obvious pore structure. The background fluorescence is also very high compared with the bead, in contrast to the other images where the background is similar or darker than the DNA within the bead. This is consistent with the data presented in Section 5.3.3 showing that DNA is not localised within the microspheres when compared to the supernatant. As the DNA has mostly diffused out of the microspheres, the DNA concentration within the microspheres is too low to accurately represent the pore structure. For the 2.5 % and 4.8 % alginate, and 1.0 % w/w agarose microspheres, the fluorescent signal appears to represent

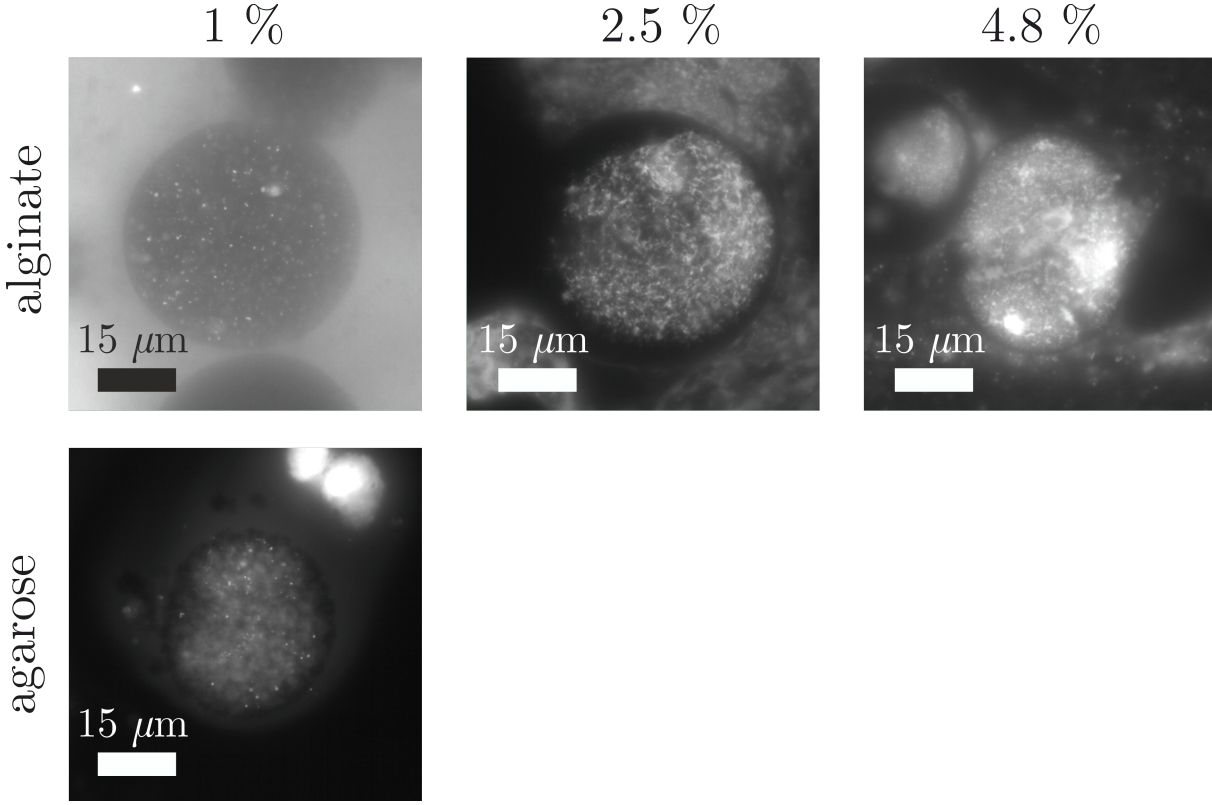


Figure 5.12: Fluorescently stained nuclear DNA trapped within 1.0 % w/w agarose and 1.0 %, 2.5 % and 4.8 % alginate microspheres. Each image is centred on one microsphere. Microspheres appear as a circular structure against a background fluorescence, with the fluorescence signal showing the DNA strands arranged within the microsphere.

pore structure. Here we see a fluorescent signal throughout the microspheres, appearing as a series of overlapping polymer chains showing the position of the DNA within the gel structure. While microspheres of differing composition appear different to each other, suggesting a different pore structure, this is difficult to quantify by eye. Fourier transforms have been done on images with similar appearances to calculate a characteristic length [243, 244, 245], hereby quantifying the pore structure.

