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Rory Giles. Novel magnetic particles for bioassays. Chemical Physics [physics.chem-ph]. Université Pierre et Marie Curie - Paris VI, 2015. English. NNT: 2015PA066313 . tel-01295485

HAL Id: tel-01295485 https://theses.hal.science/tel-01295485

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Université Pierre et Marie Curie

École doctorale de Chimie Physique et Chimie Analytique de Paris Centre

Novel Magnetic Particles for Bioassays

Par Rory GILES

Thèse de doctorat de Chimie physique

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Présentée le 25 septembre 2015

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Acknowledgements

Without doubt when I look back on my life I will consider my time spent at the Laboratoire de Colloides et Matériaux Divisés to have been a defining chapter. These four years have witnessed not only the completion of this project, but also significant personal and professional growth, none of which would have been possible without the support of a great number of people, to whom many thanks are owed.

My first thanks goes to Jérôme Bibette for welcoming me into the lab. His inexhaustible enthusiasm has provided so much inspiration, and his ability to cut straight to the heart of an issue has helped to keep the project sailing forward.

Special thanks must go also to Jean Baudry, who patiently guided me throughout this process, putting me back on the right track countless times when I have erred, and really teaching me how to organize my thoughts in a scientific manner. This thesis is a testament to his pedagogic skill!

I would equally like to thank the esteemed members of the jury of my defence: Axel Buguin for presiding over the jury, taking the time to put me at ease and generally making the defence an enjoyable process. To Leo Ijzendoorn for travelling to Paris to take part, acting as a reviewer of the manuscript, and for providing excellent feedback on my progress reports throughout the Biomax project. Thanks also to Philippe Poulin for his insightful review, and to Gilles Cauet for providing his expertise on this topic.

This project would also not have been possible without the help of Nicholas Bremond, who introduced me to microfluidic and whose innovative designs were critical for the production of the uniform droplets required in for the final application. Likewise, to Ladislav Derzsi who improved upon these chips, perfected the surface treatment and constantly astounded me with his resourcefulness and PDMS wizardry. Thanks are also owed to Djamal Zerrouki for initiating me in the mysteries of emulsification and solving the problem of the disappearing phospholipids.

I can go no further without acknowledging the many colleagues of my era in the lab. Donatien, who welcomed me into the lab and shared many of my international adventures (I regret that my skill with Cupid's bow didn't quite meet his needs). Damien too, who has been a constant and cheerful office companion, who was able to patch up the holes in my knowledge of magnetic particles when needed. Lianmei, who gave excellent Chinese lessons and always had interesting questions about the correct use of English. Thanks to Fanny for all the French proverbs and imaginary gunfights; lab time was always more exciting with her around. To Julie, it

was a privilege to be suffering with the manuscript writing in synchronisation, those meals and non-alcoholic beers made the whole process much less painful. To Anna, my Czech co-conspirator in microfluidics, who always appeared at the right times to share in experimental victories and defeats! To Klaus, my office nemesis; his coaching has really stretched my creativity for delivering friendly insults. To Gwénaëlle, who had the arduous task of spotting all those missing commas in the manuscript and for picking up the reins of this project. To Eren for mealtime company, advice and last-minute tutelage on emulsification. Not forgetting Andrzej for his unquenchable optimism, enthusiastic encouragement and variety imported snacks.

There are many more friendly faces of the LCMD past and present who will all be remembered fondly: -Denis, Leslie, Hugo, Kevin, Nicolas Atrux, Koceila, Jean-Baptiste, Céline, Anette, Shima, Nitesh, Sarah, Jairo, Noushin, Minou, Jamie, Eduard, Perrine, Leopold and especially to Florence as well for keeping the lab organised, tidy and under control! It would also be impossible to go without thanking administrative staff, Lise, Meriem, Hélène and Isabelle, since it is no mean feat to keep the paperwork moving on schedule and to the right people, especially in France!

I am indebted also to friends outside the lab who have helped me along this journey: the fine people of PMMH Joe, Pierre, Tomek, Eduardo and Jessica; all those lunchtimes could not have been the same without them! Thanks to Matt too who visited at just the right time to solve some ferrofluid issues. Also to my friends from the IHCP at the JRC in Italy, Jakub and Cesar who set me on this PhD path.

A Parallel set of thanks is also owed to everybody involved in the BioMax network; it was really an amazing experience. Thanks to Menno Prins, Jeroen Lammertyn and all the other P.I.s for orchestrating this endeavour. Thanks as well to Federica Toffalini for her diligent coordination of the network and subsequently to Dragana Spasic for continuing this role. Lastly thanks to each of the BioMax fellows! Delfina, Soledad, Stefano, Fabiola, Elena, Iulia, Malte, Tonge, Deniz, Diego, Matteo, Bedabrata and Tamara, it was amazing to meet and travel with each and every one of you, I wish all of you the best and I hope that our paths will cross again.

My final thanks go to my parents, for their love and support throughout this long challenge, as well as the supplies of Branston pickle and Marmite brought to me during their visits; such home comforts make all the difference!

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Introduction

In the domain of biotechnology, there is a high demand for novel materials with the ability to interface with biological media. Many very important breakthroughs are closely tied to the discovery of new materials or new ways to use existing materials. Often these materials are colloidal in nature (dispersions of particles 1-1000 nm) and exhibit remarkable properties of scale.

One of the most ground-breaking colloids has been nanoparticles of magnetic iron oxide. At a size range of 3 - 50 nm these particles consist of single magnetic domains which exhibit zero net magnetisation due to thermal fluctuations. Magnetisation may be induced by exposing the particles to a magnetic field which results in a very strong magnetic response. Selective magnetic behaviour makes these particles a powerful tool in both biology and medicine as it allows remote manipulation of objects within a sample. In vitro for example, magnetic particles can be grafted with ligands against a specific target, allowing them to be separated from the bulk. Moreover, integration of magnetic particles with modern biotechnological methods has led to the development of some of the most powerful next generation technologies for both DNA sequencing and biomarker quantification.

Magnetic particles suffer from two main limitations. The first problem is that colloidal stability is very difficult to achieve in biological media. Particles are subjected to high salt concentrations and a complex array of biomolecules, often resulting in aggregation. Secondly, structural anisotropy of the particles means that, in the presence of a magnetic field, there is a preferred axis of magnetisation, which results in an orientational bias. This bias impedes rotational motion of the particle which is often critical for ligand receptor reactions, resulting in a loss of reactivity.

These limitations provide the context for this work. Effort will be focused upon the development of new magnetic particles which have a higher stability and capacity to react unhindered when exposed to a magnetic field. Drawing inspiration from the receptor bearing cell membranes in nature, one route is to create particles with ligands at a liquid interface which diffuse freely. After reviewing the state of the art for magnetic particle in first chapter, the second chapter will explore emulsion theory and how functionalised fluid interfaces may be prepared. The results of formulation are then discussed in the third chapter. In the fourth and final chapter the emulsion particles are applied to a model assay system, using flow cytometry to measure the concentration of analyte objects.

Chapter 1 Colloids in Biotechnology

In this introduction, the contribution of material science to the field of biotechnology will be explored, notably through particulate materials and their applications. Discussion will then hone in on magnetic particles and how their properties have revolutionised the field of biotechnology. The theory describing magnetic particle behaviour will be covered initially, before looking in detail at the various applications. Particular emphasis will be placed on in vitro technologies, and the role magnetic particles play in such systems. This chapter will conclude by looking at other novel particle types and postulating how they may be combined with magnetic particle to create new functional materials.

1.1 Materials Science & Biotechnology

Great advances in biotechnology are often heralded by the development of advanced new materials. Materials science has a lot to offer biologists, providing access to materials that are unavailable in nature and being able to introduce novel properties into biological systems. Colloidal materials are particularly useful in this regard.

1.1.1 Colloidal Materials

Definition: A colloid is a unique state of matter in which one phase is dispersed into another as discrete particles having a size range of 1 - 1000 nm. Many different types of colloid can be classified, each combining different states of matter. For biotechnological applications one of the phases will be aqueous making the colloid either a sol (solid particles dispersed in a liquid phase) or an emulsion (immiscible liquid particles dispersed in a liquid phase). The utility of colloids comes from their unique size related properties.

Intermediate Scale: Colloids have an intermediate scale, lying in between the molecular and macroscopic. At this scale their motion is predominantly Brownian and they are able to efficiently interact with molecules. Their size generally lies above the diffraction limit of optical techniques moreover they can easily be observed or measured using light scattering. Colloids can therefore be used as a tool to observe molecular interactions.

Physical Properties: At the colloidal scale the bulk properties of materials often break down, giving rise to completely novel properties and behaviours. Properties may include:

- Quantum effects, such as those exhibited by fluorescing quantum dots [1].
- Unique electronic and optical properties, such as those of colloidal gold [2],[3].
- Unique magnetic properties (superparamagnetism) of certain metal oxides (see below) [4].

In addition, their small particle size gives them a huge surface to volume ratio, a property very useful for surface-based reactions.

Biological properties: The scale of colloids means they can often be introduced into biological systems for a wide range of applications and they are able to achieve good bio-distribution. Small particle sizes help to limit toxicity and mean that particles can be eliminated by the immune system.

Colloids of water (emulsion) can be used to replicate vivo conditions in vitro or to partition aqueous samples. Fluorocarbon oils for example can form robust aqueous emulsions, isolating cell or biomolecule populations for high throughput screening [5], or observing cell behaviours or population dynamics. This type of emulsion also forms the basis of digital QPCR [6].

1.2 Magnetic Particles

Magnetic particles have emerged as a particularly useful tool in biotechnology. The power of magnetic particles lies in their ability to apply forces very selectively within a system. Truly magnetic materials are rare in nature, so magnetic particles can be manipulated independently of the whole. As with the previously mentioned example, finely divided magnetic particles exhibit entirely different properties from bulk magnetic materials; they have no permanent magnetic moment (a property known as superparamagnetism) and so do not experience a force until a magnetic field is applied.

1.2.1 Structure & Properties

Superparamagnetism: The key property of colloidal magnetic particles is <u>superparamagnetism</u>. Superparamagnetism is a size-dependent property which arises in certain magnetic materials. The atoms in such materials have an electronic configuration which has a net spin (due to unpaired electrons), resulting in a magnetic moment. In these materials, an exchange interaction causes the magnetic moments of the atoms to align. If the magnetic moments are all parallel, the material is

ferromagnetic and if they have an alternating antiparallel arrangement, then it is called antiferromagnetic. If there are two populations of atoms with unequal magnetic moments which align antiparallel, then the material is ferrimagnetic.

Regions of isotropic magnetic alignment are called domains. The uniform alignment of the magnetic moments within a domain creates magnetic field extending out from and into the material (magnetostatic energy). The magnetic field (energy) may be reduced if the domain splits into two smaller domains, with oppositely aligned magnetic moments. However, there is an energy cost to changing the direction of adjacent dipoles. For a domain to split, the energy saved by reducing the magnetic field must exceed the cost of reorienting the dipoles. Domains therefore have a minimum size below which they can no longer efficiently split. If the particles of a ferromagnetic (or ferrimagnetic) material are small enough, they will consist of just a single domain, typically grains 3 – 50 nm in diameter.

Anisotropy of the grain's crystal structure results in an 'easy' direction of magnetisation, an axis of magnetisation which is energetically favourable. This means that the magnetisation has two stable orientations which are antiparallel along the easy axis. It is possible for the magnetisation to flip from one orientation to the other. For such small particles consisting of relatively few atoms, ambient temperatures are sufficient to cause this transition and so they are said to have no net magnetisation.

When the particles encounter a magnetic field, they will initially gain a magnetisation in the direction of the field. The particles respond to the field by attempting to rotate and align their easy axis with the field. Fixed magnetisation will persist only for as long as the field is present. If the magnetic field is removed, the grain will return to the 0 net magnetisation state. It is this property which is known as superparamagnetism. There are several different materials which can exhibit this property, although by far and away the most commonly used are the iron oxides; magnetite (Fe_3O_4), and maghemite ($Y-Fe_2O_3$) owing to their ease of synthesis and the general abundance of iron.

Superparamagnetism is completely analogous to paramagnetism where the atoms of a material each have a magnetic moment but are all randomly aligned until exposed to a magnetic field. Instead here it is magnetic grains which can reorient, so the response (the susceptibility) is far higher, hence the term 'super'.

Superparamagnetism is a useful property because it effectively allows magnetic character to be turned on and off using a magnetic field. An applied magnetic field can be used to manipulate the particles in various ways. If the applied magnetic field has a gradient, then the particle will experience a force, moving to follow the gradient. Similarly, if exposed to a rotating magnetic field, the particles will experience a torque. If two particles are at a close distance, they will experience a repulsive force perpendicular to the field but an attractive force parallel to the field which causes them to form chains.

Many superparamagnetic particles grouped together in a matrix can be used to create materials with extremely high magnetisations because, when a magnetic field is applied, all magnetisations will be in parallel. This behaviour can be seen in Figure 1.1.



Figure 1.1 - Magnetic behaviour of superparamagnetic oxide grains embedded in a polymer particle. At rest the particle has no net magnetization due to decorrelation between the magnetic moments of the grains. In the presence of a magnetic field, they align themselves with it giving a net magnetization. Figure courtesy of [7].

Low Toxicity: The potential effects of nanoparticles are highly variable, the general trend is that cellular and immune responses depend highly on the surface treatment of the particles, which dictates how they are encountered by the body. In comparison to other elements, iron oxide particles are considered to be far less toxic. The toxicity of the particles can be reduced further by coating the particles with biocompatible materials such as dextran, polyethylene glycol or silica, these coating have a second purpose of stabilising the particles under harsh biological conditions (see section 1.3.2).

1.2.2 Magnetic Particle Applications

Ferrofluids: Highly concentrated colloidal dispersions of stabilised magnetite nanoparticles act as a magnetic liquid. They were originally conceived by scientist working for NASA as a means to manipulate rocket fuel in a weightless environment. For this particular application, it was passed upon. However, in the aerospace industry, they have been found to form effective dampeners. Ferrofluids are also used to create a liquid seal around the spinning drive shaft of a hard disk. In addition, they are effective heat transfer agents – the particles obey curies law, becoming less

magnetic as their temperature increases. A magnet will thus more strongly attract cooler particles and so, can be placed near a component which requires cooling. This technique is often used to cool the voice coil in loudspeakers.

The properties of the magnetic particles make them particularly relevant for biomedical applications because their ability to respond to remote stimuli makes them an excellent non-invasive therapeutic tool.

1.2.3 In Vivo Biomedical Applications

Superparamagnetic nanoparticles work very well for in vivo applications where their small size gives them excellent biodistribution properties and means that they can be cleared from the body by the Reticuloendothelial System (RES), a network of cells and tissues which have a range of functions, one of which being the ability to clear foreign particles from the body by phagocytosis.

Magnetic Activated Cell Sorting (MACS): Miltenyibiotec is a company which develops superparamagnetic nanoparticles for cell capture. Nanoparticles are functionalised with ligands to target specific cells within a sample [8]. The cell mixture can then be run through a high gradient magnetic column so that only the targeted cells are retained. An advantage to using such small particles is their minimal toxicity, meaning captured cells can be reinjected back into a patient.

Magnetic Resonance Imaging: Magnetic resonance imaging (MRI) uses a magnetic field and powerful pulses of radio frequency to generate detailed images of the body. MRI often used in conjunction with injected substances known as contrast agents which can improve visibility of the targeted tissues. The magnetic properties of the particles make them particularly good contrast agents; an example of this is the Lumirem particles supplied by Guerbet.

Magnetofection: A technique by which a magnetic field is used to bring magnetic nanoparticles into contact with cells for the delivery of nucleic acids. Following delivery, cells uptake the nucleic acids by endocytosis. Magnetofection is a relatively new technology developed by Ozbiosciences.

There are also a number of perspective superparamagnetic magnetic particle technologies in the developmental stage:

Magnetic Hyperthermia Treatments (Future): When exposed to a rapidly alternating magnetic field, the particles experience a hysteresis causing their temperature to rise. The goal of a hyperthermia treatment is thus to deliver and concentrate magnetic particles within tumour tissues,

where an external alternating magnetic field can be applied, using the particles to raise the temperature of the surrounding cancerous cells and destroy them.

Targeted Drug Delivery (Future): Here the goal is to conjugate the particle to a drug, or drug delivery vehicle, which is then injected into the circulatory system. An externally applied magnet is used to accumulate the particles at a targeted location. Some systems are structured so that hyperthermia of the particles can trigger the release of the drug.

1.2.4 Microbeads & In Vitro Technologies

In vitro applications such as diagnostics are not limited by size, larger particle are advantageous because they are more easily detected optically and can be comprised of many superparamagnetic nanoparticles, giving them a stronger response. Micro-scale magnetic particles (also known as microbeads) are created from polymers matrices which contain dispersions of superparamagnetic nanoparticles. The principle manufacturers of such particles are Dynal (Life Technologies) and Ademtech. Biotechnological applications of microbeads include:

Assay Phase: Magnetic particles have been used to greatly improve the performance of particle based assays. In sandwich assays, magnetic actuation allows grafted particles to quickly be concentrated at the reaction site, or chained together if it the assay is agglutination based. Assays are also often based around particles grafted with DNA strands for the capture of specific sequences. Moreover magnetic particles are also far easier to separate and wash using a simple magnet instead of centrifugation. This application is explored in greater detail in Chapter 4.

Magnetic Separation: Microbeads can also be functionalised to perform separation and immunoprecipitation. Because they have a stronger response, separation is achieved very rapidly using a simple magnet.

Automation: Since magnetic beads can be manipulated remotely and they greatly reduce the number of handling steps, magnetic beads are frequently incorporated into automated devices and play an important role in several of the latest diagnostic devices.

1.2.5 Examples of Magnetic Bead Based Technologies

There are a staggering range of bead based technologies, to exemplify their importance two recent examples are described:

DNA Sequencing - 454 Sequencing: In all branches of biology, it is critical to be able to determine base pairs sequences within an organism. One of the first and most powerful next generation sequencing technologies to emerge in recent years has been 454 sequencing. The technology is based around a digital polymerase chain reaction (PCR) technique.

Fragmented DNA is captured and sequenced using beads grafted with complementary strands [9]. These fragments are captured with a ratio of one strand per bead. Emulsification (see Chapter 2) is used to isolate each bead into a water droplet. The captured DNA is then amplified using PCR. Eventually the droplets are broken and each bead with its amplified strands is captured into an individual well (~29 μ m) on a fibre optic slide (PicoTirePlate).

Each well is then also loaded with a mix of enzymes (DNA polymerase, ATP sulfurylase, and luciferase) allowing parallel pyrosequencing to be performed. Washing the sample with each nucleotide in turn generates fluorescence in the samples where those nucleotides combine with DNA. The intensity of signal is proportional to the number of complementary bases present in the DNA. The process is recorded and the plate fluorescence is read by a GS FLX system.

Overall, each bead has a separate strand and is amplified in isolation, so each well ends up with several thousand copies of the same DNA sequence, giving a very specific signal to that region. Software is able to reconstruct the sequence from the fluorescent signal pattern. In this manner, the machine is able to sequence up to 600 million base pairs (with read lengths of 400 – 500 pairs). The technique is shown in Figure 1.2.



Figure 1.2 - 454 Sequencing Technology. DNA is captured using magnetic beads (1 strand/bead), beads are emulsified to compartmentalise them for DNA amplification. Emulsions are broken and amplified bead are captured into picotitre wells along with enzymes to enable pyrosequencing. Left: Non-sequential images of the 454 process. Right: well fluorescence resulting from pyrosequencing. Images courtesy of [9] and [10].

This technique is an excellent example of the power of material science in biotechnology, using both magnetic beads and the compartmentalisation effect of water droplet emulsions. The emulsion allows individual DNA sequences to be isolated, while the magnetic beads allow the amplified clonal DNA to be directed to individual wells. Such rapid compartmentalisation is only possible using superparamagnetic beads.

Biological Assay - Philips Magnotech: At the point of writing this device is in the final stages of development [11–13]. The Magnotech is a microfluidic cartridge based device for the detection of biomarkers within biological media. It consists of two components, a cartridge and a reader. The cartridge is essentially a microfluidic chip into which the biological sample is drawn where presence of the biomarker causes a change that can be measured by the reader component.

Within the cartridge the sample mixes with a population of antibody coated magnetic beads which capture the target antigen. In a second step, the antigen coated particles are attracted by a small electromagnet to one surface of the chip which is coated with a second antibody against the target. The resulting sandwich complex sticks the bead to this surface. An opposing electromagnet is used to 'wash' the surface, pulling away any non-specifically bound beads.

The number of specifically captured beads is counted using an optical technique based upon frustrated total internal reflection, which is performed by the reader. The surface of the cartridge is designed such that, at a certain angle, a beam of light can be internally reflected without loss of signal. When beads are captured at the surface, they interfere (frustrate) with this reflection by absorbing or scattering some of the light, resulting in a loss of signal. The loss of signal will be proportional to the number of beads at the surface, providing a count for the number of antigen molecules in the sample. Sensitivity will depend on the exact target. However the device is generally able to reach sub-picomolar detection limits.

Magnetic beads are a critical component of this technology. Firstly, they accelerate the rate of immunocomplex formation. Secondly, their presence at the surface creates the signal which measures complex formation. Thirdly, they ensure that non-specifically adsorbed particles can be easily removed.



Figure 1.3 - Philips Magnotech Device. A biological sample is introduced into the chip, a target molecule is captured by antibody grafted magnetic beads. The binding magnet pulls beads to the antibody grafted surface, forming sandwiches. The wash magnet remove non-specifically bound beads. Bound beads are counted using frustrated total internal reflection, giving a signal proportional to target concentration. [14].

These examples demonstrate how magnetic beads enable some very powerful technologies. The performance of these technologies is very much tied to the properties of the beads, which must be highly magnetic, reactive and stable. In the following section, the materials science behind each of these properties will be considered.

1.3 Materials Perspective

1.3.1 Synthesis of Magnetic Particles

The first step is to synthesize the magnetic nanoparticles. There are several routes to this, which will be outlined below. Following synthesis, and depending on the requirement, nanoparticles may then be grouped together to form microbeads. In later steps, additional surface treatment can enhance particle stability. Finally, after stabilisation, particles can be functionalised with targeting ligands.

Superparamagnetic Nanoparticles: Magnetite nanoparticles can be obtained through a range of methods. Classically bulk samples of magnetite were milled with an appropriate dispersing agent [15–17]. In recent years, chemical routes have been developed such as co-precipitation reactions of ferrous (oxidation Fe^{2^+}) and ferric (oxidation Fe^{3^+}) iron salts [18],[19] or thermal decomposition [20].