A script was then written in Mathematica 13 to calculate the squared magnitude of the discrete Fourier transform (power spectrum) of an input image, using the ImagePeriodogram function. The radial average of this is then calculated, and displayed as a function of inverse pixel space (k-space). The location of a peak of the radial averaged power spectrum in k-space (k_{max}) corresponds to the characteristic length scale of the image in μm (l),

$$l = \frac{\text{FOV}}{k_{max}}, \quad (5.1)$$

where FOV is the width of the field of view in μm [243].

When this script was applied to the images collected by the Nikon microscope, no peak was seen (Figure 5.13). This was due to the initial images having too much noise from out of focus stained DNA. Any attempt to clean the images with Mathematica scripts (such as a Gaussian filter) only generated an artificial peak with a position that heavily varied depending on the values used for image analysis and were therefore arbitrary. Instead we turned to confocal microscopy to remove this noise. Going forwards, we do not consider the 1 % alginate microspheres, as these are conclusively shown to not consistently trap DNA (based on data shown in Section 5.3.3 and fluorescent microscopy), Instead we focus on 2.5 % and 4.8 % alginate and 1.0 % w/w agarose microspheres, which sustain DNA fluorescence over time.

5.4.3 Confocal Imaging

The confocal images in this Section were taken with Dr Jack Holland (Aarts group, University of Oxford). We used a Leica TCS SP5 machine confocal microscope owned by the Ligoxygakis group in the Department of Biochemistry at the University of Oxford. A HCX PL APO CS 63.0x1.40 OIL UV objective with immersion oil was used to image samples. A excitation wavelength of 488nm was used with a collection bandwidth of 503 - 800 nm in order to detect fluorescent signal originating from SYBRTM Gold labelled DNA.

Initially samples were imaged using a sample holder consisting of a cover slip placed onto the sample which was resting on a glass slide. Then being imaged with cover slip side down. This resulted in the deformation of what was expected to be a 30 μm diameter 1.0 % w/w agarose microsphere, instead having a height of around 10 μm and a diameter of 70 μm . Imaging using double-sided sticky tape as a spacer prevented this deformation of the microsphere, returning to spherical structure. This is shown by a 3D render of two hyperstacks of microsphere images using each sample holder in Figure 5.14. For the rest of this section, the sticky tape sample holders were used, this was to prevent deformation of the pore structure due to squashing of the microspheres.

As in Section 5.4.2, we first encapsulated sol-phase hydrogel, Jurkat cells and the lysis buffer developed in Section 4.2.4. The emulsions were left at room temperature for at least 1 h for lysis, before cross-linking was induced overnight. The emulsions were then broken as described in Section 2.2.4, and DNA was stained with SYBRTMGold as described in Section 2.3.3. Confocal images were taken at a z height equivalent to the equator of each microsphere. We collected

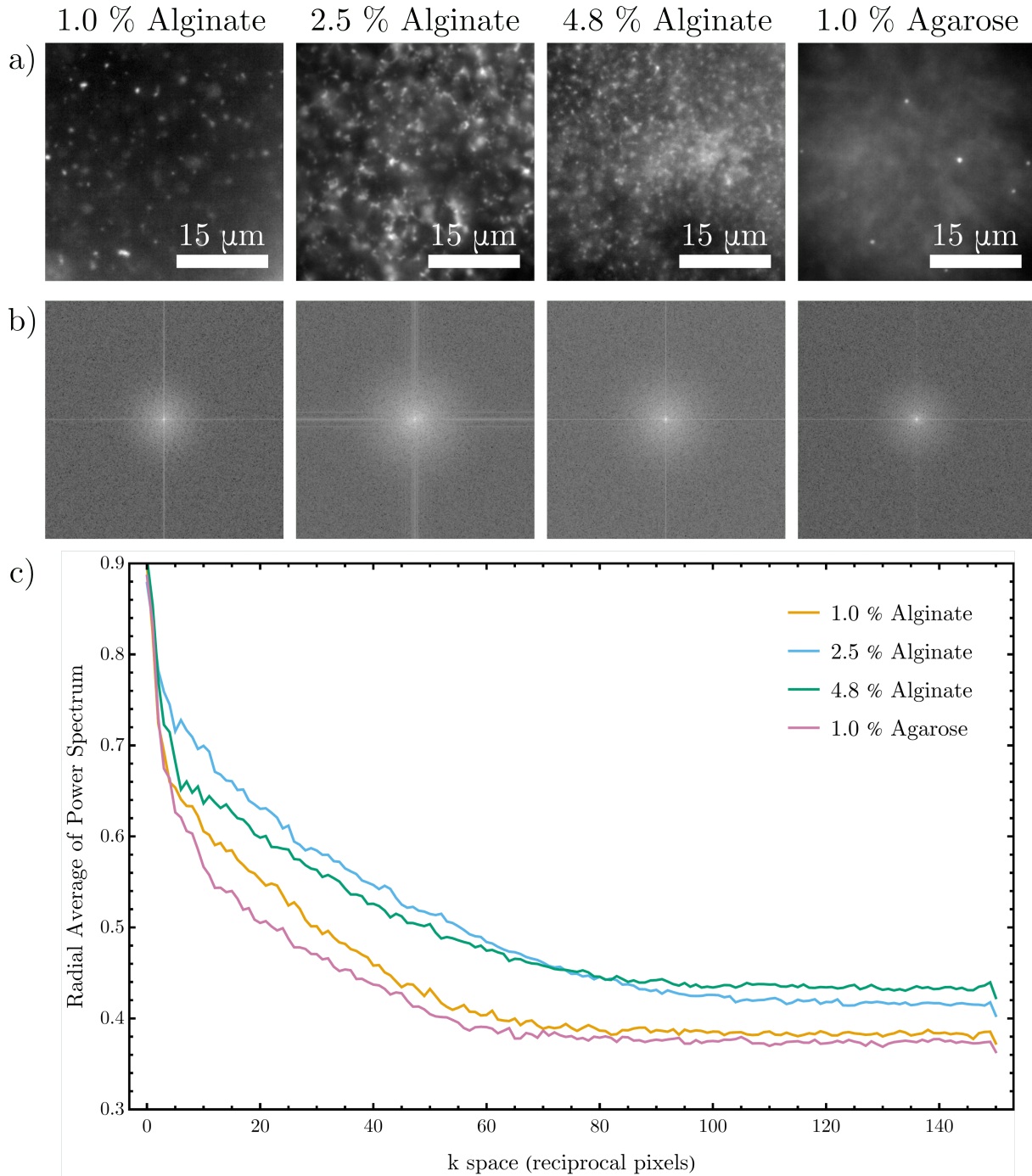


Figure 5.13: a) Cropped input images, b) their corresponding fast Fourier transform and c) the radially averaged power spectra of fluorescently stained DNA trapped within 1.0 % w/w agarose and 1.0 %, 2.5 % and 4.8 % alginate microspheres.

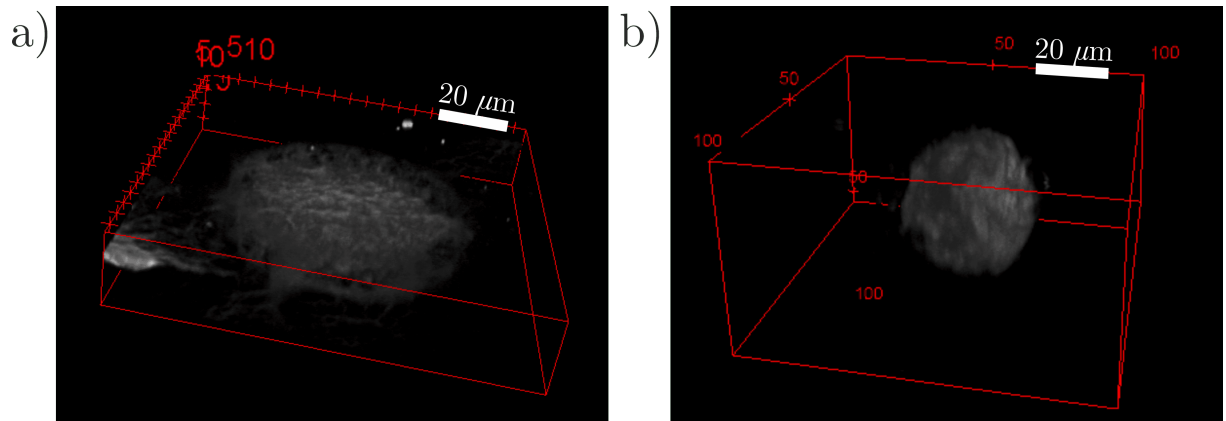


Figure 5.14: Confocal images of 1.0 % w/w agarose microspheres trapping stained DNA imaged using a) a cover slip and glass slide sample holder or b) a cover slip and glass slide separated by double-sided sticky tape.