In all cases, reaction parameters are adjusted to control particle size and keep the size distribution as narrow as possible. These reactions produce magnetite (Fe_3O_4), which gradually oxidises to the more stable maghemite (γ -Fe₂O₃). Following synthesis, nanoparticles are given a surface treatment (see 1.3.2) and functionalised. Miltenyi for example use dextran-coated nanobeads functionalised with antibodies for magnetic activated sorting of cells.

Microbeads: The main challenge of microbeads is to homogeneously disperse the magnetite nanoparticles into a polymer matrix and for this matrix to be converted into a uniform population of particles. Combining these steps is a challenge and for this reason, there are relatively few commercial suppliers of microbeads.

Two of the most well-known are Ademtech and Dynal (Life Technologies), each with their own strategy for particle synthesis. Both companies use emulsion templating to create polymeric particles (as it is one of the most reliable ways to highly uniform particles), the difference lies in the introduction of the magnetic material. Ademtech essentially emulsify ferrofluids of volatile oil, which evaporates to leave behind a solid core of magnetic nanoparticles. In a second step, this core is coated with a polymer shell [21],[22]. Dynal (Life technologies) first create monodisperse polymeric particles. A high particle porosity means that coprecipitation of iron salts results in the formation of magnetite directly within the polymer particles [23–25]. In both cases, the particles must be subsequently functionalised with the active species or precursor functional groups which facilitate bio-conjugations.

1.3.2 Particle Stability

Stability is one of the biggest hurdles facing colloids which come into contact with biological media. Achieving stability in a simple buffer can often be challenging, so complex biological fluids are considered to be particularly harsh for colloids.

Biological Interactions: The base composition of a biological medium is generally an aqueous solution containing high concentrations of various ions (blood for example contains 140 mM Na⁺ and 105 mM Cl⁻, alongside numerous others) which screen electrostatic repulsions causing particles to aggregate.

The diverse array of proteins present in biological media (10546 to date in the human plasma proteome [26]) also pose a hazard. Many of these proteins may destabilise particles through depletion or surface interactions (for more on these see aggregation, 2.1.1 and [7] for a more exhaustive study). Moreover in vivo, interacting proteins may tag particles for recognition by the Reticuloendothelial System (RES), resulting in elimination by phagocytosis. 'Bare' magnetite particles are particularly vulnerable to these processes but they also affect the aforementioned microbeads.

Destabilisation will detrimentally affect any application which uses the particle. For example non-specific aggregation within an affinity assay (such as the Magnotech device) gives rise to false positives. Figure 1.4 shows non-specifically adsorbing particles.



Figure 1.4 - Interference mechanisms of non-specifically adsorbing species in particle based immune-assays, A -non-specific bridging, B - surface adhesion.

Aggregation also inhibits particle reactivity; flocs are more limited in surface area, being larger they do no diffuse as rapidly and overall more prone to sedimentation. Great care is taken to protect particles against such interactions. Stability is usually enhanced by modification of the particles surface.

Chemical Degradation: Magnetite nanoparticles are chemically quite reactive, being susceptible to oxidation or acidic corrosion. At such small scales, a good proportion of the atoms are at the particles surface, chemical reactions with these atoms will weaken the magnetic properties of the particle. Degradation can also result in the release of iron ions released into solution, interfering with biochemical reactions. Iron ions for example can bind to and inhibit enzymatic reactions (such as those involved in PCR). These processes can be mitigated by entrapment in a polymer matrix.

Surface Treatment: The high salinity of biological media usually favours stabilisation by large uncharged molecules such as polyethylene glycol (PEG) or dextran. Long polymers such as these have a propensity to repel when brought into close approach preventing aggregation if they are attached to the particle (steric repulsion). In vitro, this effect can also be achieved using non-ionic surfactants which feature similar polymeric groups (they are usually too damaging to biological systems to be used in vivo).

Competitive adsorption (blocking) may also be used to protect particle surfaces, in such a case particles are pre-treated with inert proteins such as Bovine Serum Albumin (BSA) which bind to the surface and occupies free sites which might otherwise be claimed by more disruptive biomolecules.

Biological media such as blood are so complex that surface treatment can only reduce non-specific interactions. There is no single optimal surface treatment so stability remains a major limiting factor for colloids in biotechnology.

1.3.1 Biofunctionalisation

Biomolecule conjugation is also an important consideration; the surface of the particle requires specific functional groups for the coupling reactions which usually link to specific amino acid residues (lysine, cysteine, or tyrosine) present at the surface of the biomolecule. The structure of the polymer may inherently feature the required groups; otherwise additional components must be mixed in or applied to the surface of the particle.

1.3.2 Magnetic Particle Limitations

Other limitations relate to the reactivity of particles. For colloids such as grafted magnetic particles, reaction kinetics of are directly correlated to their size. Magnetic particles can rapidly be brought into contact with a reaction site. Rotational diffusion however, is still required at close distances for a grafted ligand to bind to its receptor (needing a specific orientation). For larger particles these motions become very small greatly increasing the reaction time.

This problem is aggravated by anisotropy of size and distribution of the embedded magnetic particles, creating an easy axis of magnetisation within the microparticle. Under an applied field, the particle will rotate to align itself with this axis, maintaining this orientation until the field is removed. This alignment restricts Brownian rotation, so even if the particle is brought near to a captured analyte, discord between the position of its ligands and its orientational preference can prevent reactions from occurring (see Figure 1.5). This effect is present even for relatively small magnetic beads; evidence of this is shown in the Magnotech system [11] where actuation of beads close to the surface is necessary to encourage efficient capture.



Figure 1.5 - When a magnetic field gradient is applied, the grafted particle is pulled to the surface. The particle rotates to align its easy axis of magnetisation with the field. This orientation may not match the alignment required for the ligand to react.

One solution to this problem is to mimic nature where cells perform specific recognition reactions using receptors embedded in fluid membranes. If ligands are able to move independently of the particle as a whole then a reaction should always be possible.

1.4 Liquid Particles

There are several different ways to create liquid particles, their unique properties gives each type a range of applications.

1.4.1 Types of Liquid Particle

The concept of liquid particles is not new, the two main types being oil in water emulsions and liposomes. Emulsions consist of stabilised droplets of an immiscible liquid phase (see Chapter 2) whilst liposomes are large multi-layered aggregates of amphiphilic phospholipids. Both types of particle are easily formed from wholly biocompatible materials thus having a wide range of in vivo applications.

1.4.2 Technological Applications

In vivo: Bilayer forming phospholipids are used to trap small aqueous volumes, acting as carriers for hydrophilic drugs [27]. Emulsions of hydrophobic materials on the other hand (such as vegetable oils) can be used to transport lipophilic drugs [28],[29]. Both types of particle may also be used as adjuvants [30], improving the efficacy of vaccines. Emulsions of various kinds of oils have also found other uses, perfluorocarbons are particularly inert whilst being able to dissolve large quantities of oxygen, allowing them to be used as artificial blood substitutes [31].

1.4.3 Biofunctionalisation

All naturally occurring liquid interfaces are functionalised with some kind of ligand or receptor. Mimicking these interactions has helped to model and study the mechanics of cell adhesion [32], cellular forces [33], rheology of actin networks [34], phagocytosis [35] and phase separation of the lipid species within a membrane [36].

Several projects have indicated that this may be a promising use of emulsion. Hadorn et al have created populations of oil droplets in water decorated with DNA oligonucleotide chains. Two populations of droplets coated with complementary oligomer chains form aggregates which are reversible upon changing temperature, electrolyte concentration and the addition of excess free nucleotides [37]. Since then Feng et al. [38] have used a similar system to demonstrate finer control of droplet assembly using a size controlled populations and oligonucleotide coated nanoparticles to induce adhesion between homogeneous populations. Emulsion droplets with conjugated antibodies have also been developed for intravenous drug delivery [39].

1.4.4 Magnetic Emulsions

In Vivo: Magnetic emulsions (produced by the emulsification of ferrofluids) offer a viable alternative to classical solid magnetic carriers. So far, they have been proposed as intravenous agents, acting as carriers for lipophilic drugs [40],[41] and MRI contrast agents function even at low magnetic particle loadings [42].

Materials: Magnetic emulsions can have a range of material uses too. As discussed previously Ademtech uses emulsification of ferrofluid for the formation of diagnostic magnetic particles [21],[22]. The chain forming behaviour of these emulsions under an applied field can be used to assemble emulsions into larger structures, which can be held together by attractive interactions induced by a polymer to create nanowires [43].

Magnetic content can also be used to create extremely monodisperse populations of droplets. Larger particles, containing a larger volume of magnetic content are more susceptible to a given magnetic field. At a low strength of magnetic field, only particles above a certain size will form chains and clusters. Chains and clusters will sediment/cream much more rapidly than single particles thus populations of particles can be sorted by size. Repeating this process several times with the separated fractions allow the creation of highly monodisperse populations of particles [44], this is completely analogous to the fractionated crystallization which can be performed to purify colloids using depletion forces (see 2.1.1) imposed by surfactants or polymers [45].

Contrary to solid biofunctionalised magnetic particles, there has not yet been any functionalisation development or applications using their liquid counterpart.

1.5 Novel Magnetic Particles for Bioassays

Magnetic particles have unique properties which are hugely beneficial to the field of biotechnology. Despite the advantages, there remain several limitations to be addressed, relating to stability within biological media and orientation trapping by applied magnetic fields. One solution would be the creation of a new generation of liquid magnetic particles. Magnetic nanoparticles in a liquid environment should limit field trapping whilst ligand diffusion at a liquid interface ensures recognition reactions can always occur. In the subsequent chapters, the production of such particles will be explored in detail, followed by a study of their properties and potential application as a diagnostic tool.

Chapter 2 Emulsions & Formulation

In this bibliographic chapter, emulsions will be introduced and explored as a means to replicate the magnetic particles employed in biotechnology (results begin in Chapter 3). The transient emulsion state will be defined and explained both in terms of thermodynamic and kinetic stability, examining in detail the mechanisms by which it destabilises, with the goal of maximising its lifespan.

The next section will elaborate upon the behaviour of surface active molecules, their role in emulsification and the various types which exist, closing with an explanation of the surfactant choices within this project. A brief discussion will be given to the dispersed phase and how it can be used to introduce more complex characteristics to the emulsion; in this case ferrofluid, making the system magnetically responsive.

Having covered the constituent materials of the emulsion, their interaction with incident light will be described and the quantitative information which can be derived from these interactions. In the final section, different emulsification methods will be evaluated, with particular focus placed upon microchannel and membrane emulsification and their relevance to this project.

2.1 Definition, Requirements & Types

To create particles presenting a liquid interface, emulsions will be used. An emulsion is a dispersion consisting of two immiscible liquids, where one of the liquids (the dispersed phase) is dispersed as droplets into the other (the continuous phase). Immiscible phases remain separated due to unfavourable molecular interactions at the interface (the interfacial tension). Phases held together by one type of molecular interaction (polar interactions) often disfavour forming other kinds of bonds and so have a higher cohesion for themselves than with the molecules of the other phase.

2.1.1 Emulsion Stability

The existence of the interfacial tension means that there is a high cost energy cost associated with creating new interfaces. The free energy change of emulsification therefore is positive overall; emulsification requires an input of energy. Being at a higher energy state, the emulsified regime is thermodynamically unstable and is driven to reduce excess interfacial tension by separating into its constituent phases. There are two main mechanisms by which separation can occur:

Coalescence: If two droplets encounter one another, at a close distance, the continuous phase between them is reduced to a vanishingly thin film. During coalescence, this film ruptures, allowing the contents of the two droplets to combine into a single larger droplet (Figure 2.1). Rupture begins with an appearance of a thermally nucleated hole (pore) in the film, most likely arising from an inhomogeneity such as a dust particle or surface active molecule (see Figure 2.1) [46]. This process will continue until all droplets have combined into the single separated phase.



Figure 2.1 - Coalescence: (A) two droplets approach, (B) A film is formed between the droplets, (C) film ruptures, (D) the two droplets coalesce.

Coalescence is dependent on the rate of drainage of the continuous phase film between the droplets, the total area of the film formed and the deformability of the droplets. The rate of coalescence is greater for larger droplets which have larger surface areas and are more deformable.

Coalescence can be impeded by the use of surface active species (surfactants), which adsorb at the interface. Surfactants at the interface of adjacent droplets prevent them from merging. Surfactants form a layer of like charges or large polymeric groups which repel each other, preventing close approach of the interfaces. Moreover, the geometry of the surfactant can determine the size of the pore requires for coalescence, if only a low curvature is permitted, the pore must be relatively large, requiring more energy and reducing the overall probability.

Viscosity also strongly affects the rate of coalescence. An emulsion is more stable when the continuous phase has a high viscosity since the droplet collision rate will be reduced as well as the rate of continuous phase drainage between approaching droplets. A highly viscous continuous phase helps during emulsification, where emulsions of both phases are continually produced and coalesce. Increasing the viscosity of one phase pushes the equilibrium towards dispersion of the other phase. Viscosity is most commonly increased by the addition of a thickening agent to the desired continuous phase.

Ostwald Ripening: Ostwald ripening is a spontaneous, thermodynamically driven process, where slight solubility of the dispersed phase into the continuous phase allows transport from smaller droplets to more energetically favourable larger droplets (Figure 2.2).



Figure 2.2 - Ostwald Ripening. A Laplace pressure gradient causes the transfer of soluble material from smaller droplets to larger droplets.

It is driven by the Laplace pressure (the pressure difference between the inside and the outside of the curved droplet interface). The Laplace pressure is given by the Laplace equation:

(1)
$$P=2\gamma/r$$

(Where, P is the Laplace pressure, $\boldsymbol{\gamma}$ is the interfacial tension and r is the droplet radius)

The pressure experienced increases with decreasing radius, which has a strong influence on the rate of diffusion of oil from the droplet. This is described by the Kelvin equation:

$$RT \ln \left[\frac{C_r}{C_{\infty}}\right] = 2\gamma V_m/r$$

(2)

(Where, C_r is the solubility of a dispersed phase particle of radius r, C_{∞} solubility a particle of infinite radius, γ is the surface tension, r is the droplet radius and V_m is the molar volume of the disperse phase)

This shows that rate of diffusion of the oil from a droplet is greater when the radius is smaller. As a consequence there is a diffusion gradient of material from smaller to larger droplets.

The final parameter which can affect the rate of Ostwald ripening is the viscosity of the continuous phase. This is described by the Stokes-Einstein equation:

$D = k_b T / 6\pi \eta r$

(3)

(Where, D is the diffusion coefficient of a droplet and η is the continuous phase viscosity).

Increasing viscosity reduces the rate of diffusion, and thus the dissolution rate of material from the dispersed phase and its diffusion rate through the continuous phase, slowing the Ostwald ripening.

Since it is a diffusive process, the ripening rate also depends heavily on the molecular weight (MW) of the dispersed phase molecules. Systems with a high MW dispersed phase, with minimal continuous phase solubility are the most resistant to this kind of destabilisation (conversely due to its low molecular weight and slight solubility in most liquids, emulsions with water as the dispersed phase are particularly vulnerable to this process).

Ostwald ripening can be slowed greatly by the addition of an continuous phase-insoluble species, to the dispersed phase [47]. The insoluble species cannot escape the droplet and so Ostwald ripening must now overcome an entropic penalty of demixing if the dispersed phase diffuses out of the droplet.

Ostwald ripening eventually becomes insignificant with larger and larger droplets; the increase in size however renders the droplets more susceptible to other modes of destabilisation. There are other destabilisation mechanisms by which emulsions can destabilise without complete phase separation:

Creaming/sedimentation: Creaming and sedimentation are processes by which dispersions of particles separate via gravity, due to differences in density. If the dispersed phase is less dense, buoyancy of the droplets will cause them to 'cream' rising to form a condensed upper layer of particles (Figure 2.3). If the continuous phase is less dense, then the droplets will 'sediment' to the bottom of the sample.



Figure 2.3 - Creaming, a difference in density between the dispersed phase and the continuous phase will result in separation. If the dispersed phase is less dense it will cream upwards.

The rate of creaming/sedimentation is described by the Stokes equation:

(4)
$$v = \frac{2r^2(\rho - \rho_0)g}{9\eta}$$

(Where, υ is the creaming (or sedimentation) rate, r is the droplet radius, ρ is the density of the droplet, ρ_o is the density of the dispersion medium, η is the viscosity of the dispersion medium/continuous phase and g is the local acceleration due to gravity.)

Creaming/sedimentation brings the droplets into close contact making, them more susceptible to destabilisation by Ostwald ripening and more particularly coalescence. Buoyancy/weight exerts a force on the droplets, pressing them together. If the droplets are sufficiently stabilised with surfactants and insoluble species, the droplets will resist coalescence and may be redispersed by agitation.

Aggregation: Aggregation (otherwise known as flocculation or coagulation) occurs when attractive forces between the droplets overcome the repulsive forces. Mutually attracted droplets aggregate together into grape-like bunches (also known as flocs or clusters). As with creaming/sedimentation, this process can accelerate coalescence or Ostwald ripening, but droplets may also persist as clusters provided they are sufficiently stabilised. Aggregation is shown in Figure 2.4.

There are several situations which result in aggregation. Droplets are stabilised by charged species, (electrostatic repulsion) are susceptible to an increase in the salt concentration (ionic strength) of the dispersed phase. Additional salt ions contribute to the charge screening, decreasing the Debye radius around the droplet. Below a certain radius, the droplets can approach close enough for the short ranged Van der Waals attraction to dominate and they clump together.

Aggregation can also be driven by *depletion*, an entropic force which can be caused by smaller soluble particles in the dispersed phase. Very small particles (depletants) are excluded from the vicinity of larger particles at close distances to one another. If two large particles approach one another at a distance smaller than the diameter of the depletant, they are driven to stick together because it frees volume that would otherwise be unavailable to the depletant (being too small to fit into that space). Depletion is more significant for larger particles because they create larger excluded volumes.

Another aggregation mechanism is *bridging*, where particles do not directly attract one another, but are joined together by an attractive species (such as a particle or polymer) which is large enough to 'bridge' adjacent droplets. This is a common destabilisation mechanism for colloids brought into contact with complex biological media and often referred to as nonspecific aggregation. Bridging can be made highly specific; if particles are grafted with antibodies for example, bridging will occur only in the presence of the antigen. This is the principle of the agglutination assay, explored in Chapter 4.



Figure 2.4 - Aggregation (coagulation/flocculation), Attractive forces between droplets overcome repulsive forces causing droplets to cluster together into flocs/aggregates.

Evaporation: If the emulsion is exposed to air and the dispersed phase is volatile, it is possible for evaporation to occur; naturally this process occurs more rapidly from a cream where oil droplets are concentrated at an air/water interface. The rate of evaporation can be reduced by using low volatility dispersed phases.

Stable Emulsions: The stability of the emulsified state is increased by using components which are minimally affected by the destabilisation mechanisms; however the system remains thermodynamically unstable and will eventually separate (it is only the timescale over which this

separation will occur which has changed). Emulsions taking a long time to separate are considered to be kinetically stable or metastable.

2.1.2 States of Emulsification

Depending on their size and composition, emulsions can be classified as one of the following:

Macroemulsions & Nanoemulsions – These emulsions consist of kinetically stabilised mixtures of two or more phases (discussed above). One phase is dispersed as droplets into the other (continuous phase). Such a system is thermodynamically unstable and the phases will eventually separate by the previously discussed mechanisms. If the emulsion consists of droplets in the micrometre size range it may be called a *macroemulsion*. If the emulsion consists of droplets below 1 µm in diameter, it may be called a *nanoemulsion*.

Microemulsion – A microemulsion (or bicontinuous emulsion) is a unique type of emulsion which is thermodynamically stable. The dispersed phase droplets range in size from 1 to 100 nm. Such systems often occur within narrow temperature ranges of surfactant rich regimes (typically not less than 20 % in mass, combining two or more surfactants). A simplified explanation is given by the Gibbs free energy equation (5).

 $\Delta G = \Delta H - T \Delta S$

(Where ΔG is the free energy of formation, ΔH is the enthalpy of formation, T is the temperature and ΔS is the change in entropy)

Under such conditions, the interfacial tension becomes very low, making ΔH effectively negligible. The free energy of formation is given directly by the entropy of the system, which is very high (the droplet dispersion is much less ordered than the separate phases), thus the free energy has a large negative value, so emulsification occurs spontaneously.

Direct versus Indirect - Emulsions are most commonly formed between water and another immiscible liquid, such as an oil and thus each of the above can be further classified depending on which phase constitutes the continuous phase, existing as either direct (Oil in Water, O/W) or reversed (Water in Oil, W/O) or bicontinuous emulsions (see Figure 2.5). The bicontinuous state is synonymous with the microemulsion state (discussed above).



Figure 2.5 - Emulsion type depends on which phase is dispersed into which. When one phase is water, emulsions can be either O/W (oil in water) or W/O (water in oil).

Nanoemulsions and microemulsions are interesting systems with a wide range of applications. However, they are not applicable within the scope of this project. The nanometric size mean they do not efficiently scatter light and would less useful for generating signal or viewing microscopically. Furthermore, small volumes limit the amount of magnetic material which can be contained within them and limiting their magnetic response. Object permanence is also a consideration - this project intends to use droplets as unchanging particles, which is not the case for microemulsions, where droplets may dissolve and reform spontaneously.

2.1.3 Project Implications

The desired emulsion is therefore a macroemulsion comprised of droplets with a scale comparable to magnetic microbeads (1 -10 μ m). For this reason, the most important factor to control is droplet coalescence which is more of a threat to stability than Ostwald ripening at this scale.

2.2 Surfactants & Stability

As discussed previously emulsions, are unstable systems, they are almost always stabilised through the use of surfactants which have several important roles.

2.2.1 Surfactant Function

Surface active species (surfactants) are amphiphilic; they contain two or more moieties, each having a high affinity for one of the emulsion phases. The dual affinity causes the surfactants to arrange themselves at the interface, lowering the interfacial tension by reducing the number of unfavourable molecular interactions between the immiscible phases. The presence of surfactants at the interface serves two functions:

- Lowering of the interfacial tension, between the phases and reducing the energy required for emulsification.
- Creating a barrier against the coalescence of droplets, kinetically stabilising the droplets.

2.2.2 Surfactant Types

Since the magnetic particles operate in aqueous systems, formulations must be based upon the O/W structure. To produce an O/W, emulsion there are hundreds of different surfactants available which generally fall into one of two categories, *ionic* or *non-ionic*. Ionic surfactants contain a charged moiety for their hydrophilicity and are either cationic, anionic or zwitterionic depending if they are positive, negative or a mixture of charges respectively (see Figure 2.6).

Ionic surfactants prevent the coalescence of droplets through the electrostatic repulsion of like charges. Non-ionic surfactants contain polar hydrophilic moieties capable of forming hydrogen bonds, allowing strong interactions with the aqueous phase. The polar groups are usually large polymers which prevent coalescence through steric repulsion.