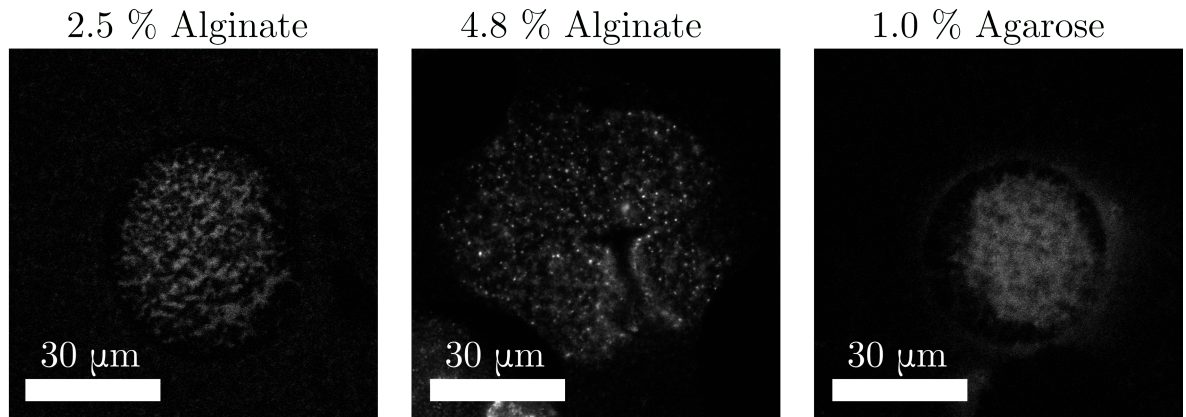


Figure 5.15: Confocal microscopy images of DNA trapped within 2.5 %, 4.8 % alginate and 1 % agarose microspheres.

images of at least 10 microspheres of each of the three types of hydrogel microsphere imaged (2.5 % and 4.8 % alginate and 1.0 % w/w agarose). Example images of each system are shown in Figure 5.15. We see a similar trend here as the fluorescent images shown in Figure 5.12. The alginate microspheres show localised fluorescence theoretically corresponding to the pore structure of the hydrogels, with increased concentration causing smaller pores and therefore smaller fluorescent spots. If the location of the DNA can be assumed to represent the pore structure of the hydrogel then the agarose samples show a larger pore size which would correspond to the reduced trapping ability shown by the Qubit™ data. This is a trend also shown by the pore size estimates in Section 3.2.4. Figure 5.16 shows the Mathematica script from Section 5.4.2 applied to cropped versions of these example images. Again no clear peaks are shown here, though the 2.5 % alginate has a potential peak around $k_{max} = 3$, which would give a characteristic