Figure 2.6 - Surfactant types, surfactants classified by the nature of their headgroup, surfactants can be charged (ionic, positive being cationic, negative being anionic and both charges are zwitterionic). Surfactants without charged groups are known as non-ionic.
Interfacial Tension: The interfacial tension (also known as surface tension) is the measure of the cohesive force of liquids and therefore also their resistance to mixing with unlike phases. Its dimension is the force per unit length (equivalent to energy per unit area) with a unit of Newtons per meter (N/m). The interfacial tension for various liquids is most often given for the air-liquid interface. The pure air-water interface, for example, has an interfacial tension of 72 mN/m at 25°C. If a surfactant is added to the water, the interfacial tension will decrease.

Interfacial tension decreases in proportion to the concentration of surfactant added, reaching a minimum value). Air-water interfacial tension minima reached by some common surfactants are shown in Figure 2.7.



Sodium dodecyl sulphate (SDS) a common anionic surfactant ~ 25 mN/m [48].



Polyoxyethylene (20) sorbitan monolaurate (Tween 20) a nonionic surfactant ~ 25 mN/m [49].



Poloxamers: A family nonionic of 'triblock- polymers' surfactants consisting of a block of polypropylene oxide, flanked by two chains of polyethylene glycol. Interfacial activity depends on the ratio of the components and generally in the range of 30 - 50 mN/m [50].

Figure 2.7 - Common surfactants and their interfacial activity.

Comparing the interfacial tensions in Figure 2.7 to the interfacial tension of the pure interface (72 mN/m) shows that surfactants can have a profound effect upon the interfacial tension, decreasing it by 50 % or more. The concentration required to do so is usually quite small and typically in the mM range or below. The extent to which the interfacial tension is lowered is determined by the

properties of the surfactant, for example its size or the absolute affinity of each moiety to its preferred phase.

2.2.3 Phospholipids

When selecting surfactants for this project, the primary concerns are biocompatibility and the ability to functionalise and remain at the interface. For these reasons, phospholipids make an excellent choice. Phospholipids consist of a hydrophilic negatively charged phosphate head-group, with (usually) two hydrophobic fatty acid chains (general structure shown in Figure 2.8).

The dual tailed structure gives the phospholipid a predominantly hydrophobic character, as monomers they are sparingly soluble (DPPC and DSPC have aqueous solubilities of 10^{-10} M and 10^{-20} M respectively [51]) helping the molecule remain at the interface. The large hydrophobicity makes them quite effective at lowering the interfacial tension between water and other phases. The presence of phospholipids can lower the air-water interfacial tension to ~24 mN/m [52].

There are a range of commercially available phospholipids, with various functional groups: Conjugated fluorescent dyes can help to reveal lipid localization, polyethylene glycol chains (PEG/PEG), which can create a steric barrier or act as a flexible linker grafted ligands and biotin which is an excellent model ligand in conjunction with the protein streptavidin. Moreover, phospholipids add a degree of biomimicry and biocompatibility to the system as they are a major constituent of cell membranes. Examples of phospholipids are shown in Figure 2.8.



Figure 2.8 - Phospholipids with a variety of functionalities: Above - 1,2-di-(9Z-octadecenoyl)-*sn*-glycero-3-phosphocholine (DOPC), Below – 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[biotinyl(polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG(2000)-Biotin). Images courtesy of [53].

2.2.4 Micelle Formation

Surfactants will generally have an unequal affinity for each phase so surfactant in excess will partition into the phase to which it has the highest affinity. There, excess surfactant molecules will exist as individual monomers. Above a critical concentration, these monomers will aggregate into complex structures, grouping phobic moieties together away from the continuous phase. The size and shape of the aggregate will depend on the type and concentration of the surfactant. Surfactant aggregation usually begins with the formation of spherical aggregates known as micelles (Figure 2.9).



Figure 2.9 – Surfactant behaviour, surfactant molecules occupy the interface, with excess existing as monomers, which aggregate into micelles above a critical concentration.

The concentration at which this self-assembly occurs is called the critical micelle concentration (CMC). The CMC usually coincides with the point of minimum interfacial tension, because it is the highest possible concentration of surfactant monomers. Adding additional surfactant results in more micelles so increasing concentration above the CMC will have no effect or only slightly decrease the interfacial tension. Emulsions are usually maintained using a surfactant at a concentration above its CMC. CMCs for the example surfactants in Figure 2.7 are 8 mM, 60 µM and 0.39 mM for SDS, Tween 20 and poloxamer 407 at 25°C. The CMC will vary with temperature and, for ionic species; it is also affected by the presence of electrolytes.

There is also a minimum temperature required for micelles to form, known as the critical micelle temperature (CMT or Krafft temperature). Below this temperature, micelles will not form; surfactant molecules will crystallise together and flocculate out of solution.

2.2.5 Reversibility

Surfactants tend to exhibit a dynamic behaviour in solution, both micelles and interface occupation may be temporary as one surfactant molecule is displaced by another or gains sufficient kinetic energy to remove itself from the micelle or interface. Reversibility is a critical parameter in this project since the emulsion will be functionalised using surface active species which are required to remain at the interface.

Reversibility will depend on the molecular weight of the surfactant and its phase affinity. Low molecular weight surfactants such as SDS and tween 20 are highly reversible, larger polymeric surfactants such as high molecular weight poloxamers show an irreversibility of adsorption which increases with increasing molecular weight [54]. For reversible surfactants, high concentrations (>CMC) will ensure the interface remains saturated.

2.2.6 The Bancroft Rule

Surfactant phase affinity often determines the type of emulsion formed- this was first observed by W. Bancroft who noted that:

"A hydrophile colloid will tend to make water the dispersing phase while a hydrophobe colloid will tend to make water the disperse phase." [55]

In other words, the system is in general most stable when the surfactant is in the continuous phase. This rule can be explained by the emulsification process, without surfactant, when the two phases are mixed, emulsions of both types are formed. The coalescence of both the direct and indirect emulsion is in equilibrium. When a surfactant is added, coalescence is prevented in one direction, resulting in persistent droplets.

2.2.1 Hydrophobic Lipophilic Balance

An approximation for the relative phase affinity of a surfactant can be obtained by calculating its hydrophobic-lipophilic balance (HLB). HLB is calculated from the molecular mass ratio of the respective hydrophilic and hydrophobic moieties:

$$(6) \qquad HLB = 20 * M_h/M$$

(Where $M_{\rm h}$ is the molecular mass of the hydrophilic moiety and M is the molecular mass of the hydrophobic moiety)

Using this equation gives a number from 0 – 20 (it can sometimes be higher), 0 corresponding to a totally lipophilic molecule and 20 being completely hydrophilic, surfactant with an HLB < 10 will be oil soluble (insoluble in water) and surfactants with HLB > 10 will be soluble in water (insoluble in oil). To stabilise an O/W emulsion, high HLB surfactants are required.

Although real systems are often more complicated, the HLB system is usually able to predict a number of behaviours. The previous surfactant examples SDS, tween 20 and pluronic F127 have HLBs of 40, 16.7 and 18 – 23, meaning they would all be suitable for the stabilisation of O/W emulsions.

2.2.2 Pickering Emulsions

Particles may be used to form emulsions in place of surfactants, if particle size and contact angle lies within a certain range, surface energy may be lowered by occupation of the liquid-liquid interface, just like a surfactant, in this manner they can enable emulsion formation. The structure of a Pickering emulsion is shown in Figure 2.10.



Figure 2.10 - Pickering Emulsion, an emulsion stabilised by particles jammed at the interface, the outer layer is no-longer fluid as a result.

Pickering emulsions are not useful here; the particles at Pickering interfaces are generally jammed and immobile, deviating from the liquid interface that is the goal of this project.

2.3 The Nature of the Dispersed Phase

As discussed previously, the nature of the oil has a strong influence in the destabilisation mechanisms through its density relative to the continuous phase as well as its solubility. Preference will be given to oils with a higher molecular weight since chain length is inversely proportional to aqueous solubility (see 2.2.3) [56]). The dispersed phase is also important because it usually constitutes the functional or active part of the emulsion. In drug delivery for example, the dispersed oil phase is used to dissolve and transport lipophilic compounds to their target.

The dispersed phase is not limited to simple liquids, interesting and complex materials can be made by emulsification of colloids, or even emulsions (to create multiple emulsions). In this project, along with the ligands, a colloidal dispersion of superparamagnetic nanoparticles (a ferrofluid) will be introduced into the dispersed phase to give the droplets a magnetic character.

2.3.1 Ferrofluids

Ferrofluids consist of stable colloidal dispersions of superparamagnetic iron oxide (magnetite, Fe_3O_4) nanoparticles within a fluid (a colloidal sol). The particles have a size range of 3 - 50 nm meaning that each consists of a single magnetic domain. At this scale, the magnetisation of the domain constantly flips under the influence of temperature. When a magnetic field is applied, the magnetic moment of each nanoparticle aligns with the field and there is a net magnetisation of the fluid.

The small size of the particles means there is no separation due to gravity. Aggregation of the particles under the applied field is prevented either using surfactants or chemical grafting to the surface.

Magnetite nanoparticles can be readily synthesized through different methods (see Chapter 1), the most classical are milling of larger particles [14-16] and co-precipitation reactions of ferrous (oxidation Fe²⁺) and ferric (oxidation Fe³⁺) iron salts [18], [19], shown in equation (7).

⁽⁷⁾ $2FeCl_3 + FeCl_2 + 8NH_3 + 4H_2O \rightarrow Fe_3O_4 + 8NH_4Cl$

Following synthesis, the nanoparticles are given an appropriate surface treatment for dispersion in the desired liquid medium. For dispersion into an oil, hydrophobic nanoparticles are required. The standard procedure for creating hydrophobic particles is to coat them with oleic acid; the carboxylic acids group chelates to the metal oxide surface [57], presenting an outward hydrophobic tail, which mixes with the oil but sterically repels between adjacent particles. The oleic acid acts as an irreversibly bound surfactant, lowering the surface tension between the non-polar oil and the charged surface of the metal oxide.



Figure 2.11 - Oleic acid coated magnetite nanoparticles, oleic acid chemically bonds to the magnetite surface. The long aliphatic carbon chain is miscible with many hydrocarbons, permitting the particles to be suspended in solvents such as octane or kerosene. The fatty acid chains also provide steric repulsion between the nanoparticles stabilising them against aggregation.

2.4 Quantifying Emulsions

Emulsions are typically characterised by their volume fraction, mean droplet size and size distribution.

2.4.1 Volume Fraction

Due to the aforementioned destabilisation mechanisms, emulsions are dynamic systems where the total number of droplets may change over time. It is therefore not very useful to measure the concentration of the emulsion by the absolute number of droplets. Concentrations for emulsions are usually given by its volume fraction (φ_i), which is the volume of the dispersed phase divided by the total volume of the system.

2.4.2 Mean Size & Size Distribution

It is important to know both the size and range of droplet sizes within an emulsion. Size and distribution may be important for the application, recording how they change over time provides information on the stability of the emulsion.

Mean Size: The mean size is simply the average droplet size of the population.

Size Distribution: Size distribution is given by the size dispersity (Đ) of the sample, which, providing the range of particle sizes follows a Gaussian distribution, is given by:

It is generally expressed as a fraction ranging from 0 to 1, the closer the value is to 0, the more uniform the sample is in size. Dispersity is also sometimes expressed as a coefficient of variation in which case the value is expressed as a percentage.

2.4.3 Light Scattering

For many applications, both particle size and size uniformity are important parameters, they are most commonly measured using light scattering. Liquid phases are usually optically clear, being permeable to light. If the liquid contains particles (sol or emulsion droplets) then light passing through the sample will encounter such an object and become deflected and/or absorbed, this phenomenon is known as scattering. Light scattering occurs because the refractive index of the particle is different to that of the dispersed phase. When light passes through an emulsion sample, the diffraction pattern it creates is related to the size and refractive index of the droplets.

Size measurement: The degree of scattering in both cases depends upon the particle size, both of these techniques may be used to infer particle size by measuring the light scattered by particle dispersion. In general a laser is passed through the suspension, when passing through the sample, light will be scattered at small angles by larger particles and larger angles by smaller particles. Mie theory is then applied to the angular scattering intensity data (using software) to provide size as a volume equivalent sphere diameter and distribution of sizes (uniformity). For accurate calculation, the refractive indices of both the dispersed and continuous phase are required.

The diameters of macro emulsion droplets generally lie in the micrometre range (μ m). The light scattering profile of a polydisperse emulsion is shown in Figure 2.12. In this project particle size and size distribution is measured using a Malvern Mastersizer 3000.



Figure 2.12 - Light scattering profile of a polydisperse emulsion measured using a Malvern Mastersizer. Left: polydisperse emulsion (Mean diameter = $6.77 \mu m$, D = 2.066).

2.5 Creating an Emulsion

An emulsion is created by generating new interfaces. The presence of a surfactant greatly lowers the energy required to create new interfaces and controls the stability of the emulsion. Surfactants in general do not greatly influence the droplet size and distribution (provided there is enough available to cover the entire interfacial area created). Droplet size depends much more on the method of emulsification.

2.5.1 Emulsification Theory

The emulsified state is at a higher energy than the separated system because of the excess of interfacial tension – it is thermodynamically unstable and will not occur spontaneously. To transition from two separate phases to an emulsified phase, an input of energy is therefore required. There are various ways to input this energy, most of them apply a form of shear stress across the (to be) dispersed phase. When the shear stress exceeds Laplace pressure, the droplet of dispersed phase is ruptured into smaller droplets.

2.5.2 Emulsification Techniques

The method by which shear stress is applied to the system can vary greatly and has a huge influence on the size and dispersity of the droplets produced. Examples include: **Mortar & Pestle** – A simple tool which allows shear to be applied by hand. The resulting emulsion has a wide size distribution; this distribution is not ideal for accurate diagnostic tests, but acceptable for rapidly assessing the quality and effects of various formulations.



Figure 2.13 - Mortar and Pestle, allows shear to be generated by hand. The resulting emulsion is highly polydisperse but can be produced very rapidly.

Couette Cell – This is a device based upon the principle of Couette flow, a laminar flow of a viscous fluid held between two parallel plates where one is moving with respect to the other. Flow is driven by the viscous drag force acting on the fluid and the applied pressure gradient parallel to the plates. The device maintains a constant Couette flow using a rotating cylinder (stator) within a precisely engineered cylindrical cavity [21],[58] (shown in Figure 2.14).

The cell can create much higher shear than can be generated by hand, and far greater control is possible, with the shear being proportional to the rotational speed of the stator. The resulting emulsions are smaller and more monodisperse. Under optimal conditions, it can achieve a dispersity as low as 0.05, however the size uniformity depends a lot on the nature of the oil [58]. The couette cell is in fact a secondary emulsification system- a premix emulsion (a lightly emulsified sample) is introduced into the system and, using controlled shear, is processed into a much more monodisperse population.

In principle, this technique may be useful for producing monodisperse emulsions; however it requires relatively large volumes at high volume fractions of dispersed phase, potentially consuming excessive quantities of reagents.



Figure 2.14 - Couette cell, applies controlled shear to premixed emulsions using a rotating cylinder (stator). It is able to achieve greater size uniformity than by hand. Left: premix injected into the cell, Right: Cell structure and cross section, image courtesy of [59].

Sonication: Sonic energy (ultrasound with frequencies >20 kHz) can be used to create very small emulsion droplets. The ultrasound is applied to the sample through an oscillating metal tip, here shear stress arises from a process known as acoustic cavitation;

"the formation, growth and subsequent collapse of microbubbles caused by the pressure fluctuations of the acoustic wave. A collapse event causes extreme levels of highly localised turbulence – an implosion on a microscopic scale. It is the accumulation of many thousands of these miniature implosions that forms the basis of ultrasonic homogenization." [60]

This process is extremely disruptive, resulting in the production of tiny droplets. Ultrasonic cavitation is so efficient at breaking up the dispersed phase that droplet size becomes limited by the relative concentration of the surfactant, which may be insufficient to cover such large interfacial areas. Sonication is therefore the technique of choice for producing nanoemulsions. Droplets produced by sonication are typically tens to hundreds of nanometres in diameter, with a dispersity of 0.1 - 0.15. A sonication device and the cavitation process are depicted in Figure 2.15.

The main drawback of sonication is that it generates a lot of heat and the sample must often be cooled. Furthermore, the stress placed upon the oscillating tip causes degradation, often polluting the sample with metal particles. This project is focused upon macroemulsions, thus sonication will not be employed.



Figure 2.15 Probe - Sonication, Sonic energy is applied to the sample via a resonating metal tip, such rapid vibration within the sample crates microscopic bubbles which instantly collapse releasing a lot, of energy – a process known as cavitation. Image courtesy of [61].

High Pressure Homogenisation: In high pressure homogenization, the two phases (or a crude premix) are forced to flow through an inlet valve into a mixing chamber under high pressures. The mix is subjected to elongation, shear flows, cavitation and impacts, which fragment the dispersed phase into small droplets.

Size distribution can be reduced by passing a sample through the homogenizer multiple times. The large energies involved usually result in the creation of nanoemulsions, a size range which is considered sub optimal for the application of this project. Moreover high pressure homogenizers are usually industrial scale devices, designed to be used with large volumes, this is not practical for the specialized materials used in this project.



Figure 2.16 - High pressure homogenisation, homogeneous samples are created by forcing a rough premixed emulsion through a pore of defined size under high pressure. Image courtesy of [62].

This project has very precise requirements for the emulsification technique which should be capable of producing monodisperse emulsions using low volumes (<2 mL) quantities of oil. These constraints preclude most of the above techniques. Fortunately, there exists an emulsification technique where a membrane with a uniform pore size can be used to create size calibrated emulsion droplets. This technique is known as membrane emulsification.

2.6 Membrane & Microchannel Emulsification

In membrane emulsification, the (to be) dispersed phase is fed though a microporous membrane into a rapidly cross-flowing continuous phase. Microchannel emulsification covers a several techniques which use micrometre scale channels to mix the two phases.

2.6.1 Membrane Emulsification

As the dispersed phase passes through the membrane, droplets will grow from pore openings until they reach a certain size and detach, resulting in a drop-by-drop production, this process can be seen in Figure 2.17. The technique was developed by Nakajima et al. in the late 1980s using a special porous glass (Shirasu porous glass - SPG). SPG is created from the spinodal decomposition and acid treatment of a calcium alumino boro silicate. The glass is named after the Shirasu volcanic ash from which it is refined.



Figure 2.17 - Membrane emulsification, oil is forced through pores into a crossflowing continuous phase (left). SEM image of Shirasu glass structure (right), image courtesy of [67].

Droplet size is controlled by the pore geometry [63] and shear flow of the continuous phase [64],[65]. If the pores are uniform in size, and constant shear is maintained (continuous phase flow), the resulting emulsion will be highly uniform. The SPG membrane is typically able to achieve a variation coefficient of around 10 % [66].

2.6.2 Microchannel Emulsification

Membrane emulsification lead to the development of microchannel (microfluidic) emulsification, which initially created a similar effect using microchannels produced via semiconductor technology (silicon photolithography and etching) [68]. Lithography permits a much greater control over pore geometry and has allowed a more detailed study of this kind of emulsification.

It was subsequently found that the system could be improved firstly by switching to oblongshaped channels [69] and then further by having an asymmetric architecture, with a circular narrow channel introducing the dispersed phase into the oblong 'slit' before it encounters the continuous phase [70], similar to a step (coining the term step-emulsification). These modifications have been used to achieve coefficients of variation of less than 2 %.

Droplet formation is capillary-driven, triggered by confinement-release; the distorted finger of dispersed phase proceeding into the continuous phase is spontaneously broken into a spherical droplet by interfacial tension [71]. Distortion is controlled by the aspect ratio of the oblong slit and there exists a threshold of approximately 3, below which only large and non-uniform droplets are formed [72]. Moreover, there must be a sufficiently large height difference between the inlet and

continuous phase channels (large channel must have twice the height of the inlet slit) for the droplet size to be independent of step height [73]. There also exists a flow velocity threshold above which droplet size is no-longer controlled by channel geometry, becoming large and polydisperse [74].

The change in pressure created as the droplet emerges into the larger chamber results in a shear stress and the droplet is broken by the stream. With a constant pressure applied to the dispersed phase, this process occurs at an extremely regular frequency and the size of the droplet is determined by the aspect ratio of the step and main chamber. This results in an emulsion with a very narrow size distribution.

Overall the low volume processing and narrow size distribution make microfluidic step emulsification an excellent choice for this project.

Flow Focusing: Microchannel chips permit other kinds of emulsification. In flow focusing [75], shear stress is applied to the dispersed phase by co- or counter flowing streams of continuous phase. Compared to membrane/step emulsification, it is more dependent on the flow ratio of the various phases. The flow-focussing method will be neglected in this project in favour of step-emulsification – changing the dispersed phase oil in a geometry controlled device will have less influence on the size of the droplets produced and will require less balancing of the flow rates.

2.6.3 Microfluidic Chip Fabrication

Microfluidic chips can be made out of various materials. Transparent materials are favoured because they allow the channel interior to be observed using a microscope. The most commonly used material is a thermally curable polydimethylsiloxane (PDMS) elastomer. PDMS chips are much easier to create than other material because the liquid elastomer can be very easily moulded using silicon wafers. A relief of the desired microchannel geometry is first patterned onto the silicon wafer using photolithography (Figure 2.18). Once created, a silicon wafer can be used to create any number of PDMS chips.

Photolithography: To create the microchannels photolithography, is used to template a relief onto a silicon wafer. In brief, a photo-curable polymer (photoresist) is homogeneously deposited on the wafer surface. UV light is then applied through a mask of the design (curing only the desired pattern). Excess photoresist is removed, leaving only the cured relief on the wafer. The finished

mould is then used to template the relief into the liquid PDMS, which cures into a hardened elastic material in the presence of heat and crosslinking agent (cured in an oven for 2 hours).

The cured, patterned PDMS can then be activated using plasma, to disrupt the silicon-oxygen bonds at the surface. These bonds will gradually reform, but if the chip is placed against another, siloxane surface (PDMS or glass) the chip will irreversibly bond to that, allowing the creation of sealed channels. Figure 2.18 shows the photolithography process.



Figure 2.18 - Basic photolithography procedure for forming a PDMS mould, spincoating allows uniform deposition of a photo-curable polymer (photoresist) onto a silicon wafer. The resist is patterned using a UV light applied through a mask. Unreticulated material is removed and the resulting relief is used as a template to print patterns into PDMS elastomer. Plasma treatment of the PDMS activates the surface allowing it to irreversibly bond to glass, forming closed microchannels. Image courtesy of [76].

2.6.1 Limitations of Microfluidic Emulsification

Despite the advantages offered by microfluidic emulsification, there are a number of limitations which should be taken into consideration:

Solvent Compatibility: The drawback of microchannel emulsification is that the commonly used PDMS elastomer exhibits a limited material compatibility. Certain solvents infiltrate and swell the PDMS elastomer, this swelling was recently demonstrated to be related to the solubility parameter ratio of the elastomer and the solvent, a higher ratio results in less swelling [77]. Naturally, even a

small amount of swelling is disastrous for a device where monodisperse production is dependent upon the geometry. A change in the channel dimensions will result in a change the size of the droplets produced. Swelling will be controlled by testing oil compatibility.

Surface Treatment: Microchannel/step emulsification requires that the channel surface and the dispersed phase have opposite affinities (i.e. O/W emulsions, the channels should be as hydrophilic as possible) to minimise wetting by the dispersed phase. During plasma bonding, PDMS is initially rendered hydrophilic during the plasma treatment of the bonding process. Over time the hydrophilicity diminishes as the Si-O bonds reform, allowing the oil to wet the surface.

To maintain the hydrophilicity, the PDMS surface can be treated with the hydrophilic polymer polyvinylpyrrolidone (PVP K90) shortly after plasma treatment [78], which reacts with the surface and provides a hydrophilic steric barrier. In brief, after bonding, the chip is flushed with a 3 % w/v solution of PVP for 2 hours. Excess PVP is rinsed away using water (2 hrs). Finally, the chip is dried overnight using airflow (protocol developed in group by Ladislav Derzsi). A bonded and surface treated chip can be seen in Figure 2.19.



Figure 2.19 - A bonded PDMS chip

Production Rate: The size of the channel (μ m) means that compared with bulk techniques, the throughput is very low. The overall rate of droplet production (necking time) is determined by the continuous phase viscosity/interfacial tension ratio (with a minor correction determined by the absolute viscosity ratio of the two phases) [79]. Typical production rates range from 2 – 15kHz [80], depending on the nature of the dispersed phase, this gives an overall production yield of 0.002 - 0.015 mL per hour. This yield is very low compared to other emulsification techniques, but can be improved by several orders of magnitude by using a chip with multiple channels in parallel.

2.7 Emulsions & Formulation Conclusion

Emulsion science is a well-established field which has enabled the production of emulsions for a huge range of applications. There is almost no limit to the materials which can be combined for the creation of novel emulsions. Emulsification is more limited by production technique since there is almost always a trade-off to be made between size, polydispersity, rate of production and cost.

In this project, the goal is to create a complex emulsion with well-defined properties, consisting of biofunctionalised droplets which contain a magnetic material. Emulsions will be functionalised using phospholipids, while ferrofluid will be added to provide the magnetic character. Rapid emulsification can be performed by hand to test the basic properties of the emulsion (stability, magnetic response and ligand receptor interactions). Having found an ideal combination of components, the formula can then be used with a microfluidic emulsification device for the production of size calibrated droplets. The following chapter will detail this formulation process in a step-by-step manner.

Chapter 3 Formulation Results

3.1 Introduction

In this chapter, it is demonstrated how the principles discussed in chapter two can be applied to produce functional magnetic emulsion droplets. Formulation begins with the creation of stable emulsions. Complexity is then gradually increased as various components are added to the system. The first major component to be introduced is the phospholipid layer which contains the functional ligands of the droplet. Fluorescent labelling permits the lipid behaviour to be observed, revealing lipid-cosurfactant interactions and ultimately allowing a stable formulation to be found. The stable formulation is then applied to a microfluidic device which is used to produce size-calibrated functionalised emulsions.

Ligands are added to the emulsion by introducing biotinylated lipids into the oil, which are able to capture streptavidin at the droplet interface. Capture efficiency is probed using fluorescentlylabelled streptavidin, highlighting several subtle interactions between the cosurfactant and the ligand. Size calibrated droplets are used to perform a quantitative analysis of capture efficacy, comparing ligand and analyte concentrations. In the final, section magnetic content is introduced into the system using a ferrofluid. Ferrofluid stability is demonstrated as an important parameter for both microfluidic production and droplet reactivity.

3.2 Preparation of a Bulk Emulsion

Before advancing to more complex components and emulsification techniques, it must first be shown that stable emulsions can be produced bearing a layer of phospholipids at the interface.

3.2.1 Using a Cosurfactant

To perform reactions between droplets in the aqueous phase, the emulsion must be oil in water (O/W). For the particles to behave in the desired manner, the functionalised surfactant must remain in the oil droplet or at the interface; free ligand in the continuous phase will react with the target, interfering with agglutination. For functionalisation, phospholipids have been selected as an appropriate surfactant, owing to their availability, high lipophilicity and biomimicry.

Phospholipids have higher affinity for the oil phase than for the aqueous phase. This ensures ligands are not lost into the continuous phase. The drawback of this is that lipophilic surfactants drive the formation of reverse emulsions (recalling the Bancroft rule) while, in this project a direct emulsion is required. To compensate for this effect, a secondary surfactant (known as a cosurfactant) will be added to the aqueous phase to encourage the formation of an O/W emulsion.

In terms of properties, the cosurfactant should also be uncharged, since biomolecules owe their activity to a precise three dimensional structure arising from charge interactions. These interactions can be disrupted by extreme pH or the presence of charges species, thus it is desirable to avoid the use of ionic surfactants. Formulation will therefore be focused on non-ionic surfactants, which have the added advantage of being far less sensitive to pH or salt concentration.

3.2.2 Preparation of a Bulk Emulsion

For basic formulation testing, bulk emulsions are prepared using a mortar and pestle. The oil phase is added dropwise to a volume of continuous phase containing $1 \, \% / v$ non-ionic surfactant and $10 \, \% / v$ polyvinylpyrrolidone (PVP K90) as a thickener, (chosen for its rapid solubilisation). Shear stress is applied by periodically grinding the two phases with the pestle until a homogenous emulsion is achieved.

3.2.3 Emulsion Stability

Before other factors are considered, a useful emulsion should have a high degree of stability. It was found that stable emulsions could be created from a range of oil and surfactant combinations including mineral oil, soybean oil, medium chain alkanes (10 – 16 carbons). Cosurfactants used include tween 20, pluronics (poloxamer 407), pluronic F68 (poloxamer 188), synperonic P84, the Brij series and triton X-100. Destabilisation kinetics were not studied in detail, creaming occurred quite rapidly but could be reversed by agitation of the sample. Microscopic analysis of the emulsions however showed little change over several months.

3.2.4 Phospholipid Preparation

As the functionalising agent, the first step is to ensure stabilisation of functional phospholipids at the interface. Oils containing phospholipids are prepared by a modified lipid film rehydration technique. Phospholipids (dissolved in chloroform) are added to a small glass container, chloroform is removed

under a stream of nitrogen, creating a dry lipid film. Finally the oil phase is added to the container and the lipid film is dispersed by a combination of vortexing, heating and sonication. This oil can then be emulsified to produce a phospholipid containing emulsion.

Phospholipid Solubility: Phospholipid solubility proved to be a reoccurring issue, many of the phospholipids exhibited a limited solubility in the oil phase. To better understand the solubility behaviour, a range of phospholipids were investigated. It was found that lipid solubility is heavily influenced by the degree of acyl chain saturation. Adding lipids to a sample of oil at 1.3 mM it was found that fully saturated phospholipids such as 1,2-dioctadecanoyl-sn-glycero-3-phosphoethanolamine (DSPE) are insoluble, whilst lipids with some degree of unsaturation including 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), soybean lecithin, L- α -phosphatidylcholine (Egg PC) and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) can be dissolved fully.

This relationship between solubility and acyl saturation is most likely due to a variation in the critical micelle temperature of the phospholipids (recalling section 2.2.4), with saturated lipids such as DSPE being below the CMT at room temperature. Acyl chain saturation is known to influence the phase transition temperature (PTT) of the phospholipid [81].

As amphiphilic compounds, phospholipids are driven to assemble together in monolayers or bilayers with heads and tails aligned. These layers can exist in two phases, depending on the relative mobility of the phospholipids. At low temperatures, the layer is said to be in a gel phase. The acyl chains are fully extended and closely packed with the lipids locked together. If the temperature is raised sufficiently, the acyl chains lose can gain sufficient energy to overcome the interactions holding them in place. They become fluid and can diffuse freely throughout the layer. The temperature at which this occurs is called the phase transition temperature (PTT). The PTT is influenced by the chemical structure of the lipid, particularly by the length and saturation of the acyl chains which determine geometry and ability to interact with neighbouring lipids.

Saturated hydrocarbons consist of chains of carbon atoms. Bonds around the tetravalent carbon atom adopt a tetrahedral configuration to minimise electrostatic repulsion. Single carbon bonds are able to freely rotate so, connected into a chain, these tetrahedrons arrange into staggered 'zig-zag' to avoid bond overlap. Alternating between just two orientations gives the chain an overall straight structure, combined with the flexibility of the single bonds allows the chains to pack closer together. Close packing increases the Van der Waals attraction and therefore melting point, which increases proportionally to the carbon chain length (conformations shown in Figure 3.1).



Figure 3.1 - Structural conformations of single bonded carbon chains. Left-tetravalent arrangement of carbon in methane molecule. Centre- Newman projection showing end-on view of C-C bond and orientation of bonded groups. Right- structure of a chain of single carbon bonds (C_6H_6 , Hexane).

In an unsaturated hydrocarbon, two or more of the carbon atoms are joined by a double bond. Rotation is restricted around the double bond, so two isomers are possible. It is a *cis* isomer if both carbon chains occur on the same side of the double bond and *trans* if they occur on opposite sides (configurations show in Figure 3.2).

In the trans configuration, the orientation of the C=C is not significantly different from the C-C bonds, so the trans isomer has similar properties to the saturated chain. The cis configuration forces a break in the regular 'zig-zag', putting a 'kink' into the chain. The presence of such kinks means the chains pack together less efficiently resulting in weaker Van der Waals interactions and lower melting points than the saturated forms.



Figure 3.2 - Cis/Trans Isomers of Oleic Acid. The Cis-isomer (below) has functional groups (carbon chains) on the same side of the double bond- giving a 'kinked' structure. Trans-isomer (above) has functional groups on opposite sides, giving a similar 'zig-zag' structure to the saturated chain.

In nature, all unsaturated fatty acids (and therefore phospholipids) are cis. In lipids, the effect of the double bond is particularly pronounced compared to other parameters; decreasing the acyl chain length by one carbon may reduce the melting point by up to 10°C, adding a double bond may decrease it by up to 63°C [81].

Results indicate that saturation has a similar effect on the CMT and oil solubility. At room temperature, efficient packing of the saturated acyl chains means that the lipid-lipid bonds are stronger than the lipid-oil bonds so the phospholipid is insoluble. Unsaturated chains cannot pack so efficiently, lipid-lipid interactions are weaker than oil-lipid interactions and dissolution of unsaturated phospholipids occurs. Lipid structures with their relative PPTs are shown in Figure 3.3.



Figure 3.3 - Examples of Phospholipid Saturation, Top: 1,2-di-(9Z-octadecenoyl)-snglycero-3-phosphocholine (DOPC), featuring unsaturated cis-isomer fatty chains, lowering it's critical temperature (-17°C) and increasing its oil solubility. Bottom: 1,2distearoyl-sn-glycero-3-phosphoethanolamine (DSPE), featuring saturated fatty acid chains, efficient packing gives a high phase transition temperature of 74°C. Images courtesy of [53].

From a commercial perspective, unsaturation is disadvantageous, since double bonds are prone to oxidation, limiting the lifespan of the product. Many of the functional lipids are based upon the saturated DSPE to maximize its shelf-life. This makes them difficult to dissolve, especially when they have hydrophilic functional groups such as PEG chains.

A solution to this problem was found by exploiting lipid mutual miscibility. Saturated and unsaturated lipids will generally mix together. Thus an excess of unsaturated lipid (Egg PC, POPC, DOPC, or soy lecithin) is added along with the functional lipid to ensure dissolution, typically at a mass ratio of 100:1 unsaturated lipid to functionalised lipid.

3.2.5 Visualisation of the Phospholipids

Following emulsification, it is important to verify that the lipids are at the interface. To achieve this, fluorescently tagged lipids can be introduced into the lipid mixture. Two such lipids are 1-myristoyl-

2-{6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl}-*sn*-glycero-3-phosphocholine (NBD PC) and Oregon Green[®] 488 1,2-Dihexadecanoyl-*sn*-Glycero-3-Phosphoethanolamine (shown Figure 3.4). Typically 1 w/v% of fluorescent lipids is introduced into the oil.



Figure 3.4 - Fluorophore tagged Phospholipids: Left - Oregon Green[®] 488 1,2-Dihexadecanoyl-*sn*-Glycero-3-Phosphoethanolamine, tagged on the phosphate head group. (image courtesy of [82]. Right - 1-myristoyl-2-{6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl}-*sn*-glycero-3-phosphocholine (14:0-06:0 NBD PC), tagged on the fatty acid chain. (image from [53]).

Images of an emulsion containing fluorescent lipids can be seen in Figure 3.5. When lipids are at the interface, the distinctive pattern can be seen with a maximal fluorescence towards the outside of the droplet. This is because the microscope has a focal plane which captures only a lateral slice of the droplet. The lateral interface fluorescence can be observed whilst interface above and below cannot be observed directly (though some out of focus fluorescence can be observed).



Figure 3.5 - Bulk emulsion containing fluorescent phospholipids. Localisation of the phospholipids is seen using fluorescently labelled lipids into. Emulsion containing Oregon Green lipid (left) and NBD (right). [100x magnification, 200ms exposure].

It was observed that in the presence of certain surfactants (such as tween 20 and SDS), the fluorescent signal of the emulsion diminishes very rapidly (within 1 hour- faster than would be

expected by ambient bleaching). If the emulsion is formulated with certain poloxamer surfactants, then fluorescence persists. These effects are shown in Figure 3.6.



Figure 3.6 - Emulsion fluorescence behaviour after 3 hours. Fluorescence diminishes rapidly in the presence of tween (Top Left: Tween 20 + Oregon Green lipid, Bottom Left – Tween 20 + NBD lipid). Fluorescence is unperturbed when a poloxamer cosurfactant is used (Top Right – PF127 + Oregon green lipid, Bottom Right, PF127 surfactant + NBD lipid). [100x magnification, 200ms exposure].

The rapid loss of fluorescence is attributed to mixed micelle formation:

Mixed Micelle Formation: The hydrophobic portions of both SDS and Tween 20 consist of carbon chains very similar to the phospholipids. It is likely that the carbon chains of the surfactant and the phospholipid can interact, with the lipid able to participate in surfactant micelles. Thus an exchange can take place between the phospholipids at the interface and the micelles of surfactant in the solution. The result is that surfactant monomers in the continuous phase (which is at much higher concentration than the phospholipids) exchange with phospholipids at the interface. The displaced lipids are solubilised into surfactant micelles, in a process known as mixed micelle formation [83].

This result is unsurprising as such surfactants are often used to lyse cells in biology [84],[85]. Such surfactants insert into the cell membrane, disrupting its structure, before causing its complete breakup through mixed micelle formation. This same effect appears to be occurring with lipid functionalised emulsions.

The persistence of fluorescence when the emulsion is formulated with certain poloxamer surfactants (tradename pluronic/synperonic) indicates that phospholipids are not removed from the interface. An explanation for this apparent compatibility is that the polypropylene glycol chain (hydrophobic group) of the pluronic is chemically different from the carbon chains of the phospholipids (being more polar) and so it does not efficiently mix.

Poloxamer surfactants are said to adsorb at interfaces by 'rejection-anchoring' [86] – the polypropylene glycol is neither soluble in the oil nor the water (phobic to both phases), preferring to remain at the interface between the two. This chemistry may also disallow it from mixing with the acyl chains of the phospholipids, preventing the formation of mixed micelles and substitution of the phospholipids at the interface.

This effect is known elsewhere in biology; contrary to other non-ionic surfactants, poloxamers have been shown to have beneficial interactions with cells, protecting them from shear stress during sparging [87]-[88]. Poloxamer 188 has even been observed to promote cell membrane repair after electropermiabilisation [89] and various other membrane injuries[90]. Further formulations will therefore be stabilised using poloxamer surfactants.

3.2.6 Poloxamer Stability Testing

Having discovered poloxamer phospholipid compatibility, a range of poloxamer surfactants were screened for their ability to form stable emulsions, using the protocol given in section 3.2.2. It is found that stable emulsions could be produced with P84, P123, P105, F68, F127, and F108. The general trend indicates that stable O/W emulsions are achieved using larger and more hydrophilic poloxamers (having a HLB value >10).

3.2.7 Bulk Formulation Summary

A stable functionalised bulk emulsion is produced using 0.5 mL of oil (chosen from alkane C10 – 16, Mineral Oil, Soybean Oil) containing 1.3 mM unsaturated lipid (soy lecithin, DOPC, POPC, Egg PC) and 10 μ M fluorescently labelled lipid (NBD, Oregon Green). Using a mortar and pestle, the oil in mixed dropwise into a continuous phase containing 10 w/v% PVP K90 (mw 360 000) and 1 w/v% poloxamer

surfactant (Pluronics P123, P84, F68, P105, F127, F108). This stable formulation can then be applied to a microfluidic emulsification system for the production of highly uniform emulsions.

3.3 Microfluidic Emulsification Results

Microfluidic droplet production was achieved using a parallelised chip designed in the group by Nicolas Bremond. Droplets are formed by passing the oil under a step into a crossflowing continuous phase. A cross-section of a single step can be seen in Figure 3.7. A range of geometries are possible with a H_1 height varying from $1 - 2 \mu m$. Variation of this height changes the diameter of the resulting droplets which lie between 6.5 and 10 μm . Depending on the oil, a single channel has a production rate of 20 – 50 Hz.



Figure 3.7 - Step geometry, shear is created by droplets emerging from the step into a flowing continuous phase. The size of the droplet is primarily controlled by the geometry. The result is a highly monodisperse population of droplets. $H_1 = 1 - 2 \mu m$.

To increase production, the chip employs a highly branched oil inlet channel, equilibrating the pressure of the dispersed phase as it passes through a membrane containing 1000 parallel channels (Figure 3.8).



Figure 3.8 - Parallelised step emulsification chip. Using a highly branched channel, pressure can be evenly distributed to 1000 channels, vastly increasing the production rate. Channel dimensions (width = 10 μ m, height = 2 μ m).

The chip is able to produce highly uniform droplets with an average coefficient of variation (CV) of 7 – 12 % leading to the crystalline structure shown in **Figure 3.9**. Crystallisation of spherical objects can only occur when the CV lies below a critical value of 6 – 12% [91]. The device has a total production rate of 15 – 40 kHz/device. On average the device processes oil at a rate of 35 – 50 μ L/h.



Figure 3.9 - Monodisperse droplets. Extreme regularity of the channels results in a highly uniform emulsion. Droplet production is shown on the left. Droplets are uniform enough to show crystalline periodic arrangement (centre). Size uniformity can be seen using from the light scattering profile, CV = 7.9 %.

3.3.1 Oil compatibility & Swelling

The most commonly used PDMS compatible oils are fluorocarbons. These, however, are unsuitable for this project as their inertness prevents them from solubilising either the phospholipids or the ferrofluid. The best compromise was found to be 2-octyl-1-dodecanol, a long chain aliphatic alcohol

which exhibits minimal swelling, stable long term production whilst being able to solubilise both ferrofluid and phospholipids.

3.3.2 Surfactant Limits

Another issue with the microchannel emulsification is that it necessitates the use of a low molecular weight surfactant (Tween 20 or SDS) – Poloxamer (P84, F127) alone results in wetting and unstable droplet production (shown in Figure 3.10). The necessity of a low molecular weight surfactant has not been studied in detail, but is believed to arise from the rate of diffusion to the interface, being smaller; these surfactants simply diffuse much more rapidly to the newly formed interface. SDS also has a higher surface activity than the pluronic surfactants. SDS seems to be the optimal choice because of its low molecular weight (containing no bulky PEG chain).

SDS also has a relatively high CMC (0.23 w/v%). This means a high concentration of surfactant monomers can exist in solution without threatening the removal of phospholipids though mixed micelle formation. It is found that 0.5 w/v% of SDS enables emulsification whilst avoiding removal of lipids from the interface via mixed micelle formation. SDS micelles can be removed in a second step by replacing the continuous phase or dilution to below the CMC.



Figure 3.10 - Wetting of the oil phase. Droplet emulsion production is ruined if the oil phase wets the chip. The oil phase is no-longer effectively shorn and large volumes bleed into the channel. Wetting occurs when the pluronic surfactant is used alone. The large size of the polymer and lower surface activity prevent effective emulsification.

3.3.3 Glass Chip

A large issue with microfluidic emulsification has been the limited compatibility of the PDMS with the oil phase. This issue was addressed by the use of glass microfluidic devices, bearing the same channel geometry as the PDMS device. Glass has a much higher resistance to organic solvents, allowing the emulsification of oils such as decane. Using oxidising agents such as piranha etch (H_2O_2 + H_2SO_4) the glass device may be cleaned after use and used for another production run.

Glass vs PDMS Devices: Based on solvent compatibility, the glass device appears to be the superior choice, however glass devices are expensive and the condensed structure of the glass means it takes a lot longer for the air to be expunged from the channels during introduction of the oil phase.

In this project, both devices have been employed. A PDMS device has been used to emulsify mineral oil, soybean oil and octyldodecanol. A glass device was used to emulsify decane and other members of the alkane family.

3.3.4 Calculating droplet concentration

With a uniform population of droplets, it is possible to make a good estimate for the number of objects in a sample. Concentration is calculated from knowing the total volume of the droplets in the sample (the volume fraction) and the volume of a single droplet.

In general the volume fractions of samples produced by microfluidics are unknown because production is controlled by pressure and not flowrate. However, samples of known volume fraction can be taken from the cream layer of an emulsion because spheres (droplets) always randomly pack with the same efficiency, 63.4 % [92], meaning a volume fraction φ = 0.634.

Being uniform, all droplets have the same radius. The total number of droplets is thus obtained by dividing the volume of oil in the sample, by the volume of a single droplet.

The cream is commonly diluted 40x to volume fraction $\varphi = 0.016$, which ensures droplets are dispersed enough to be observed individually under the microscope. Droplet diameter is measured to be 8.5 µm diameter (r = 4.25 µm), assuming size uniformity a 10 µL sample of $\varphi = 0.016$ emulsion will contain 4.93 x 10⁵ (0.82 fM), this can later be compared to object counts provided by flow cytometry (see Chapter 4). An accurate estimation for the number of droplets is useful for calculating ligand and possible protein coverage (see 3.3.6).

3.3.5 Functional Droplets

Functionalised droplets can be produced by processing an oil containing phospholipids in the same manner as section 3.2. Figure 3.11 shows a microfluidic soybean oil emulsion containing \sim 1% Oregon green labelled phospholipids (1.3 mM DOPC + 10 μ M of Oregon green lipid).