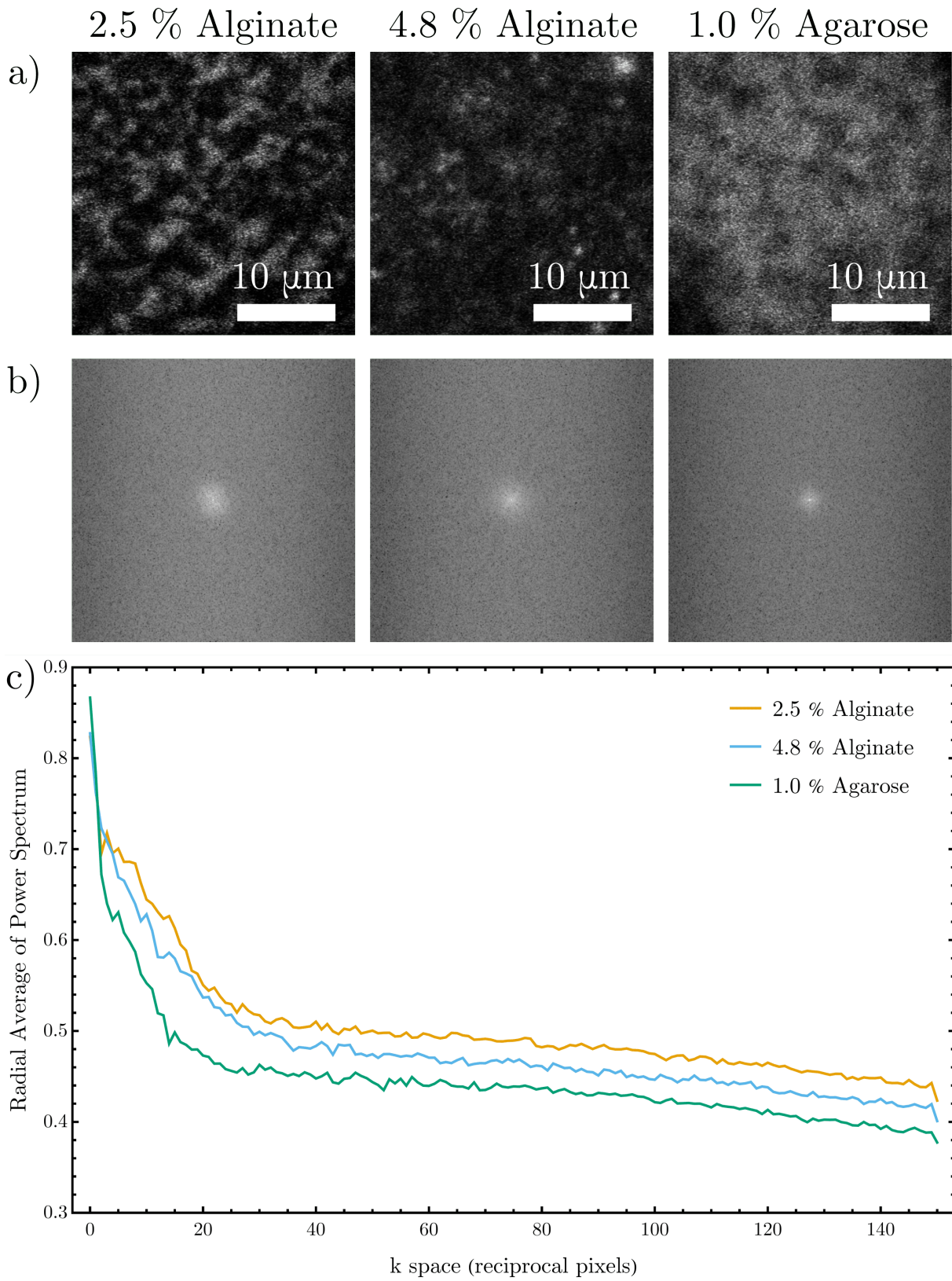


Figure 5.16: a) Cropped confocal input images, b) their corresponding fast Fourier transform and c) the radially averaged power spectra of fluorescently stained DNA trapped within 1.0 % w/w agarose and 2.5 % and 4.8 % alginate microspheres.

length around $9.2\text{ }\mu\text{m}$ using Equation 5.1. Other images of 2.5 % alginate microspheres either resulted in a similar k_{max} or no peak was seen. This is not a value that is very close to reported pore diameters for alginate, which are on the 10 - 100 nm scale [203, 204, 205], or with the pore size of 41 nm estimated from the plateau modulus in Section 3.2.4. However this length scale is reasonably consistent with the fluorescent structures seen in the 2.5 % alginate images in Figures 5.15 and 5.16. This suggests that the pore structure is not well described by the location of trapped DNA. This deviation from reported values strongly suggests that imaging trapped and fluorescent DNA within hydrogel microspheres is not a reliable method to determine pore sizes of hydrogel microspheres.

5.5 Chapter Summary

We set out in this Chapter to quantify the trapping behaviour of hydrogel microspheres shown in Chapter 4. We started by showing that the ability of microspheres to trap polystyrene microparticles increases with increased volume concentration of alginate. This is consistent with the pore size of the gels decreasing with increased concentration. We also showed that large particles trap less well than smaller particles, but this can be overcome by increasing concentration of alginate chains. We compared this to the ability of microspheres to trap Jurkat cells, and showed that polystyrene microparticles are a poor model for Jurkat cells in this context. Jurkat cells were shown to be well trapped within alginate microspheres.

We next used Qubit measurements to investigate how well DNA is trapped within microspheres. Here we showed that increasing the concentration of alginate chains increased the proportion of DNA localised within the hydrogel microspheres. We also showed that using a salt buffer to screen the like-charge repulsion between alginate chains and DNA increased the retention of DNA within microspheres for a longer time period. This shows that to maximise the DNA retained for the assay developed in Chapter 4, we should instead use 4.8 % alginate and a screening salt. Finally we proposed using imaging techniques to characterise DNA trapped within microspheres. Both fluorescence and confocal imaging techniques provided qualitative evidence which confirmed the Qubit observations. Unfortunately, using Fourier transforms to analyse these images to determine pore sizes of the hydrogel proved unsuccessful. In future, the pore sizes of these hydrogels should instead be determined using higher quality imaging techniques such as cryo-scanning electron microscopy before applying the Fourier analysis.

Chapter 6

Conclusions and Outlook

The overarching aim of this thesis was to investigate the possibility of using the pore structure of hydrogel microspheres to create a size-based filtration technique to add to the droplet microfluidic toolbox. This would contribute towards the long-term goal of developing an assay to detect and sort genetic material based on the presence of a very rare target sequence. The main application of this would be to sequence HIV provirus found within latently infected T cells, while retaining single-cell resolution. The aim with this assay is to reduce the amount of unnecessary sequencing of non-proviral DNA, saving time and expense while also providing single-cell resolution. The specific goal of this thesis was to produce suspensions of hydrogel microspheres which could trap genomic DNA at a single cell resolution, and then identify the microspheres containing a specific target sequence of DNA. We saw in Chapter 4 that the current limitation in developing such an assay in droplets was the inhibition of PCR after successful cell lysis. We proposed a solution which could use a hydrogel microsphere to trap the large genomic DNA, then allowing washes to dilute out small inhibitors such as cell lysates. Providing these microspheres maintained single cell resolution, the microspheres could be re-encapsulated for PCR, with successful detection of HIV target sequence resulting in a fluorescence signal allowing sorting.