Figure 3.11 - Microfluidic emulsion functionalised with fluorescent phospholipids. 100x magnification bright field (left), 200 ms blue fluorescence excitation (right).

3.3.6 Overall Microfluidic Formulation

The overall formulations are as follows:

A reservoir of functionalised oil (Mineral Oil, Soybean Oil, 2-Octyl-1-dodecanol) containing 1.3 mM unsaturated lipid (soy lecithin, DOPC, POPC, Egg PC) and 10 μ M functionalised lipid (NBD, Oregon Green) is prepared with containing 3 w/v% ferrofluid (typically 2 mL in an Eppendorf, secured within a 15 mL falcon tube). The continuous phase consist of 0.5 w/v% SDS and 0.5 w/v% P84 (other pluronics are acceptable).

3.4 The Ligand/Receptor Pair

To model antibody-antigen reaction, the biotin streptavidin pair has been chosen (shown in Figure 3.12). Streptavidin is a tetrameric protein of 52.8 kilo Daltons. It has an extremely high affinity for the molecule biotin, with a dissociation rate Kd $\approx 10^{-14}$ mol/L [93]. This is one of the strongest known interactions of this type in the natural world. The high affinity has been attributed to cooperative bonding with the ureido group, forming five hydrogen bonds (3 to the carbonyl and one per –NH group). Polarization of the biotin's urea group by a charged aspartate residue results in a stabilisation larger than the sum of the individual hydrogen bond energies [94].

Its high affinity ensures there will be few problems with reaction kinetics and efficiency. The multiple binding sites of the streptavidin protein make it an excellent analogue for the epitopes on an antigen which permit crosslinking between particles for immuno-agglutination (see Chapter 4). Another advantage is that this system allows further functionalisation later on using biotinylated biomolecules such as antibodies.



Figure 3.12 - The biotin (left) streptavidin (right) pair has an extremely high binding affinity, eliminating and binding issues. Streptavidin is tetrameric, being able to bind up to four biotins, thus enabling bridging between particles.

The ubiquity of this pair means there are a wide range of commercially available biotin and streptavidin products. Fluorescently labelled streptavidin allows easy microscopic observation of protein localisation and image analysis can provide information about concentration and reactivity.

3.4.1 Buffers & pH

Many of the protein interactions are charge-based, charge interactions which give the protein its precise three-dimensional structure, as well as charge interactions between one protein and its receptor. There is usually an optimal pH range under which the protein will function. Significant deviations from this pH range may result in a loss of function and even denaturation of the protein.

To maintain the desired pH, buffers are used; a combination of a weak acid/base and its conjugate salt. If the pH would decrease (with increasing H+ concentration) then the excess protons are accepted by the conjugate. If the pH is raised by excess OH- ions then weak acid molecules will dissociate to protonate them. In this project, three buffers are employed:

- Phosphate buffered saline (PBS) a solution of sodium phosphate, sodium chloride, potassium phosphate and potassium chloride, it is easily prepared by the dissolution of tablets. PBS is also isotonic to the human body and buffers to the same pH (pH 7.4).
- 2-(*N*-morpholino)ethanesulfonic acid (MES) has a more acidic buffering range (pH 5.5 6.7).

• 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) – has a neutral pH buffering range (pH 6.8 to 8.2), for situations where it is preferable to avoid the use of PBS.

3.5 Adding Ligands to the Interface

Using the optimised formulation, the fluorescent phospholipids may be substituted with biotinylated lipids, in order to investigate ligand protein interactions. After incubation with fluorescently labelled streptavidin (streptavidin Alexa series), optical microscopy can be used to observe the protein captured at the interface.



Figure 3.13 – Fluorescent image of biotinylated Emulsion droplets incubated with excess streptavidin alexa 555. [100x, 100ms].

Biotinylated emulsions incubated with fluorescent streptavidin appear very similar to emulsions containing fluorescently labelled lipids. As before the diameter of the droplets is generally greater than the width of the focal plane so, when in focus, a lateral cross-section of the droplet is seen. Fluorescence is greatest towards the outside of the droplet, where the labelled streptavidin has been captured by the interfacial ligands.

3.5.1 Measuring Protein Capture

An estimation of the degree of capture can be made by measuring the intensity profile of the droplet. The intensity maximum is found by plotting the histogram of a line-scan made using image analysis software (ImageJ). A line is drawn across the droplet in the image and the grey value of each pixel is plotted as a function of position (see Figure 3.14). The absolute intensity is obtained by subtracting the background from the peak maximum. When there is no capture, there is no signal peak and only background noise is observed.



Figure 3.14 - Fluorescence image comparison of biotinylated emulsions incubated without (top left) and with fluorescently labelled streptavidin (top right). Using image analysis software (ImageJ), a line-scan is made across the droplet, and the grey value is plotted as a function of pixel position on the line (bottom).

Using this technique, a sample mean intensity is obtained by averaging the intensity of many droplets. Absolute quantification is difficult but this measurement allows protein capture to be assayed under a range of conditions.

3.5.2 Kinetics of Capture

Having demonstrated that it is possible to capture and compare the amount of protein captured, it is important to look at the kinetics of capture to determine the requisite time for maximum signal to be reached, allowing experiments to be reliably compared.

Protocol: Calibrated microfluidic emulsion (mineral oil containing 1.3 mM DOPC and 10 μ M DSPE-PEG2000-Biotin samples are diluted in 100 μ L volumes of PBS (150 mM, pH 7.4) to $\phi = 4 \times 10^{-4}$, followed by the addition of 1.89 nM (total reaction concentration) of streptavidin. Samples are allowed to incubate for a variable amount of time on a soleil (1, 20, 40 and 60 minutes). Following incubation, microscopy is used to capture fluorescent images of the emulsions. Image analysis is performed using ImageJ to measure the intensity profile for multiple (10) droplets, from which the mean intensity is calculated. Results are shown in Figure 3.15.



Figure 3.15 - As incubation time increases, more streptavidin is captured at the interface, increasing the signal.

Fluorescence intensity is observed to increase over time, meaning that sufficient time must be allowed for the protein to be captured. The graph should be expected to plateau when capture is maximal. Here no plateau was observed in the timeframe of this experiment. To speed up reaction kinetics for future reactions, it was decided to use smaller volumes of more concentrated reagents. The above reaction was performed in 100 μ L, further reactions were performed in a reaction volume of 10 μ L and given 60 minutes to complete.

3.5.3 Effects of PEG Spacer Length and Cosurfactant PEG Length

Capture of streptavidin was originally observed with an emulsion of mineral oil, stabilised by poloxamer 407 (pluronic F127), with DSPE-PEG2000-Biotin as the ligand. The droplet interface layer is complex, consisting of a mixture of PEG chains from both the poloxamer surfactant and the spacers of the biotin lipid. It was hypothesised, that by changing the relative PEG lengths of either of these components would have an impact on the ability of the ligands to capture the protein.

Two effects may be anticipated. Firstly, a competition for space at the interface, changing the cosurfactant may allow more or less space for ligands at the interface. Secondly, steric hindrance might be expected to interfere with protein capture if the PEG of the cosurfactant is long compared to the ligand. To probe these effects, the following experiments were performed:
Influence of the Poloxamer Cosurfactant: Members of the poloxamer family vary according to either the length of the polyethylene glycol chains or the polypropylene glycol chain. The relative weights of these two chains determines the hydrophilicity/hydrophobicity of the surfactant (an excellent overview of these surfactants can be found in [50]). It is highly probable that, at the interface, these chains will influence protein capture. To investigate this, biotinylated emulsions were formulated using the stable emulsion forming poloxamers discovered in 3.2.6. Structural and surface active properties of these emulsions are summarised below in Table 1.

Poloxamer	EG units	PG units	EG/PG ratio	Surf. Tens.*	HLB
P84	19 (x2)	43	0.88	42	12 - 18
P123	19 (x2)	69	0.55	34	7 – 12
P105	37 (x2)	56	1.36	39	12 - 18
F68	76 (x2)	29	5.24	50	>24
F127	99 (x2)	65	3.0	41	18 – 23
F108	132 (x2)	50	5.28	41	>24

Table 1 - Properties of the poloxamer surfactants used. Surfactants are arranged in order of increasing molecular weight. *Surface tension in mN/cm of a 0.1 % solution, at 25°C, EG and PG units are the numbers of each monomer in each polymer chain (data provided by [50]).

Protocol: Bulk emulsions were formulated according to the protocol in 3.2.2 with various poloxamer surfactants and 1.3 mM DOPC + 10 μ M of biotinylated ligand (DSPE-PEG2000-Biotin) in the oil phase. Emulsions are diluted in PBS (150 mM, pH 7.4) buffer to φ = 0.001, followed by the addition of 1.89 nM or 18.9 nM of streptavidin. Samples are allowed to incubate for 1 hour on a Soleil.

After incubation microscopy is used to capture fluorescent images of the emulsions. Image analysis is performed using ImageJ to measure the intensity profile for multiple (10) droplets, from which the mean intensity is calculated. The results of this experiment are shown in Figure 3.16. Cosurfactants are arranged in order of increasing PEG length.



Figure 3.16 - Mean capture intensities of emulsions formulated with various poloxamers. Poloxamers are organized in order of increasing molecular weight from left to right. Emulsions formulated using 10 μ m DSPE-PEG2000-Biotin (ϕ = 0.001, PBS buffer).

With the exception of pluronic P123, all poloxamers permit protein capture to some degree. Comparing the two graphs, it can be seen that a 10x increase in streptavidin does not result in a 10x increase in signal. This is a strong indication that the concentrated 18.9 nM system is close to the maximum ligand binding capacity.

In both regimes, the highest capture intensity is achieved using the F68 cosurfactant, whilst the others display varying degrees of capture efficiency. It can be deduced that there are other interactions at play here. A tentative explanation may be provided when looking at the hydrophobicities of the surfactants and their surface activities. Highest capture correlates with the F68, the most hydrophilic of the poloxamers, having the highest EG/PG ratio (5.24), and highest surface tension (its higher affinity for the water means it remains less at the interface and reduces interfacial tension to a smaller degree). By having a lower affinity of the interface, F68 has a lower competition with the phospholipid ligands, which become more present, increasing capture efficiency.

The opposite argument can be made for P123, which has the lowest EG/PG ratio (0.55) and lowers the surface tension to the greatest degree, because of its large hydrophobic PPG block. In the case of P123, this affinity is so strong that phospholipids are displaced and are not present at the interface.

Further evidence for the effect of surface activity is provided by comparing P84 to P123. Both surfactants have a similar structure with same PEG chain lengths of 19 units, but different PPG lengths. Contrary to P123, P84 emulsions are able to achieve capture so the difference seems to be best explained by the changing PPG length/hydrophobicity. Results can be replotted with capture intensity as a function of the interfacial tension shown below in Figure 3.17.



Figure 3.17 - Mean capture intensity plotted as a function of poloxamer surface tension. Emulsions formulated us 10 μ m DSPE-PEG2000-Biotin (ϕ = 0.001, PBS buffer).

At both protein concentrations, results are remarkably linear, with the exception of a couple of outliers (F127/F108 at low strep. conc. and P105 at high strep. conc.). It can be seen that, as the surface tension increases (decreasing interfacial activity), the capture intensity increases.

From this, it would seem that the optimal choice would be the F68 cosurfactant, however, a balance must be struck between ligand activity and emulsion stability. The lower surface activity of the F68 results in a less stable emulsion with F68 emulsions having been observed to be the least stable amongst this group. P84 was chosen as the cosurfactant of choice, providing a decent stability, demonstrating capture and having the shortest EG chains of the series.

Influence of the Ligand Spacer Length: Another potentially important parameter is the length of the spacer molecule which connects the biotin to the head of the phospholipid which can vary greatly. The majority of spacers are PEG chains of varying length. PEG is favoured because of its properties. It has a high hydrophilicity, improving the solubility of attached objects, whilst being non-immunogenic and biocompatible. The polymer is also highly flexible and so can improve reactivity by providing additional orientation freedom to tethered ligands. Thus increasing the length of the PEG is expected to increase the reactivity of the ligand, improving capture.

Interactions with the cosurfactant PEG chains are also expected, since there may be steric competition for space at the interface. Commercially available PEG spacers range from hundreds to tens of thousands of monomers in length. For this experiment biotin-PEG-lipids with PEG spacer molecular weights of 600, 1000, 2000, 5000, 10000, and X (several atoms in length) will be investigated. The lengths in ethylene glycol (EG) monomers and nm of these spacers are show in Table 2.

Structure	Length (EG monomers)	Spacer Length (nm)
PEG	1	0.35
DSPE-X-Biotin	~6*	~2
DSPE-PEG600-Biotin	14	4.9
DSPE-PEG1000-Biotin	23	8.05
DSPE-PEG2000-Biotin	45	15.75
DSPE-PEG5000-Biotin	113	39.77
DSPE-PEG10000-Biotin	227	79.45
Pluronic P84	19 (x2)	6.65 (x2)

Table 2 - Summary of PEG spacers and their relative lengths. Length in monomers obtained by dividing molecular weight of the spacer by molecular weight of the monomer (44.05 g/mol). Length in nm is obtained by multiplying the length in monomers by the length of a single PEG monomer (0.35 nm [95]). *Spacer X is not PEG, but its equivalent length can be calculated.

Protocol: Bulk emulsions were formulated according to procedure 3.2.7, replacing the fluorescent lipid with 10 μ M DSPE-Y-Biotin (Y = PEG 600, 1000, 2000, 5000, 10000, and spacer X).

Emulsions are diluted in PBS (150mM, pH 7.4) buffer to $\varphi = 0.001$ (100 µL sample volume), followed by the addition of 1.89 nM of streptavidin. Samples are incubated for 1 hour on a Soleil. After incubation, microscopy is used to capture fluorescent images of the emulsions. Image analysis is performed using ImageJ to measure the intensity profile for multiple (10) droplets, from which the mean intensity is calculated. The results of this experiment are shown in Figure 3.18.



Figure 3.18 - Streptavidin capture efficiency as a function of ligand spacer length. Performed with Bulk P84 stabilised mineral oil droplets, $10 \mu M$ of DSPE-PEG2000-Biotin ligand in PBS buffer (150 mM, pH 7.4).

Results show that the shortest spacers, X and 600 capture very little streptavidin. Increasing the length from MW 600 to 1000 increases the capture intensity greatly. Intensity increases slightly for the 2000 spacer and begins to drop as the length is increased to 5000. Finally the signal falls again to almost nothing with the 10000 spacer. This pattern seems to indicate there is an optimal length of spacer for efficient capture (1000 – 5000 Da).

The initial jump in intensity can be explained when the relative PEG lengths of the spacers are compared to the PEG length of the pluronic P84 cosurfactant. P84 has PEG chains of approximately 836 Daltons, corresponding to a length of 19 monomers per chain. The monomer lengths for the 600 spacer and the 1000 spacer are 14 and 23 respectively. It seems that the P84 PEG chains are sufficiently long to screen the X and 600 ligands from the protein, so no capture is observed.

The PEG 1000 spacer with a chain length of 23 EG units is longer than the P84 chains (EGx19) and so extends beyond this screening layer and is able to efficiently capture streptavidin. For P84, there is therefore a spacer length threshold of ~23 EG units required for efficient capture (shown in Figure 3.19).



Figure 3.19 - Relative contour lengths of PEG spacers in ethylene glycol units, the dashed blue line represents the PEG length of the P84 cosurfactant.

This finding ties in with literature which has shown that ligand spacer length plays a critical role in tethered ligand interactions. A similar length threshold has been observed when similar biotinylated lipopolymers are used to functionalise cells. In this case, the PEG spacer must extend outside of the cell's glycocalyx [96], which is analogous to the surfactant layer in this experiment. In addition for tethered ligand reactions between lipid bilayers, it has been demonstrated that it is the full extension length of the ligand which is important for receptor binding [97],[98].

Efficient capture should be expected for all spacers longer than 19 EG units. Interestingly for spacer lengths beyond PEG 2000 capture efficiency begins to drop and the explanation for this is less clear. However increasing the molecular weight of the hydrophilic PEG polymer will increase the HLB of the phospholipid ligand. It is possible that above a certain length the phospholipid becomes sufficiently hydrophilic that it is too easily dissolved into the continuous phase or that it can no-longer be solubilized into the oil phase in meaningful amounts. Increasing size of the polymer chain may increase steric competition with the cosurfactant which may 'squeeze' the ligand out.

A similar length threshold may also have been expected with the various poloxamers used in the first experiment. Interestingly, capture is still observed even when the PEG length of the surfactant greatly exceeds the length of the PEG 2000 spacer ligand. This might be explained by a conformation argument. Statistically, longer flexible polymers adopt coiled conformations measured by their radii of gyration. This means that increasing the cosurfactant PEG length will only slightly increase the thickness of the steric layer. During full extension of the ligand should be able to reach beyond the coiled cosurfactant PEG chain. It is also important to note that the standard deviation for the fluorescence intensity (shown by the error bars) is high for each PEG length, this may result from poor mixing or sub optimal conditions chosen for these experiments.

Using the optimal spacer and cosurfactant combinations (Pluronic P84 + DSPE-PEG1000/2000-Biotin) the formulation can then be used with a microfluidic device to produce of homogenous magnetic emulsions (magnetic content will be introduced later on).

3.5.4 Quantifying Streptavidin Capture

Droplets produced via microfluidics should possess similar surface densities of ligand and having a narrow size distribution. Uniformity allows strong estimates to be made for the number of droplets from knowing both the volume fraction and average radius. Using the fluorescent streptavidin capture intensity technique, a strong estimation of the amount of streptavidin captured per droplet can be made (assuming all streptavidin is captured by the droplets). This provides a more quantitative analysis of the relative protein capture and giving useful information about ligand protein interactions and capture efficiency.

Protocol: To find the saturation point $\varphi = 0.016$ of calibrated microfluidic emulsion (mineral oil containing 1.3 mM DOPC and 10 µM DSPE-PEG1000-Biotin) was incubated with various concentrations of streptavidin in PBS (150 mM pH 7.4) for 1 hour using a soleil. As before, fluorescent microscopy is used to record images which are analysed using ImageJ to obtain the relative capture intensity. Mean relative intensities (averaged from 10 droplets) are plotted as a function of streptavidin concentration, shown in Figure 3.20.



Figure 3.20 - Measured capture intensity plotted as a function of protein concentration for a biotinylated emulsion incubated with streptavidin. Capture reaches a maximum at a certain concentration.

Initially, the relative fluorescence intensity increases with increasing streptavidin concentration. Intensity gradually reaches a plateau where additional streptavidin has no effect on the signal. At this point the interface has become saturated with streptavidin.

Saturation may be either be geometric or depend on the availability of the ligand. In the case of geometric saturation, the total surface area is fully occupied and there is no free space to accommodate further streptavidin. If it depends on the concentration of available ligand, at a certain concentration, all ligands of the droplet will have captured a protein.

Emulsions with a very narrow size distribution allow the total surface area of the droplets to be calculated and compared to the calculated footprint of the streptavidin at the saturation concentration. The dimension of a streptavidin protein are known to be $4 \times 5 \times 6$ nm [99], this gives a footprint of 3×10^{-17} m² per protein. The average diameter of a droplet is measured to be 8.5μ m, giving a surface area of 2.27×10^{-10} m² per droplet. A reaction sample is calculated to contain 4.93×10^{5} droplets with a total surface area of 111.9×10^{-6} m².

The signal reaches a plateau (saturation) at 75.8 nM protein in the sample, (equivalent to 4.56×10^{11} protein molecules in the sample) covering a total surface area of 13.7×10^{-6} m². This value is approximately 12.2 % of the total surface area of the droplets therefore it can be concluded that saturation does not have a geometric basis.

It is also interesting to compare the number of streptavidin molecules to the number of biotinylated ligands per droplet. The standard formulation uses a concentration of 10 μ M of biotinylated lipid, for 8.5 μ m droplets, this means there are 1.94 x 10⁶ ligands per droplet.

At saturation, there should be 9.25×10^5 streptavidin per droplet, and so there are ~2 biotin ligands for every streptavidin protein. At first glance, this implies that not all of the ligand has reacted. It may be that not all of the ligand is present at the interface, some remaining bound in reverse micelles within the oil phase. A second possibility is that the biotin reacts with the streptavidin in a 2:1 ratio, which is possible because of the multiple binding sites available on the streptavidin. To verify this, a similar experiment can be performed with the ligand concentration as the variable.

3.5.5 Effect of Ligand Concentration

Variation of the ligand concentration with respect to the protein will allow the capture ratio at saturation to be observed in greater detail, proving how saturation relates to ligand availability.

Protocol: Microfluidic emulsification is used to produce emulsions with concentrations of DSPE-PEG2000-Biotin ranging from 0.16 – 10 μ M. Emulsions of φ = 0.016 are prepared by dilution into MES Buffer (pH 5.5 100 mM NaCl) in a 10 μ L reaction volume and incubated with streptavidin for 1 hour on a soleil. The experiment is repeated for each emulsion with three different concentrations of streptavidin (9.5, 18.9 and 37.9 nM). Relative fluorescence intensity is calculated using image analysis. Results are shown in Figure 3.21. Ligand concentration is recalculated relative to the sample volume, for direct comparison with the protein.



Figure 3.21 - Dose response curves for emulsions with varying concentrations of biotin ligand at different streptavidin concentrations. Blue = 37.9 nM, Red = 18.9 nM, Green = 9.5 nM, dotted lines show their concentration with respect to the biotin.

As the concentration of biotin is varied, a similar saturation curve emerges for each concentration of streptavidin. The data for these fits is rather scattered so it is difficult to draw a strong conclusion. Overall, increasing the concentration of the biotin ligand increases the amount of streptavidin which can be captured, up to a maximum.

For each curve, saturation appears to begin close to the point where biotin and streptavidin are equimolar (shown by the dotted lines). This indicates firstly that most of the biotin in the droplets is available for reaction at the interface. Secondly, this means that saturation seen in the streptavidin dose-response curve (Figure 3.20) is most likely due to occupation of all available biotin ligands.

In these samples ligand appears to be saturated by the protein at a 1:1 ratio, previously it was calculated to be approximately 2:1. The disagreement most likely arises from differences in experimental conditions such as sample age. This combined with the large standard deviations and relatively few data points for each curve mean that there is insufficient resolution to verify the absolute ratio. It is however encouraging that the concentration of both ligand and receptor are calculated to have similar orders of magnitude.

3.5.6 Ligands - Conclusion

It has been shown that ligands can be successfully added to the droplets, capture is observed using a fluorescently labelled protein. The efficiency of capture can be gauged by measuring the relative intensity of the fluorescent protein captured at the interface and is used to explore a range of reaction conditions. A simple kinetic study show that sufficient incubation time is required for efficient capture.