We started by investigating the production and properties of two hydrogel systems, alginate and agarose. We established a method of producing agarose microspheres using tubing and slide heaters to keep agarose samples above the melting temperature to allow flow through microfluidic devices. These devices then used flow focusing junctions to create water-in-oil droplets, which were cooled below the gelling temperature to produce an emulsion of cross-linked microspheres. Breaking the emulsion produced the desired suspension of agarose microspheres. Alginate microspheres were produced using EDTA as a chelation agent, allowing us to control the timing of

cross-linking to occur after generation of water-in-oil droplets containing sol-phase alginate and calcium EDTA. Subsequent de-emulsification produced a suspension of alginate microspheres. We then checked whether these microspheres would behave as required for the proposed assay. We developed washing methods for both agarose and alginate microspheres, by showing how sedimentation of the microspheres can be achieved. We showed that the microspheres are stable with respect to the timescale and temperature scale required for this assay. We also studied the swelling of microspheres upon de-emulsification, aiding control of microsphere size by creating droplets of a specified size.

We also investigated the viscoelastic properties of bulk hydrogels, showing that the hydrogels could be made more "solid like" by increasing w/w % of polymer chains, viscosity of the initial sample, and extent of cross-linking (by varying acetic acid concentration in alginate). We also showed that reducing the gelling temperature of agarose produces less "solid like" samples, and that agarose samples are typically more "solid like" than alginate. We predicted that more mechanically "solid like" hydrogels will trap DNA and particles more effectively, a concept we returned to in Chapter 5.

We then proceeded to use agarose and alginate microspheres within the HIV latent reservoir assay. Here we attempted to detect human DNA lysed from within Jurkat cells, as a model for detecting proviral HIV DNA from latently infected T cells. We first developed a method to lyse Jurkat cells without damaging the structure of the hydrogel microspheres. Initial attempts at trapping Jurkat cells within microspheres and then lysing them proved unsuccessful. Instead the cells were lysed within a droplet containing sol-phase hydrogel before cross-linking was induced. Breaking the emulsion and staining the resulting suspension with a DNA specific stain showed the successful trapping of genomic DNA. We then investigated how changing microsphere size and dimensions of a re-encapsulation device affected successful re-encapsulation of hydrogel microspheres. Here we showed that encapsulation of 30 μm alginate microspheres within a 35-50 re-encapsulation device resulted in little to no observable damage to microsphere structure. We then re-encapsulated alginate and agarose microspheres containing trapped DNA, showing DNA remains trapped after the re-encapsulation process. We observed that while 2.5 % alginate microspheres seemed to trap a majority of the DNA, when DNA was trapped within 1.0 % agarose microspheres there was a significant fluorescent signal outside the microspheres. This was the first evidence that alginate microspheres are more effective at trapping DNA than agarose microspheres.

We next investigated whether the hydrogel structure would inhibit the PCR of trapped DNA.

Microspheres trapping genomic DNA were re-encapsulated in new water-in-oil droplets alongside PCR reagents. As has been shown in previous literature [10], PCR thermal cycling causing melting of the agarose structure, so inhibition was low, and PCR was successful. Despite literature showing methods to overcome alginate inhibition of PCR, these methods were not successful in this context, and successful PCR amplification of DNA trapped within alginate microspheres was not achieved.

As we had now shown the potential of alginate and agarose microspheres as micro-reactors trapping long chain DNA, it became useful to quantify this behaviour. This would potentially allow future application of the microspheres to other bioassays requiring single cell resolution. We started by showing how increasing concentration of alginate was required to trap large polystyrene microparticles, but smaller microparticles remained trapped at a lower concentration. We contrasted this with Jurkat cells, which remained trapped at lower concentrations despite their larger size, suggesting polystyrene microparticles are a poor model for Jurkat cells when examining their trapping behaviour. It is possible that the different surface chemistries are responsible for this behaviour.

We expected DNA to diffuse out of the microspheres over time, driven by the higher entropy of unconfined DNA strands. We used a Qubit DNA concentration measurement technique to observe the proportion of DNA trapped within microspheres of different compositions over time. We showed that increasing alginate concentration increased trapping effectiveness, confirming the viscoelastic prediction. However, alginate microspheres were shown to trap DNA more effectively than agarose, in contrast to the viscoelastic results, suggesting this is not a behaviour that can be predicted by mechanical properties alone. We also showed that salt to screen the electrostatic repulsion between the microsphere and DNA also improves trapping ability. Combining all these observations suggested that for optimal DNA retention within microspheres, 4.8 % alginate microspheres with a screening salt should be used.