Variation of the poloxamer cosurfactant reveals a strong negative correlation between interfacial activity and capture efficiency suggesting a competition between the ligand and surfactant. Capture is also modulated by the length of the spacer; at short lengths, the spacer appears to be screened by the cosurfactant and no capture is observed. Strong capture is apparently observed when the spacer is sufficiently long to reach beyond the steric layer of the cosurfactant. A further increase in spacer length results in reduced interaction.

Using an optimal ligand and cosurfactant combinations, size calibrated droplets can be produced using microfluidics. Homogeneity of size and ligand coverage means that the saturation point can be determined by screening a protein concentration range. Saturation is calculated to occur well below the theoretical geometric maximum. At the saturation point, biotin is calculated to be in excess compared to the streptavidin. Ligand protein interactions are further probed using an emulsion series with variable concentrations of ligand, incubated with fixed streptavidin concentrations. Dose-response curves show that saturation occurs when ligand and receptor are within an equimolar ratio. Saturation is therefore related to the availability of the ligand at the interface.

The next step is to demonstrate and if possible measure the independent mobility of ligands at the interface.

3.6 Evidence of Fluidity

A key deliverable for this project is to create particles which have a demonstrably reactive liquid interface. This is achieved by looking at the behaviour of objects captured at the interface. The most clearly visualised objects are fluorescently labelled protein and protein grafted beads. Their investigated are discussed in the following experiments.

3.6.1 Adhesion Plaque Formation

An interesting phenomenon which highlights fluidity is the formation of adhesion plaques between biotinylated droplets incubated with streptavidin. Streptavidin has four binding sites and so may be used to crosslink biotinylated droplets (discussed in more detail in Chapter 4). Crosslinking is performed simply by incubating droplets at a streptavidin concentration which is below the saturation point. This ensures there are unbound ligands available for crosslinking between droplets.

A typical experiment uses $\varphi = 0.016$ of size calibrated microfluidic emulsion (mineral oil containing 1.3 mM DOPC and 10µ M DSPE-PEG1000-Biotin) incubated with 37.8 nM of streptavidin Alexa 555 in 10 µL PBS (150 mM pH 7.4) for 1 hour using a Soleil. Crosslinking and plaque formation are observed by measuring the fluorescence profile of droplets in contact and how they change over time. Results are shown in Figure 3.22.



Figure 3.22 - Patch Formation, streptavidin is initially fluid at the interface but becomes cross-linked and fixed at the contact zone between droplets. As more streptavidin diffuses in and becomes crosslinked the zone becomes enriched, greatly increasing the fluorescence signal. *Images: 100x magnification, 2000 ms exposure*

After incubation, droplets initially have a homogeneous fluorescent signal from the captured protein. A line-scan of two droplets in contact (A) shows that the contact zone has similar fluorescence intensity to the outer regions of both droplets (the contact zone is slightly higher due to signal overlap). After 2 hours, the contact zone appears much brighter than the outer regions of the droplets (Image B). If a line-scan is performed (plot B) the relative intensity of the contact zone is shown to be many times higher than the outer regions of the droplets. Intensity of the contact zone has increased while the intensity of the outer regions has decreased, suggesting a redistribution of the protein at the droplet interface. Fluorescent streptavidin is enriched at the contact zone, forming the bright 'patch' (or plaque).

Redistribution can only occur if proteins and ligands are mobile as a result of the fluid interface. Captured protein diffuses around the interface, until it becomes cross-linked at the point of contact between droplets. Cross-linking immobilises the protein at the contact zone between the droplets. Additional captured streptavidin diffuses in, also becoming immobilised. As more and more streptavidin in immobilised, the contact zone becomes enriched, resulting in the observed florescence amplification. This is a phenomenon was originally observed in cells which show a redistribution of receptors thanks to membrane fluidity [100]. This has since been replicated and studied using a functionalised emulsion system [101],[102].

3.6.2 Bead Capture & Mobility

More direct evidence for interfacial fluidity is provided by reacting emulsion samples with streptavidin-coated beads. A bead is far larger than an individual streptavidin protein, thus the motion of a single captured object at interface can be observed microscopically.

Beads are captured by incubating 1 μ g of MyOne Streptavidin C1 Dynabeads (Diameter 1 μ m) with $\phi = 0.016$ of size calibrated microfluidic emulsion (mineral oil containing 1.3 mM DOPC and 10 μ M DSPE-PEG1000-Biotin) incubated 10 μ L MES (100mM NaCl pH 5.4) for 1 hour using a Soleil. After incubation the sample is viewed using a microscope, captured beads are located and their motions are recorded using a 60 second time sequence. Selected images can be seen in Figure 3.23.



Figure 3.23 - Capture of streptavidin coated beads, their larger size allows their interfacial movement to be easily tracked. Sequential images show the relative positions of the beads – changing positions indicates interfacial fluidity, time between images = ~12 s. (*Capture of MyOne carboxy C1 streptavidin coated beads Bright field, 100x magnification*)

Beads can clearly be seen captured at the droplet interface. Single beads can be seen to move around the droplet, however, the droplet has a rotational diffusion and for a single bead it is difficult to distinguish between this diffusion and the diffusion around the interface. Fluidity can be verified by tracking the relative positions of two beads. If the positions of the beads change relative to one another then it is clear they are independently mobile, which can only occur if the interface is fluid. This is seen in the above time sequence.

3.6.3 Interfacial Fluidity Conclusion

Interfacial fluidity permits the diffusion of ligands around the droplet, confirmed experimentally using two different techniques. Ligand fluidity is expected to greatly enhance the reactivity of these droplets.

3.7 Magnetic Content – Ferrofluid

Having created a stable emulsion with a functionalised interface, the next step is the introduction of magnetic content into the oil. This is achieved using a hydrophobic ferrofluid.

3.7.1 Preparation of the ferrofluid

Ferrofluid was initially prepared by the coprecipitation procedure outlined in 2.3.1, (references [18], [19]). To save time, it was decided to switch to using dilutions of a pre-synthesized ferrofluid provided by Ademtech. The pre-synthesized ferrofluid consists of oleic acid coated magnetite nanoparticles (70 %w/v) suspended in a mixture of octane and oleic acid (15 %w/v).

Octane is highly volatile and soluble enough in water to render Ostwald ripening problematic. To circumvent this, the stock ferrofluid is diluted into less volatile oils such as mineral oil, soybean oil or a medium chain alkane (10 – 16 carbons). Experimentation showed that a concentration of 3 % w/v imparted sufficient magnetic character for the droplets to be easily be manipulated using a magnetic field.

3.7.2 Ferrofluid Stability

Changing the carrier oil was found to greatly affect ferrofluid stability, giving rise to some interesting observations. Stability tests were performed by diluting ferrofluid to 1 % w/v in a range of oils with varying compositions.

Effect of the Hydrocarbon Chain Length: It was decided to investigate the stability of the ferrofluid in longer hydrocarbons from the alkane series, ranging from decane (10 carbons) to hexadecane (16 carbons). Increasing the carbon chain length increases the Van der Waals interaction between carrier oil molecules, decreasing volatility and water solubility. Ferrofluid stability was assessed by visual and microscopic observation. Results are shown below in Figure 3.25 and Figure 3.26.



Figure 3.24 - Effect of carrier oil on ferrofluid Stability. Increasing ferrofluid aggregation is observed with increasing carrier oil chain length. [*Chain Lengths:* A = Decane (*C*10), B = Dodecane (*C*12), C = Tetradecane (*C*14), D = Hexadecane (*C*16), E = Mineral Oil (*mixed*)].

Visual inspection of the series shows that, as the hydrocarbon chain length is increased, samples exhibit increasing turbidity and sedimentation (seen by inversion of the samples). In contrast to the longer chains, the ferrofluid appears to be completely stable in decane.



Figure 3.25 - Microscope images of ferrofluid aggregates, Carrier oil/oleic acid induced aggregation as observed by microscope [100x magnification].

Microscopic observation confirms the increasing aggregation of the samples.

The exact cause for this aggregation is unknown. It is most likely related to intermolecular forces between the carrier oil and the oleic acid coating of the iron oxide nanoparticles. Increasing the hydrocarbon chain length increases the strength of the Van der Waals forces between the alkane molecules resulting in increased melting and boiling points. This effect may decrease the oils ability to solubilise the oleic acid layer as the chain length increases, resulting in the observed aggregation.

Interestingly, literature reports that hexadecane is a commonly used dispersant for oleic acid coated magnetite [103], suggesting a secondary cause of aggregation.

Effect of the Concentration of Free Oleic Acid: The Ademtech ferrofluid contains oleic acid in a large excess (\sim 15 %w/v), indicating that it too plays role in stabilisation. To test this, 1 %w/v of ferrofluid was diluted into samples of hexadecane mixed with increasing proportions of oleic acid (0, 1, 5, 10, and 20 %v/v). Results are shown in Figure 3.26.



Figure 3.26 - Effect of oleic acid concentration on the stability of the ferrofluid. In oils where the ferrofluid is initially unstable, increasing the concentration of oleic acid first destabilises the suspension (0-10 %), then at higher excess restabilises it (20 %w/v). [1wt % Ferrofluid in Hexadecane : Oleic Acid - A = 100:0, B = 99:1, C = 95:5, D = 90:10, E = 80:20, F = 0:100].

As the proportion of oleic acid increases, aggregation initially worsens, but beyond a certain oleic acid, the ferrofluid appears to be re-stabilised (at 20 %v/v). This would indicate that the magnetic particles are more stable in oleic acid, however if pure oleic acid is used as the carrier oil, the ferrofluid is again unstable. Literature corroborates that excess dispersing agent (such as oleic acid) can result in destabilisation [104], one author attributes this effect to depletion [105], however this does not explain reversal of aggregation at high OA concentrations. In these experiments without addition of any oleic acid, it is estimated that a ferrofluid of 1 %w/v iron oxide particles, has a free oleic acid concentration of ~0.3 %w/v.

Restabilisation of the ferrofluid at high oleic acid concentrations is reminiscent of the overdosing behaviour observed with polymeric bridging flocculation of colloids[106]. It is known that analogous fatty acid in non-polar solvents form dimers [107],[108], it may be that the extended form of the dimer large enough to bridge between magnetite nanoparticles.

The influence of the hydrocarbon chain length and the concentration of free oleic acid appear to be interrelated, however further experiments are required to fully characterise these effects.

3.7.3 Consequences of Aggregation

When aggregation does occur, it has negative consequences for both the emulsification process and the resulting emulsion.

Aggregation in the Microfluidic Chip: Firstly when an unstable ferrofluid is used in microchannel emulsification aggregates sediment and rapidly accumulate at certain points of the chip, building up, changing its geometry and eventually blocking channels completely (images shown in Figure 3.27).



Figure 3.27 - Ferrofluid aggregates in the microfluidic chip. Microscopic aggregates can be seen passing through the channels, (left and centre). Deposition of aggregates (right) eventually results in membrane blocking.

Pickering emulsion formation: It is also observed that droplets formed from unstable ferrofluid later become amorphous and rigid. The explanation appears to be the formation of Pickering emulsions, (shown in Figure 3.28) where droplets are stabilised by particles which partially wet both phases and jam at the interface. Here the particles move from the bulk of the oil to the interface, becoming jammed. Pickering emulsion formation is dependent on several factors including particle hydrophobicity shape and size. Here it seems that Pickering formation is promoted by decreasing solubility of the magnetite nanoparticles in the carrier oil. A consequence of the solid layer of aggregates is that the interface of the droplet is no-longer fluid.



Figure 3.28 - Pickering Emulsions. Amorphous droplets observed in ferrofluid containing emulsion samples. The amorphous shape appears to be the result of particle jamming at the interface (Pickering) which creates the observed distorted structures.

Stable Formulation: Various other oils were screened for ferrofluid stability and compatibility with PDMS, an optimal balance was found using 2-Octyl-1-dodecanol. Emulsions of decane were also produced using a glass microfluidic device.

3.8 Formulation Results Summary

In summary, the overall structure of the emulsion consists of an oil phase dispersed in an aqueous phase. The oil contains hydrophobic super paramagnetic iron oxide to impart magnetic properties to the droplets whilst a mixture of phospholipids (some functionalised for protein capture) which arrange themselves at the interface structure shown in Figure 3.29. Stability and function of the emulsion is controlled by mutual compatibility of the components; the carrier oil must be able to dissolve the phospholipid and disperse the ferrofluid, whilst immiscibility of the surfactant and the phospholipid maintains functionality of the interface.



Figure 3.29 - Structure of integrated emulsion. The emulsion consists of droplets of carrier oil containing both hydrophobic magnetite nanoparticles and dispersed phospholipids. The phospholipids assemble at the interface presenting protein capture ligands. The emulsion is stabilised by a poloxamer triblock copolymer surfactant.

Important criteria can be summarised as follows:

Emulsification Technique: Bulk emulsions are easily obtained using a mortar and pestle, allowing formulations to be quickly analysed and compared.

Monodispersity: This is controlled by the step emulsification, performed using a PDMS chip obtained by photolithography. The parallelised chip gives a high throughput and, under good conditions, is stable for extended periods of time. The porous PDMS elastomer is prone to swelling which necessitates the use of certain oils and precludes the use of others. An alternative to the PDMS chip is a glass chip of the same geometry, allowing emulsification of the swelling oils.

Functionalisation: Functionalisation is achieved by the introduction of phospholipids into the oil phase. Dispersion of the phospholipids is controlled by their interactions with the oil. Low phase transition temperature is requires for dispersion which is found in phospholipids with unsaturated acyl chains. Many of the functionalised lipids bear saturated chains so solubilisation is encouraged by mixing them with bulk unsaturated lipids. Lipid presence at the interface can be observed using NBD and Oregon green tagged fluorescent lipids. Fluorescent lipids reveal problematic cosurfactant-lipid interactions, that certain surfactants form mixed micelles and remove the lipids from the interface. Certain members of the poloxamer family have been identified as surfactants which can stabilise the emulsion without disrupting the phospholipids at the interface.

Biotinylation is achieved by using phospholipids grafted with PEG-Biotin. Capture of protein by the ligand at the droplet interface is revealed through the use of fluorescently tagged streptavidin which can be seen using fluorescence microscopy and measured with image analysis software to indicate the relative capture efficiency under a range of conditions. Using size calibrated emulsions and a known protein concentration, it is possible to estimate the capture efficiency and compare it to the number of ligands available. By changing the ligand concentration, it is found that the droplet saturation point is determined by ligand availability at the interface. Protein/ligand ratio is high, indicating good ligand interfacial ability.

Protein affinity is found to be affected by both the type of poloxamer cosurfactant used to stabilise the emulsion and the length of the ligand spacer. For a fixed length of spacer, protein affinity appears to diminish with increasing surface activity of cosurfactant. Changing the length of the PEG spacer indicates for shorter spacers there is a critical length for efficient capture, appearing to correspond to the PEG length of the cosurfactant.

Magnetic Content: Magnetic content is introduced by the addition of hydrophobic magnetite into the oil phase (creating a ferrofluid). Stability of the ferrofluid depends on the nature of the oil and the concentration of oleic acid. Stability of the ferrofluid is a critical parameter; aggregate formation interferes greatly with the geometry based step emulsification, blocking channels. Aggregation within the emulsion droplets results in Pickering with aggregates becoming jammed at the interface, creating a solid layer.

Stability: Stability is provided by both the poloxamer cosurfactant, which provides a large degree of stability and the choice of the oil. The choice of oil is largely determined by mutual compatibility with both the ferrofluid and PDMS, however where possible low volatility/solubility oils have been chosen to limit emulsion destabilisation through evaporation and Ostwald ripening. With sufficient surfactant, no coalescence is observed. Creaming remains the only significant destabilisation process and would be extremely difficult to prevent, a possible route is by increasing the magnetite content.

The resulting emulsion can be seen in Figure 3.30 which shows functionalised emulsion droplets which are able to capture streptavidin and to form chains under an applied magnetic field thanks to their magnetic content.



Figure 3.30 - Images of Magnetic Chaining of stable ferrofluid emulsions. Bright field and fluorescence images of magnetically chained emulsion. Fluorescence is from protein capture, magnetic chaining of the functionalised emulsion is used to induce specific binding between droplets. (100x magnification, fluorescence = 2000 ms exposure Green channel).

In the following chapter, this emulsion will be investigated as a possible diagnostic tool by performing agglutination reactions (controlled specific aggregation) and measuring the response at various concentrations of analyte.

Chapter 4 Application – Agglutination Assay

4.1 Introduction

In the previous chapter, emphasis has been placed upon the creation of novel functionalised magnetic liquid particles. Although a potentially wide range of applications are envisaged, here, focus will be placed upon how such particles may be used in assay technologies for the quantification of biomolecules. Discussion will first cover the principles behind the particle based assay and how assay performance can be greatly enhanced through the use of magnetic particles. Assay detection limits are subsequently examined and related to the material limitations of the particles, which necessitate a new generation of magnetic particles.

The emulsion developed in the previous chapter will be explored as a potential assay particle. Assay functionality will initially be demonstrated by performing agglutination with bulk emulsions, using a model ligand/receptor pair. The qualitative nature of bulk agglutination reveals the need for advanced aggregate counting techniques.

In the final part, flow cytometry is introduced and explored as a technology for counting single droplets and aggregates, thus determining the amount of analyte present in a sample. Emulsion behaviour in a flow cytometer is first considered to develop a protocol. Measurements are then performed using size calibrated emulsions and a range of model analytes.

4.2 In Vitro Diagnostics & Particle Assays

4.2.1 Biomarkers

In the fields of medicine and biotechnology, there is an increasing demand for the ability to accurately characterise and quantify the physiological state of a given biological system. This is particularly relevant in medicine where many diseases are identified by late term symptoms; early detection is advantageous because treatment can be far more effective. When diagnostic methodologies are created, the presence of a disease is usually correlated to a biological marker (biomarker), defined as:

"objectively measured indicators of biological processes or response to a therapeutic intervention:" [109].

A biomarker usually takes the form of a biomolecule whose presence results from the disease process, including proteins, hormones, DNA or RNA. Determining the presence and quantity of the biomarker can provide a lot of information about the nature and state of the disease.

4.2.2 Bioassays & IVD

A technique used to detect and measure a biomarker is known as a bioassay. Bioassays are usually performed in vitro, analysing tissue or fluid samples taken from a patient. This branch of medicine is also often known as in vitro diagnostics (IVD). IVD covers a staggering range of techniques for all types of diseases, however, many of these techniques are costly and time consuming, requiring specialize equipment and personnel, limiting the number which can be performed. There is a commercial interest in the development an improvement of bioassays, to make them small and simple to uses such that they can be performed at the point of care, with minimal training, circumventing hospital visits, with a minimal cost and providing results as rapidly as possible.

4.2.3 Targeting Biomarkers

For any assay, the greatest challenge is to isolate the biomarker target from a complex biological medium- biological samples contain thousands of proteins and biomolecules making it extremely difficult to target just one. As of yet, nothing developed by humans can match the selectivity of the targeting molecules employed by nature, known as antibodies. Antibodies are Y-shaped proteins, secreted by plasma cells. They form a crucial part of the adaptive immune system binding with extreme selectivity a single target known as an antigen. The bound antibody-antigen state is known as an immunocomplex.

The function of an antibody is to bind to the target tagging it for recognition by other elements of the immune system or otherwise interfering with its function, effectively neutralizing it. The region of the antigen to which the antibody binds is known as an epitope, if several antibodies have affinities for different epitopes of the same antigen, they are said to be polyclonal. If a population of antibodies all bind to the same epitope they are said to be monoclonal, the discovery of monoclonal antibodies [110] has been critical for the development of IVD.

4.2.4 Affinity Assays

Due to their small size, individual antibodies and immunocomplexes are quite difficult detect. To facilitate detection, they are usually conjugated to something which can generate a reliable signal, such as an isotope, particle, or enzyme which generates a measurable product. These latter two are particularly common because they can be performed with relatively simple apparatus.

Enzyme-Linked Immunosorbent Assay (ELISA): ELISA is a technique which uses antibodies bound to enzymes. In its most basic form, Direct ELISA, antigens are captured at a surface followed by the introduction of enzyme linked antibodies into the sample. The sample is washed to remove excess antibodies, leaving only the immunocomplex at the surface. In a final step, a solution containing the enzyme's substrate is introduced. Action of the enzyme upon the substrate (usually a kind of dye) results in a colour change which can be measured spectroscopically. Accurate quantification is achieved by performing the ELISA over a broad range of dilutions.

The sensitivity of the assay can be increased through the use of secondary antibodies which bind to the first (primary) antibody. The initial surface capture of the antigen can also be made specific by functionalising it with antibodies, this is called 'sandwich' ELISA because the antigen is sandwiched between two antibodies. The various forms of the ELISA assay are shown in Figure 4.2.



Figure 4.1 - Enzyme-linked immunosorbent assay (ELISA), an antigen is detected using antibodies coupled to enzymes. Figure courtesy of [111].

Particle Assays: Particle assays employ colloidal suspensions of particles coated with antibodies. When an antigen is introduced into the sample, antibodies bind to it, forming sandwich complexes between two particles, a process known as specific aggregation or agglutination. Particle aggregation causes a change in the optical properties of the system. Simple assays rely on changes which can be seen using the naked eye. For greater sensitivity, light scattering or turbidimetry can be used

depending on the size or concentration of the particles. A change in absorbance or scattering will in general be proportional to the degree of aggregation which can be linked to the concentration of analyte.

The very first particle assays used pathological cells with antibodies as the bridging component [112],[113]. Shortly afterwards, erythrocyte agglutination was discovered, initially used for blood typing and later developed into an assay (hemagglutination) [114]. Drawbacks associated with cell based assays (membrane cross reactivity, sampling inconsistencies, loss of potency over time and irreproducibility) lead to the development of antibody grafted latex particles [115]. Possible reactions of grafted particles are shown in Figure 4.2.



Figure 4.2 - Agglutination Assay. Particles substrates can be used in various ways to create a signal in immune-complex reactions. A) Cluster formation, particles specifically agglutinate into large clusters which can be observed. B) Particles captured specifically at a surface.

High antigen concentrations result in multiple crosslinking events, forming large clusters of particles. At low concentrations, there will be less than one antigen per particle so only doublets will be observed.

Dose - Response: The signal generated by an agglutination assay greatly depends on the ratio of antibody to antigen. Agglutination is maximal when antibody and antigen are in the correct proportions. If the antibody is in complete excess, there will be very few aggregates, this is called the prozone. If the antigen is too concentrated then, all surface antibodies will be occupied and aggregates cannot form, this is called the post zone. The concentration over which this dose-response occurs is called the dynamic range. These regimes are shown in Figure 4.3.



Figure 4.3 - Dose Response Curve based on the concentration of antigen incubated with antibody coated beads. Figure courtesy of [116].