Finally, we attempted to use the location of trapped DNA within a microsphere to characterise the pore structure of the hydrogels. This was qualitatively able to confirm earlier observations of the inefficiency of using agarose microspheres to trap DNA compared to alginate, and that trapping ability increased with concentration of alginate. Unfortunately, attempts to quantify this using Fourier transforms to produce a characteristic length were unsuccessful.

There are several key areas where further research would be of great benefit to the field. In terms of the latent reservoir assay, work can focus on overcoming inhibition of PCR due to

alginate presence, as literature has shown this is possible. We have obtained fluorescent labelled populations of target DNA retained within microspheres, so a method to sort and sequence these should now be investigated, using microfluidic sorting methods or by adapting conventional cell sorting techniques. In order to further study the ability of microspheres to trap DNA, work should focus on determining the pore size of these microspheres (using higher quality imaging techniques such as scanning electron microscopy). Then the effect of changing the length of trapped material with respect to the pore size can be fully investigated, allowing fine tuning of this trapping method to other specific bioassays.

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Appendix

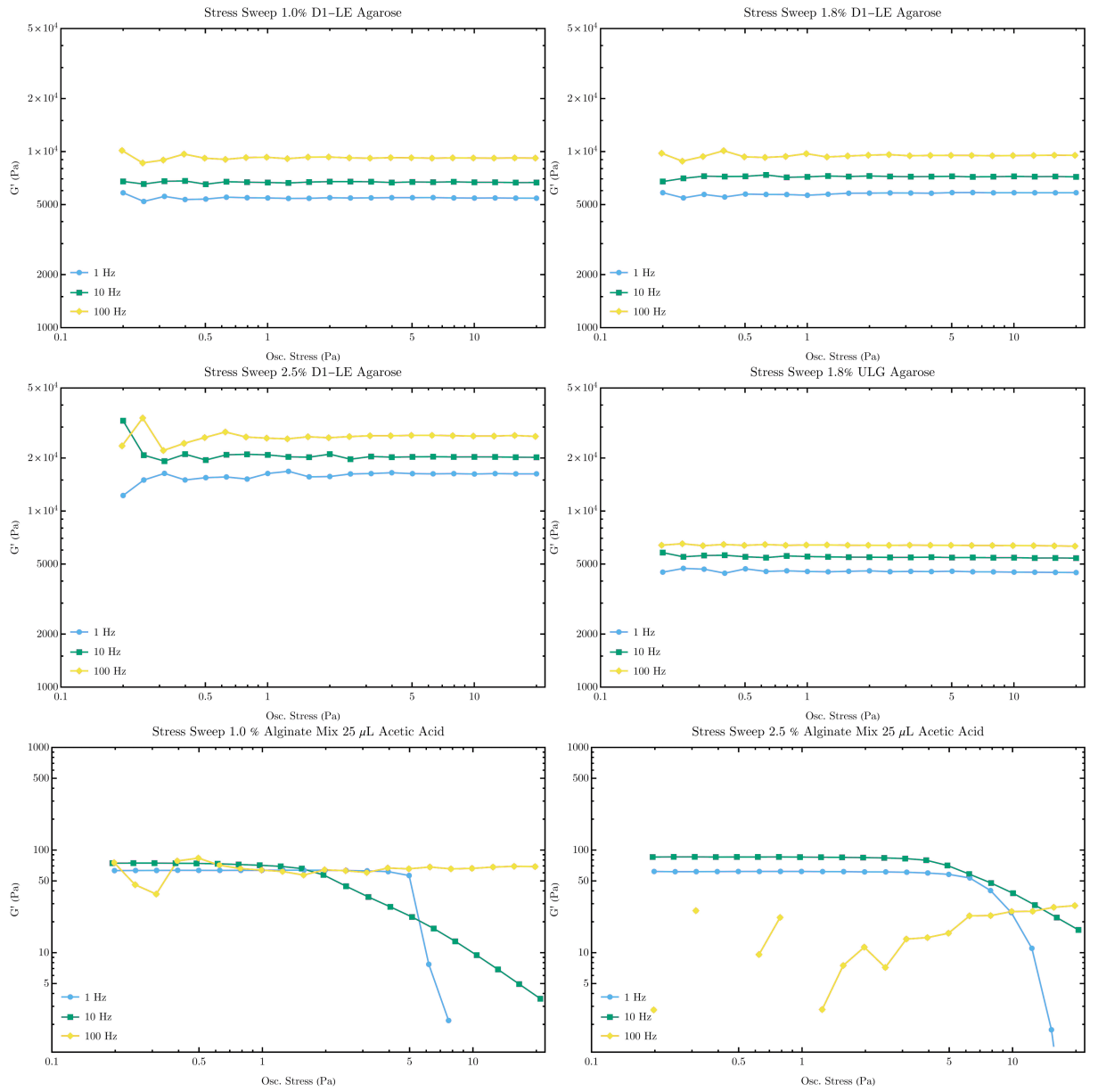


Figure 6.1: Stress sweeps for samples 1 to 6 in Table 3.1.