4.2.5 Affinity Assay Limitations

Affinity assays suffer from a number of limitations:

Non-specific binding: Both antibodies and particles are susceptible to non-specific binding. In ELISA primary antibodies may stick to the surface without antigen, giving a false positive. In agglutination, particles may aggregate non-specifically, creating false signal.

Kinetic Issues: The sensitivity of an assay is determined by the smallest quantity of analyte which can be detected. At dilute concentrations, the average separation between reagents increases, increasing the time required for reaction.

For an ELISA reaction, the rate of reaction is simply given by the time it takes for the antibody to diffuse to the antigen (or vice versa) which increases proportionally with dilution.

In an agglutination assay, the reaction can be considered to proceed in 3 steps:

- 1) Binding of the target in solution to the receptor.
- 2) Translational diffusion of an assay particle to proximity of another.
- 3) Rotational diffusion to allow intra-particle reaction.

Since the target is very small compared to the beads, the rate of diffusion is comparatively high, so step 1 is rapid and the rate is limited by steps 2 and 3, as the much larger assay particles have a far lower rate of diffusion.

In both cases, kinetic issues may be addressed through the use of magnetic particles.

4.3 Magnetic Particle Based Assays

Chapter one introduced magnetic particles and covered their many applications. One such application is as assay particles. In both ELISA and agglutination, the particle's characteristics provide a significant advantage.

4.3.1 Magnetic ELISA

Magnetic ELISA was the first assay to make use of magnetic particles [117]. Here, the magnetic particles are used as the solid support for the grafted antibodies in lieu of a simple surface. As particles, they can be dispersed into the sample volume, greatly reducing the average distance between the antibody and the antigen.

Following reaction, the magnetic character of the particle allows them to be collected during the subsequent wash steps. Overall, using magnetic beads as the solid phase in an ELISA assay allow the assay time to be reduced from the order of T > 10 hours to less than 60 minutes.

4.3.2 Magnetic Agglutination:

Magnetic agglutination was first explored here in the LCMD [118], where it was shown that magnetic particles can also be used to accelerate specific aggregate formation in an agglutination assay. In essence, a magnetic agglutination assay exploits the particles propensity to gather into chains in the presence of a magnetic field. This eliminates the very long diffusion time required for inter particle specific recognition reactions. The magnetic agglutination process is shown in Figure 4.4. Elimination of the particle diffusion step greatly reduces the assay time, development of this technique has shown picomolar detection can be achieved in as little as a minute [119].



Figure 4.4 - Magnetic Agglutination – monodisperse magnetic beads grafted with antibodies are incubated in the presence of the target, aggregate formation is accelerated using a magnetic field which causes the particles to assemble into chains. Figure courtesy of [118].

4.3.3 Magnetic ELISA versus Magnetic Agglutination

The choice of magnetic affinity technique will depend on the time and sensitivity requirements of the assay. Magnetic ELISA has an overall sensitivity advantage, with detection limits of 10 fM, its major drawback is that more handling steps are required so it takes longer. Magnetic agglutination conversely is simpler and much more rapid due to minimal handling, but is less sensitive with a detection limit in the picomolar range. The simplicity of agglutination means that it may be possible to improve this detection limit. To do so, the limitations relating to the magnetic particles should first be analysed.

4.3.4 Magnetic Particle Limitations for Agglutination

The magnetic particles in the agglutination assay are subject to the same limitations outlined in 1.3.2. Here, these limitations are reiterated, outlining their influence on the assay.

Non-specific aggregation: Even under optimal conditions, sample impurities or inhomogeneities may result in nonspecific aggregation between particles. It is many times more difficult to maintain

colloidal stability in biological media. High electrolyte concentrations may cause destabilisation through charge screening, allowing particles to aggregate by Van der Waals attraction. Moreover, the large numbers of biomolecules present may have charge interactions with the particles surface potentially forming non-specific bridges if two particles come into contact. High concentrations of biomolecules may also induce depletion.

Non-specific aggregation is evidently detrimental for the agglutination assay since it is impossible to distinguish between specific and non-specific aggregates. The limit of detection is determined using the amount of signal which can be attributed to false positives created by nonspecific aggregates.

In particle assays, non-specific aggregation can usually be controlled using non-ionic surfactants which bind to the particles surface and form a steric layer against protein adsorption or collision between bare particle surfaces.

Blocking agents may also be used, which consist of inert species which bind to all potential non-specific binding site on the particle, preventing more disruptive species from interacting.

Size and Rotational Diffusion: In a magnetic agglutination assay, the translational diffusion (step 2) is controlled magnetically so only, rotational recognition (step 3) remains as the rate limiting step. The rotational diffusion is Brownian and therefore related to the size of the particle, this means there is a size threshold beyond which particles can no-longer efficiently react (rotational motion becomes too feeble in comparison to the magnetic field coupling - see below).

Larger particles would in principle be advantageous because they are easier to detect, so a smaller concentration of beads may be used for a given concentration of grafted antibodies.

Magnetic Trapping: Interaction with the magnetic field can also influence the rotational Brownian motion. The superparamagnetic nanoparticles within the particle core are slightly inhomogeneous in size and distribution within the polymer matrix. Nanoparticles are also randomly aligned. The result of this is that the magnetic particle will have an easy axis of magnetisation which is the vector sum of the easy axes of the embedded superparamagnetic nanoparticles. As the magnetic field persists, the microbead will rotate to align this net easy axis of magnetisation with the magnetisation induced by the field.

At a certain threshold, the force of field alignment overcomes Brownian rotational diffusion and the particle is held orientationally immobile. For larger particles which have little Brownian motion and proportionally more magnetic content, this threshold is very low [120]. Unless the orientation of the ligand matches exactly to the field imposed orientation, reaction is no longer possible.

This limitation can be mitigated to a degree by switching the magnetic field on and off, giving the particle time to reorient after each application of the field.

A small amount of reactivity can also be regained by coupling ligands to the surface using long flexible polymeric tethers such as polyethylene glycol, which provide more orientational degrees of freedom [121].

4.3.5 Emulsions as an Assay technology

The novel emulsion particles created in chapter 3 should be able to address these issues as they present a liquid interface such that the ligands can diffuse freely independent of the motion of the particle meaning they will have sufficient orientational freedom to react even under an applied magnetic field. Changing the nature of the interface may also limit non-specific binding.

4.4 Bulk Agglutination

For a successful assay, the protein captured by the particles must be able to bridge particles into doublets and larger aggregates. Aggregation leading to the formation of large clusters was initially observed at high volume fractions ($\varphi = 0.02$) of bulk emulsion mixed with streptavidin. Cluster formation appears to be related to the volume fraction of emulsion employed; at higher volume fractions there are more particles and the average distance between them is smaller so aggregate formation is rapid.

Protocol: Bulk biotinylated emulsion volume fraction $\varphi_i = 0.02$ was suspended in PBS (20 mM NaCl pH 7.4), total 100 µL, and incubated with 18.9 nM streptavidin for 40 minute on a soleil. Aggregation is assessed by microscopic observation.



Figure 4.5 - Protein induced agglutination. Tetravalent streptavidin allows crosslinking of ligands between droplets, multiple droplets bind together forming aggregate clusters. (Bright field images, 40x magnification).

The above experiment may be repeated with lower volume fractions of emulsion (φ_i = 0.001, a 20x dilution), and a reduced streptavidin concentration (1.89 nM, a 10x dilution) shown in Figure 4.6.



Figure 4.6 - Streptavidin-induced aggregation can be repeated at lower volume fractions, capture is shown using fluorescent streptavidin. 100x magnification, Left - Bright field, right fluorescence 2000 ms), since the volume fraction is much lower the size of the clusters formed is much smaller.

Reducing both the volume fraction and streptavidin concentrations results in smaller clusters, due to the decreased number of particles and increased average distance between them. Further dilutions with lower limits of detection may be possible, however, at high dilutions, locating aggregates with a microscope becomes more and more difficult. A major limitation is the large size distribution of the sample which makes it very difficult to calculate the number of droplets in the sample.

4.4.1 Bulk Agglutination Conclusion

These initial experiments show that the principle of agglutination is working well with the formulated emulsion. Agglutination of the bulk emulsion is the first step towards assay functionality, providing a binary result for protein detection, and in this case being sensitive to 1.89 nM of streptavidin.

For a polydisperse emulsion, there is no easy way to quantify the aggregation and relate it to the protein concentration so the assay is qualitative. Further development requires monodisperse particles (this has been achieved using microfluidics – see Chapter 3) and a means to rapidly count the number of aggregates in a dilute sample. A new approach is therefore required. One technique which shows a lot of potential for this application is flow cytometry.

4.5 Flow Cytometry

4.5.1 The Flow Cytometer

One of the most powerful tools available for particle counting is a flow cytometer. Developed originally for counting algae, the cytometer is now a standard tool in most biological labs for analysing cell populations. The flow cytometer is a microfluidic device, which draws objects into a flow-focussed stream and passes them one-by-one through a laser beam/detector pair which allows the scattering of *each* object to be recorded.

The device features several detectors; one directly opposite to the laser which measures forward scattering (FSC) of the particles passing the beam, a perpendicular detector, for measuring the side scattering (SSC - light scattered orthogonally to the source) as well as detectors for various wavelengths of fluorescent light (Figure 4.7).



Figure 4.7 - Flow Cytometer. The flow cytometer measures the forward and side scattering profiles of a microfluidic stream of objects passing a laser beam [122].

The various scattering profiles can be combined to create a dot plot diagrams, with each event detected plotted using its relative side scattering and forward scattering intensity, creating a two dimensional map of the population distribution (Figure 4.8).



Figure 4.8 - Flow Cytometry Histograms. Typical histograms generated by 3 µm Partec calibration beads, plotting the number of objects counted against the scattering signal. Forward scattering as a function of counts (right), Side scattering as a function of counts (centre). Plotting side scattering as a function of forward scattering gives the Dot plot (pseudocolour) to the right.

If the objects have a narrow size distribution, they will appear as a small area on the plot. The plot can further be enhanced to a pseudocolour diagram. The software uses an algorithm to apply colour to the dot plot, according to the incidence of occurrence; red being the zone area of highest concentration, transitioning to yellow to green and then blue – the zone of lowest concentration.

4.5.2 Differentiation of population by size

The flow cytometer detects objects using light scattering (discussed in Chapter 2). It is a powerful tool for differentiating differently sized populations of objects. Mie theory describes how the intensity of light scattered as a function of the scattering angle changes with the radius of the object. The FSC detector is triggered by light scattering within the range of $1 - 10^\circ$, whilst the SSC detector is triggered by light scattered at $90 \pm 50^\circ$. Figure 4.9 shows that the scattering within these regions is (and the overall scattering intensity) very different depending on the radius of the object.



Figure 4.9 - Mie Scattering Diagrams. Diagrams depict scattering profiles for a 1 μ m sized object (left/red) and a 5 μ m object (right/blue), as can be seen the size of the object dramatically affects both the scattering intensity and the scattering angle. Profiles calculated for spheres with a refractive index of 1.5.

The relationship between FSC (or SSC) and size is not simple, so a cytometer cannot be used to measure the absolute size in a simple way [123]. However relative differences in size can be seen by the FSC and SSC profiles, allowing for the counting of differently sized sub-populations within a sample. Flow cytometry is particularly effective for analysing cell populations and a flow cytometer is present in almost all medical laboratories for determining the cell count for the various white blood cell populations in blood samples.



Figure 4.10 - Cell Sorting by Flow Cytometry. The position of the cell in the dot plot depends on its size and granularity (number of particulate objects within), allowing populations to be differentiated. Fluorescent antibodies can be used to target only a specific cell type if there is a lot of overlap in the scattering regions. (image courtesy of [124])

Cells are categorized by size and features such as granularity (particulate objects within the cell) which also contributes to variations in the scattering profile (see Figure 4.10). Moreover, particular sub-populations can be identified using surface receptors such as CD4 for T-cells in the case of HIV infection.

This should make it a powerful diagnostic tool when applied to agglutination based assays as presence of the target protein will cause droplets to aggregate. To the cytometer, clusters of droplets will appear as a single large object which produces a different scattering signal compared to any single droplets passing. The concentration of the analyte can thus easily be linked to the number of aggregates.

An example of population differentiation is given by Figure 4.11, which shows the scatter plot generated by Dynal 3 μ m M270 beads. The particle sample contains a certain number of aggregates (non-specific aggregation arising from instability of the particles) which can be seen in the two distinct populations of the Dot plot. Using analysis software (Flowjo, Flomax) it is possible to treat the data using gates, allowing the user to isolate a certain area of the Dot plot, (or histogram range), counting only the objects which lie within this defined range. Due to the size differences, single objects and aggregates populate distinct regions of the plot therefore their absolute numbers of each can be counted. Knowing the flowrate and the runtime (here 1 μ L/s and 120 s respectively), the absolute concentration of the objects can be determined for the M270 beads and a concentration of

20 fM is found (including both singlets and aggregates). This is much lower than the 50 - 60 fM estimated by the supplier. Such a count would be impossible to do using bulk techniques.



Figure 4.11 - Flow profile of 3 µm M270 Beads. Pseudocolour dot plot of Dynal M270 beads giving a characteristic profile when passed through the cytometer. Gating allows the number of object in each region to be calculated, giving 18282 single beads and 1971 doublets, Here aggregates represent 9.7 % of the signal. (Measured conc. = 20 fM, according to supplier = 50 - 60 fM).

Prior Research: It should be noted that several studies have trialled flow cytometry as a means to measure magnetically induced aggregation in [125] and [126]. The detection limit of these investigations could not exceed the pM range owing to limitations of the magnetic beads. It is hoped that the novel magnetic particles created in this project will be able to surpass these detection limits.

*Non-specific Aggregation: Aggregation seen in the bead population above is problematic for particle assays, in the presence of an agglutinating analyte it is difficult to say which aggregates are caused by the target and which arise non-specifically. It remains to be seen if this can be avoided using emulsion populations.

4.5.3 Advantages of Flow Cytometry

Ubiquity: The cytometer is such a powerful tool for counting and analysing cell populations that it is present in almost all biological and medical laboratories.

Absolute Counting: The advantage of the system compared with other techniques is that it provides the absolute number of objects passed per unit volume, having a much higher precision than bulk techniques such as optical density of a sample, which provides only an estimate for the number of
objects. It also has a higher throughput than counting by eye (hemocytometry) which is also prone to error because of the sampling and large dilutions which are often required.

It is also able to detect far lower concentrations of objects than are measureable by other techniques which usually provide molar concentrations. It is therefore a very powerful tool, and when used as an assay, it should be able to reach very low limits of detection.

Complex systems: The one-by-one counting of flow cytometry gives it a huge advantage over classical light scattering techniques which can only provide information on concentration but no information as to composition if for example multiple cell populations are present in a sample.

Agglutination: One-by-one counting also has the advantage in measuring the exact number of particle aggregates, even at very low concentrations – a limit of bulk techniques (aggregation of <10 % of the particles will show little change in signal for a bulk optical technique). Flow cytometry eliminates this because each object is measured individually, it is impossible for aggregates signal to be 'drowned-out'.

4.5.4 Flow Cytometry Limitations

As with any technique, flow cytometry is also subject to a number of limitations:

Noise: The slight noise in the distribution of a population (the area it takes up) seen in the scattering profiles arises from small inhomogeneities in the object size distribution (no population is 100 % uniform). There are also small fluctuations in the position of each object within the focusing stream, (either closer to the laser or the detector). Side scattering is especially sensitive to these phenomena.

Absolute Size: The number of scattering angles measured by the flow cytometer is limited compared to bulk methods. Moreover, the relationship between size and FSC (or SSC) is complex, making it very difficult to determine the absolute size from the scattering profile.

Object Coincidence: Flow cytometry is sensitive to extremely low concentrations of objects. A drawback to this is that there is an upper limit to the object concentration. Each object is detected by passing a laser through a fixed volume in front of the detector (Figure 4.12). An object passing through this zone is recorded as one 'event'. If the concentration is too high then the detection zone will contain several objects, resulting in a miscount. Count is lost because several objects are recorded as a single event. The Partec Cyflow ml device used in this project can reach a count rate of 1000 objects per second before the onset of coincidence.



Figure 4.12 - Object Coincidence. A + B represent the cytometer flow channel with objects (in yellow passing through). In A objects pass through the detection volume one at a time, the count is accurate. In B object concentration is high; multiple objects occupy the detections volume simultaneously, counting as a single event (object coincidence).

Object Size: There is both a minimum and a maximum size for objects which can be detected. The lower limit is set by the lowest scattering intensity which can be detected by the cytometer.

"for particles with sizes well below the wavelength of the excitation light, the light scattering intensity falls off with nearly the sixth power of the linear dimension of the particles."[127]

This means that objects cannot usually be smaller than several hundred nanometres. Maximum object size is defined more simply by the width and height of the flow channel. For the Partec Cyflow ml system, the size range is $0.2 - 200 \mu$ m. With a diameter of 8.5μ m, the calibrated emulsion droplets easily fall within this size range.

Shear Stress: The sample stream passing through the flow cytometer has a strong elongational flow, putting shear stress on the sample, this can sometimes be useful for dispersing samples however in the case of agglutination, it has the potential to break aggregates, reducing signal.

4.6 Flow Cytometry and Emulsions

To begin with, it is important to see if a useful signal can be generated using emulsion droplets. The comparative signals of the bulk emulsion and the monodisperse step-emulsion are seen in Figure 4.13. The pattern displayed by the bulk emulsion highlights the effect of size upon the scattering profile. Extreme non-uniformity of size ($\Phi = 2.1$) gives rise to a broad profile, the scattering scales

with the size of the object, but complexity of optics means that the profile is not entirely linear and has a characteristic 'S' shape. The uniformity of the step-emulsion can be seen, in comparison, some noise is still observed, because the scattering profile is highly sensitive to even small variations in particle size. The relationship between particle size, size distribution and flow cytometry scattering profile is something studied before [123].



Figure 4.13 - Pseudocolour Dot Plots of Bulk and Step emulsions with corresponding light scattering profiles. The Bulk emulsion (Left) is extremely non-uniform in size (D = 2.1), giving rise to a complex S-shaped dot plot, arrow R show direction of increasing radius. The 'Step' emulsion (Right), with D = 0.079 produces a dot plot with objects confined to a much smaller region.

4.6.1 Aggregation of the Bulk emulsion

Now the flow cytometry profiles of the emulsions are known, it is possible to repeat the previous agglutination experiment (section 4.4) and pass aggregated samples through the cytometer to observe how the profile changes.



Figure 4.14 - Dot plots of bulk emulsion incubated with streptavidin. Left: Blank control, centre: emulsion incubated with 1.89 nM streptavidin, right: emulsion incubated with 18.9 nM streptavidin. [60s acquisition time, 1 µL/s flowrate].

As can be seen in Figure 4.14 there is a clear change in signal. As streptavidin is first introduced there seem to be more objects to the centre of the curve. Increasing the streptavidin concentration further results in larger aggregates (larger than any object in the Blank run) which appearing to the upper right region. The doublets and higher order aggregates do not scatter light like spheres, appearing to the side of the 'S' curve, seen as a cloud which grows with increasing streptavidin concentration.

It can be concluded that agglutination of emulsion droplets can be followed using flow cytometry. However, the object size distribution is too extreme to extract useful numerical data. Subsequent tests are performed using the microfluidic step emulsion. From this point forward, all measurement will be made using size calibrated emulsions.

Before showing the results obtained from size-calibrated emulsion droplets, there is an important limitation to consider when counting emulsion droplets using flow cytometry.

4.6.2 Limitations of Measuring Emulsions

Variable Object Count: As with particles, gating can be used to count the number of objects in a given sample volume to determine its concentration. It is found that for a fixed dilution of emulsion, the number of objects counted depends heavily on the flowrate of the measurement (Figure 4.15).



Figure 4.15 - Objects counted for a fixed concentration of emulsion at different flow rates. Black line = object count of a fixed concentration of emulsion measured. Red line = Partec calibration beads. Blue dashed line = theoretical emulsion count. [0.5 μ L cream into 50mL, random sphere packing φ_i = 0.64 therefore φ_i = 6.4 x 10⁻⁶, 0.8 mL sample volume per run].

At higher flowrates, the number of objects counted reaches a maximum object count is 24.8×10^3 objects per mL (or 41.2 aM). Using the measured droplet diameter (8.5 µm) and assuming random sphere packing ($\phi_i = 0.64$), the objects concentration of 0.5 µL of cream in 50 mL is computed to be 19.7×10^3 objects per mL (or 32.7 aM), depicted by the dashed blue line in the figure. The proximity of the two values indicates that the plateau reached by the higher flow rates is most likely the real object count.

The strong dependence of object count on flowrate most likely results from the creaming of the emulsion. This is verified by comparing the object count for Partec calibration beads, (the red line), which shows little change with flow rate.

The injection probe (Figure 4.16 and Figure 4.17) of the cytometer has a low position within a sample, approximately 2.5 mm from the bottom of the tube. The creaming emulsion will eventually reach a point where it is inaccessible to the injection probe and so is effectively lost. The flow rate determines the amount of time required to aspirate the volume of sample required for volumetric counting (200 μ L for the Cyflow mL device). Aspiration of 200 μ L takes 400 s with a flow rate of 0.5 μ L/s and 25 s for a flow rate of 8 μ L/s. At higher flowrates, there is much less time for creaming and so fewer objects are lost and the count becomes more accurate.

The rate of creaming can be determined using the Stokes equation (4):

$$\mathbf{v} = \frac{2r^2(\rho - \rho_0)g}{9\eta}$$

Where, υ is the creaming (or sedimentation) rate, r is the droplet radius, ρ is the density of the droplet, ρ_o is the density of the dispersion medium, η is the viscosity of the dispersion medium (continuous phase) and g is the local acceleration due to gravity.

For mineral oil droplets ($\rho = 0.838 \text{ g/mL}$) with a diameter of 8.5 µm, the creaming rate is 6.38 µm/s. Because of the flow, it is hard to calculate exactly the flow speed which should be used, however it takes a droplet 392 s to cream up to the height of the injection probe from the bottom of the tube. This is equivalent to a flow rate of 0.5 µL/s (for a 200 µL volume) and marks a lower threshold below which almost no objects will be observed.



Figure 4.16 - Flow Cytometer Volumetric Counting. Counting is initially triggered when the sample interface passes the upper 'start' electrode' and ends when the 'stop' electrode. The aspirated volume is represented by the yellow band, this volume is 200 μ l for the Cyflow ml.



Figure 4.17 - Sample Injection Probe Depth Relative to sample volume. Changing the sample volume changes the depth of the probe (ΔD), in the low volume sample (left) the cream remains close to the injection probe, in the high volume sample right (cream will accumulate far from the injection probe).