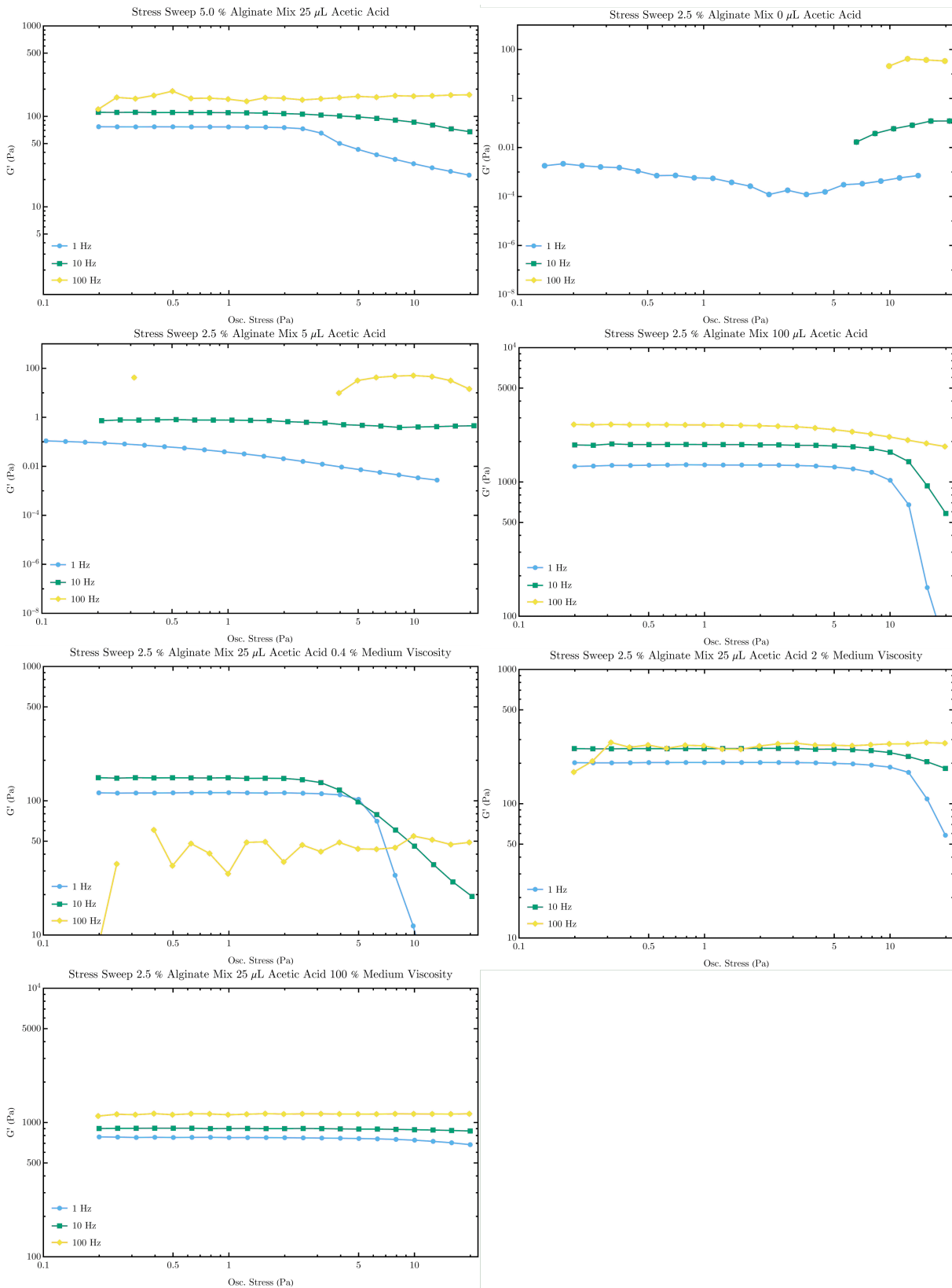


Figure 6.2: Stress sweeps for samples 7 to 13 in Table 3.1.