Creaming can be controlled in a number of ways. Creaming rate might be changed by matching the densities of the oil and use of smaller objects, unfortunately due to the formulation constraints and the fixed size of the membrane, this is difficult to control. Sample volumes can be minimized to ensure more of the sample remains accessible to the injection probe and the time taken before counting begins is shorter.

The curve in Figure 4.15 indicates also that flow rate should be maximized; however the possible effects of shear must also be taken into account. At higher flow rates, the elongational shear placed on the droplets is much greater, thus as the flow rate increases so does the risk that aggregates will be torn apart by shear.

4.6.3 Conclusion

Flow cytometry permits the quantification of size calibrated emulsion droplets. Limitations arise from the tendency of the droplets to cream up within the sample tube, changing the observed count rate over time. This problem is exaggerated by the low position of the sample injection probedesigned to measure cell populations which sediment over time. The creaming velocity of the emulsion, combined with the low position of the injection probe means that the total volume of sample which can be measures is limited. Furthermore higher flow rates are required to counter the rapid creaming of the droplets.

4.7 Measuring Agglutination using Flow Cytometry

Having characterised the behaviour of the emulsion during cytometric measurements, agglutination experiments can now be performed.

4.7.1 3 μm M270 Streptavidin Beads

To begin probing the reaction and response, it is useful to use model analytes large enough to be quantified by the cytometer. This allows the concentration of both the emulsion and the analyte to be accurately compared. Figure 4.18 shows the individual flow profiles of the 3 μ m M270 beads and the calibrated emulsion droplets.



Figure 4.18 - Pseudocolour diagrams of SSC vs FSC for Dynal 3 μ m M270 beads (left) and calibrated magnetic emulsion droplets (right). Both objects show subpopulations, with non-specific aggregates present in the 3 μ m bead sample (9.7 % of the signal) and smaller objects present in the emulsion sample (most likely tiny droplets formed by Ostwald ripening, representing 7.5 % of the signal).

These beads have a clear characteristic profile via the flow cytometer and thus can be easily quantified. Since the beads are magnetic, reaction efficiency is assured, as both beads and droplet will mutually attract when a magnetic field is applied. The reaction between the objects can be seen microscopically (Figure 4.19), the next step is to count the aggregates using flow cytometry.



Figure 4.19 - Microscope image of reaction between 3 µm Dynal M270 beads and calibrated magnetic emulsion. [*Bright Field, 100x magnification, MES pH5.5, 100 mM NaCl, protocol below*]

4.7.2 Reaction Protocol

Reactions are performed using the following reagents and protocols:

Emulsion: Emulsions used in this section are size calibrated (~8.5 μ m), produced using microfluidic step emulsification (section 3.3). Emulsions contain 10 μ M of biotinylated lipid and 3 w/v % ferrofluid.

General Reaction Volume: A slug of calibrated emulsion droplets is drawn from the cream layer (typically $1 - 5 \mu$ L) and diluted 40x (using 10 mM MES pH 5.5, 100 mM NaCl) to give a reaction concentration which can effectively be taken for microscopic observation.

The typical reaction volume is 10 μ L, placed within a 600 μ L Eppendorf (a volume small enough to resist gravity within the tube during mixing, avoiding excessive shear which might break aggregates). The chosen concentration of analyte (streptavidin or streptavidin coated beads is added to the reaction volume) and the sample mixed using a Soleil for 1 hour, to ensure complete reaction. For magnetic samples, reaction between beads is induced by placing the Eppendorf tube next to a bar magnet, pulling all the objects into contact for a total of 5 minutes.

Flow Cytometry Dilution: The reaction volume is too small to pass through the cytometer and also too concentrated. For cytometry runs, the sample is diluted with 750 µL buffer (the minimal amount required for volumetric counting). To avoid disruption of the aggregates the buffer is added slowly at an angle to minimise the impact and turbulence of the arriving droplets, until the Eppendorf is full. The diluted reaction volume is then gently transferred to the cytometry sample vial (pouring) and remaining dilution buffer is added carefully. The sample tube is then introduced into the cytometer port and the run is initiated.

Flow Cytometer Parameters: Flow cytometry experiments were performed using a Partec Cyflow ml machine. Previous experiments using emulsions had shown that a flowrate of at least 2 μ L/s must be used to avoid sample loss through creaming. Most experiments were performed using this flowrate to avoid placing excessive shear on aggregates.



Figure 4.20 - SSC vs FSC pseudocolour dot plots for fixed calibrated emulsion concentration mixed with increasing concentration of M270 beads. To the upper right of the emulsion profile (red dashed line), increasing scattering from aggregate clusters can be seen, eventually to the lower left (black dashed line) excess 3 µm beads are observed. [*emulsion conc.* = 32.6 fM, MES buffer pH5.5, 100 mM NaCl]

Looking at the sequential SSC vs FSC pseudocolour dot plot of emulsions incubated with increasing concentrations of beads, two prominent changes are visible. An increase in the signal of the pure beads (lower left) and an increase in signal to the upper right of the plot. The increase in to the right is due to larger objects, and so can be attributed to the increasing presence of aggregates.

The border between the aggregates and singlets is a little hard to determine and is more evident by comparing the side scattering histograms only (Figure 4.21). Using gating the object counts of single droplets can be compared to the numbers of aggregates.



Figure 4.21 - SSC histograms for calibrated magnetic biotinylated emulsion mixed with Dynal 3 μ m M270 Beads. Red line = Gate used to calculate singlet (S) and aggregate (A) numbers. R = ratio of aggregates/singlets.

In principle, it should be possible to follow the reaction by counting purely the object counts of either the aggregates or the singlets. Figure 4.22 shows the curves obtained purely from plotting the object count as a function of analyte concentration.



Figure 4.22 - Plots of the number of singlet and aggregate objects counted as a function of M270 bead concentration.

Both curves show the anticipated trend: As the concentration of beads increases, there is an increase in the aggregate population with a corresponding decrease in single objects population. Both curves also show some variation in object count between runs. This variation may arise from pipetting errors (extremely low volumes) combined with the creaming of the emulsion which may affect cytometry counting or the numbers of droplets collected when a sample is aspirated.

To reduce the impact of the observed noise, the object counts can be normalised, by plotting their respective ratios to one another (Aggregate/Singlet ratio) as shown in Figure 4.23. After normalisation, the trend is very linear, showing little noise. This means that the aggregate and singlet populations are changing proportionally to the bead concentration and the noise is a problem related to the absolute count.



Figure 4.23 - Plot of Aggregate/Singlet ratio for 32.6 fM of emulsion droplets mixed with 3 µm M270 beads. Blue fit: Actual data ($\mathbf{Y} = 6.8 \times 10^{-3} \mathbf{X} - 1.48 \times 10^{-2}$), red fit: Ideal aggregation ($\mathbf{Y} = 30.7 \times 10^{-3} \mathbf{X} - 8.13 \times 10^{-8}$).

This curve can be compared with an idealised curve which is plotted assuming that each bead present in the sample will give rise to a doublet (for [beads] < [droplets]). The maximum possible slope of the prozone can be computed by dividing the absolute number of beads by the absolute number of objects (depicted by the red line in Figure 4.23). For this first M270 bead experiment, there is a difference between the actual slope (6.8×10^{-3}) and the ideal slope (30.7×10^{-3}), the actual/ideal ratio being 0.22. Reactivity is less than ideal, but has a similar order of magnitude, indicating efficiency.

There are a number of reasons why the slope may deviate from the ideal. It is highly likely, for example, that a certain number of the aggregates may be broken by shear when taken into the cytometer. A certain number may be lost due to creaming (remembering aggregates cream more rapidly than single droplets). It is also possible for a single droplet to capture multiple beads, resulting in less than one aggregate per bead. Lastly, the flow profiles show a population of small objects present in the sample, likely to be tiny droplets resulting from Ostwald ripening, these may react with the beads first, diminishing their reactivity with the larger droplets.

Standard Deviation: Due to noise coming from dust and a minor population of larger objects in the sample, the blank emulsion sample also gives a count in the aggregate region; the ratio given by this count provides us with our uncertainty value and can be used to determine the lower limit of detection. For the concentration of droplets used during these measurements, the standard deviation of the aggregate/singlet ratio calculated form 8 blank runs was found to be 3.4×10^{-4} (this value is so low that the error bars cannot be accurately shown on the plot).

4.7.3 Limit of Detection

The sensitivity of any assay is determined by its limit of detection (LOD), which is the lowest quantity of analyte which can be distinguished from a measurement where no analyte is present (i.e. a blank/control run, within a stated confidence limit (usually 1 %). It is determined by the signal to noise ratio. By convention, a signal to noise ratio of 3 determines the LOD, with the noise (σ). If the noise follows a normal probability density, (being representable by a Gaussian distribution) and σ represents the standard deviation, 3σ will cover 99.7% of events, arising from non-specific aggregation. Thus a LOD based upon a signal to noise ratio of 3 has a 0.03% chance of false positives.

The LOD is affected by two factors:

- the intensity of the noise, σ
- the slope of the curve

To determine the limit of detection, flow profiles are obtained for several blank samples (10 runs minimum) of emulsion (containing no beads/streptavidin). Using the same aggregate / singlet gates, the numbers of objects appearing in each region are recorded and thus an ASR noise value can be obtained. This ASR ratio can then be used as σ for the determination of the LOD.

Limit of Detection M270: Using the maximum slope of the actual curve, and the standard deviation (σ) of the ratio ASR, it is possible to compute the lower limit of detection (see 4.7.3), which is taken to be three times the standard deviation divided by the maximum slope of the curve. 3σ here equals 1.02×10^{-3} , which corresponds to a limit of detection of 0.15 fM. It was decided to switch to smaller Dynal 1 µm MyOne C1 (MOC1) beads, which have a higher stock concentration. Additionally, the smaller size of the MOC1 promotes efficient mixing.

4.7.4 1 µm Dynal MyOne Carboxy Streptavidin Beads

The smaller 1 μ m MOC1 beads should react more rapidly than the M270 3 μ m beads during the incubation step. These beads are also magnetic, ensuring a strong interaction in the magnetic reaction step. The MOC1 beads are supplied at higher concentration and should be able to reach the plateau of maximum aggregation. As can be seen in Figure 4.24, the beads are large enough to give a distinct profile for counting by gating.



Figure 4.24 - SSC vs FSC pseudocolour dot plots of emulsion and MOC1 beads. Side scattering vs forward scattering dot plots of pure emulsion (left) and pure MOC1 beads (right).

Following the same protocol used for the M270 beads, several concentrations of MOC1 beads are incubated with a fixed concentration of emulsion to prepare a dose response curve. Figure 4.25 shows a selection of pseudocolour dot plots with their corresponding SSC histograms used to calculate the S, A and R numbers.



Figure 4.25 - Selected pseudocolour dot plots and corresponding SSC histograms of a fixed concentration of calibrated magnetic emulsion mixed with increasing concentration of MOC1 beads. Dashed red line = singlet/aggregate gate. [*Emulsion conc.* = 50.9 fM, MES buffer pH5.5, 100 mM NaCl].

Exactly as before, increasing the concentration of MOC1 beads gives rise to an increasing number of aggregates, appearing as larger objects to the upper right corner of the dot plots (and a shoulder on the to the right of the SSC histograms). Singlets and aggregates are counted using gating, Table 3 summarises the gated object counts for the complete set of MOC1 concentrations.

[MOC1 Beads] fM	Aggregates (A)	Singlets (S)	A/S Ratio (R)
0	261	48979	0.006
8.49	535	29950	0.018
15.8	1819	18069	0.10
37.6	2986	13929	0.21
53.3	5533	21888	0.25
89.3	2625	9323	0.28

 Table 3 - Singlet and Aggregate counts for fixed concentration of emulsion mixed with increasing concentrations of MOC1 beads.

Again, the aggregate/singlet ratio can be plotted as a function of bead concentration and compared to an ideal slope (based on the assumption that one bead generates one aggregate).



Figure 4.26 - Aggregate/Singlet vs Bead concentration profile for MOC1 beads incubated with 50.9 fM of calibrated emulsion droplets. Black crosses: Actual data ($\mathbf{Y} = 1.13 \times 10^{-2} \mathbf{X} - 78.31 \times 10^{-3}$), Red fit: Ideal aggregation ($\mathbf{Y} = 1.96 \times 10^{-2} \mathbf{X} - 1.49 \times 10^{-8}$).

As before, there is an increase in aggregation with bead concentration. MOC1 beads are at a higher concentration than the M270 beads, a plateau of maximum aggregation begins to appear. Encouragingly, the plateau arrives very close to the [MOC1]/[droplets] = 1, the expected point of maximum aggregation. This fits well with what is shown by the dot plots, with very few single beads

remaining even at higher concentrations. At the highest concentration (89 fM), gating shows 381 single beads remain, out of a possible 85995, a capture ratio of 0.996.

A surprising detail is that even with an almost 2:1 ratio of beads to droplets, there are still single droplets present in the sample. The slope for the real data is measured at the steepest point of the curve, in this experiment; the slope is closer to the ideal, 1.13×10^{-2} for the real data and 1.96×10^{-2} for the ideal (data is 0.576 of the ideal value). The limit of detection for the actual data is found to be 0.9 fM of MOC1.

Dynamic range: With an upper limit to the concentration of objects which can be detected, the dynamic range for MOC1 detection can be said to lie between 0.9 – 100 fM of beads.

Overall it can be seen that both 3 μ m M270 beads and 1 μ m MOC1 have a similar response. Figure 4.27 shows the overlaid dose-response fits (as a function of numbers of beads/droplets). The initial slopes of both data sets are close in value, being 6.8 x 10⁻³ and 1.13 x 10⁻² for the M270 and MOC1 beads respectively (ratio 1.66).



Figure 4.27 - Combined A/S vs [Beads] plots for 3 μ m M270 and 1 μ m MOC1. Blue line = 3 μ m M270 beads, red = 1 μ m MOC1 beads.

The next step is to try to measure an analyte closer in scale to single proteins.

4.7.5 40 nm FS40 Neutravidin FluoSpheres

Protein scale objects can be approximated first using Life technologies 40 nm neutravidin (a streptavidin analogue)-coated FluoSpheres. These beads are non-magnetic, so capture will be controlled only by diffusion. With such a small size means capture should be efficient. With a diameter of approximately 40 nm, it is impossible for the cytometer to detect these beads;

concentrations are based purely on information provided by the manufacturers. Since the object concentration is less well known, a much wider range of concentrations are screened. Selected flow profiles are shown in Figure 4.28.

Formulation Note: At this stage, the original decane based emulsion displayed signs of degradation (Ostwald ripening). For subsequent measurements a fresh emulsion was used, based upon 2-Octyl-1-dodecanol (201D) which is much less soluble in water. Changing the oil is unlikely to significantly impact reactivity. Dot plots of the 201D emulsion show no presence of the smaller droplets observed previously. 10 blank runs give an aggregate/singlet ratio standard deviation of 3.67×10^{-3} and thus a 3σ value of 1.1×10^{-2} , higher than previously but still a very low value for a particle assay.



Figure 4.28 - Selected pseudocolour SSC/FSC dot plots of a fixed concentration of calibrated magnetic emulsion mixed with increasing concentration of FS40 beads with corresponding SSC histograms. Dashed red line = singlet/aggregate gate. [*Emulsion conc.* = 35.1 fM, MES buffer pH5.5, 100 mM NaCl].

FS40 beads show the same behaviour as before, with increasing visible aggregation in both dot plots and SSC histograms. Once again, gating provides the counts for the numbers of each population (Summarised in Table 4).

[FS40 Beads] (fM)	Aggregates (A)	Singlets (S)	A/S Ratio (R)
0	434	67131	0.006
7.55	1247	65769	0.019
18.9	2313	49089	0.047
37.8	3721	19711	0.19
56.7	4610	16158	0.29
75.5	5870	15325	0.38
<mark>189</mark>	<mark>6660</mark>	<mark>42381</mark>	<mark>0.16</mark>
378	6917	16246	0.43
<mark>567</mark>	<mark>1543</mark>	<mark>5843</mark>	<mark>0.26</mark>
755	3545	7640	0.46

 Table 4 - Summary gate counts of emulsion + FS40 beads. Anomalous results are highlighted in yellow (see below).

The flow profiles show an apparent decrease in number of objects counted as the concentration is increased. This may be explained by increasingly higher orders of clustering which consumes more and more droplets as the concentration of the analyte is raised. The dose–response curve of this data is shown in Figure 4.29.



Figure 4.29 - Number of aggregates/singlets vs concentration of FS40 beads added.

There is a rapid increase in aggregation followed by an apparent plateau. It should be noted that there are a couple of data points with lower than expected aggregate/singlet ratios (189 fM and

567 fM), highlighted in Table 4. The source of this noise is unclear, one possibility is inhomogeneous mixing of the beads and emulsion, being small and diffusing rapidly the beads may react with the emulsion before the sample is fully mixed, causing deviations from the expected result. Larger clusters resulting from higher bead concentrations are also expected to be more vulnerable to shear (shown in Figure 4.30).





Figure 4.30 - Aggregation induced by FS40 beads. Comparison of blank emulsion sample (left) and emulsion mixed with 75 fM FS40 beads (right). Large clusters are formed in the presence of the beads. [Bright field 40x magnification, emulsion conc. = 35.1 fM, MES buffer pH 5.5, 100 mM NaCl].

Again the initial slope can be plotted and compared with an ideal fit, see Figure 4.31.



Figure 4.31 - Aggregate/Singlet vs Bead concentration profile for FS40 beads incubated with 35.1 fM of calibrated emulsion droplets. Actual data, black crosses + blue slope: $Y = 5.28 \times 10^{-3} X - 1.78 \times 10^{-2}$ fit based on ideal aggregation, red slope: $Y = 1.45 \times 10^{-2} X + 1.12 \times 10^{-8}$

The slope of the FS40 beads (5.28×10^{-3}) also falls short of the ideal aggregation slope (1.45×10^{-2}) , being 0.36 of the value. It is interesting to see that the bead/droplet equivalence point

occurs far before the plateau. This may indicate a large discrepancy between the FS40 concentration given by the supplier and the actual stock concentration. It may also be that, being smaller and having fewer proteins coating the surface, more FS40 beads are needed per droplet to effectively bridge to another droplet. The LOD for FS40 beads is found to be 2.1 fM, with an ideal LOD of 0.75 fM. Overall, the dynamic range for FS40 bead appears to be 2.1 - 75.5 fM.

In this experiment, both LOD and dynamic range are more tentative due to the inability to measure the exact concentration of the FS40 beads. Provided the manufacturers' concentration is accurate, the result is encouraging since a similar LOD has been reached using non-magnetic beads with a scale comparable to individual proteins. In the final step, streptavidin itself will be assayed as a model analyte.

4.7.6 Streptavidin

emulsion droplets incubated with streptavidin are show in Figure 4.32. **Blank Emulsion** Emulsion + 9.47 nM strep Emulsion + 37.9 nM strep 102 ŝ ŝ ŝ

Emulsions are reacted with streptavidin using the same protocol as the beads. Selected plots for



Figure 4.32 - Selected plots of a fixed concentration of calibrated magnetic emulsion incubated with increasing concentration of Streptavidin Alexa 555. Dashed red line = singlet/aggregate gate [emulsion conc. = 46.4 fM, MES buffer pH5.5, 100 mM NaCl].

Streptavidin shows the same aggregation behaviour as the previous objects. It is immediately obvious that the concentration of streptavidin required to induce aggregation is far higher than the concentrations of beads used in the previous samples (nM compared to fM). This concentration is consistent with the nM concentration of streptavidin required for aggregation in bulk (section 4.4).

The reason for this is unknown. As with the FS40 beads, the concentration of streptavidin cannot be measured directly, however is unlikely to be several orders of magnitude different from the manufacturers' measurement. The small size and comparative weakness of an individual bond, may mean that many more streptavidin are required to crosslink two droplets than the FS40/MOC1/M270 beads.

Aggregation is first seen at 3.79 nM added streptavidin, assuming homogenous coverage of the 46.4 fM of droplets this is equal to 8×10^4 streptavidin per droplet. Even if only a small percentage of them are available for crosslinking, this is far more than the protein coverages of the FS40 beads (geometric maximum of 250 proteins per bead). FS40 beads show aggregation at less than 1 bead/emulsion droplet so less than 200 streptavidin should be required for crosslinking, and would imply a picomolar detection range for the pure protein. Gating gives the full dose-response profile (see Table 5).

[Strep] (nM)	Aggregates (A)	Singlets (S)	A/S Ratio (R)
0	408	88949	0.004
3.79	2108	68238	0.031
9.47	2950	72269	0.041
18.9	4427	18777	0.24
28.4	1683	5252	0.32
37.9	2772	11777	0.24

Table 5 - Summary Table of Aggregates/Singlets for emulsion reacting withstreptavidin.

The plot of this data is unlike (Figure 4.33) what has been observed previously, although it is difficult to say if this is due to measurement noise or not.



Figure 4.33 - Aggregate/Singlet ratio vs concentration of added streptavidin.

Another explanation which may be evoked for apparent reduction reactivity with pure streptavidin is same droplet-crosslinking, (self-CL) shown in Figure 4.34. Essentially, the flexibility of the linker and fluidity of the ligands means that two or more ligands from a single droplet are able to reorganise and reach around to react with multiple binding sites on a captured streptavidin. Reactivity is therefore quenched due to a reduction in the number of binding sites available for crosslinking.



Figure 4.34 - Possible self-crosslinking mechanism. A and B show the an ideal crosslinking scenario. Self-crosslinking is shown in C where multiple ligands from the same droplet bind to each streptavidin, 'neutralising it'. Having lost binding sites, the droplet will be less reactive.

This may prove to be one of the drawbacks to a liquid interface which affords extreme mobility and orientational freedom to the ligands, since a biotin ligand will always be able to diffuse in and react with a streptavidin site. To verify this, it should be possible to graft the streptavidin directly to the droplet using peptide bond formation with carboxylic acid groups. Agglutination can then be induced using biotinylated analytes such as BSA-biotin.

4.7.7 Cytometric Agglutination Summary

Results are summarised in Table 6. The general trend seems to be femtomolar detection, surpassing the picomolar detection limit for magnetic agglutination assays and rivalling the 10 femtomolar detection limit of a commercial device dedicated to the detection of TSH. Further optimisation may be able to attain a much lower LOD.

Analyte	LOD	Dynamic Range
Strep	-	-
FS40 (40 nm)	2.1	2.1 – 75.5 fM
MOC1 (1 µm)	0.9 fM	0.9 – 100 fM
M270 (3 µm)	0.15 fM	2.3 – 50+ fM

 Table 6 - Summary of LODs and Dynamic ranges for objects investigated.

4.8 Agglutination Conclusion

Bulk Agglutination: Using bulk polydisperse emulsions, it is possible to induce agglutination using streptavidin. The polydispersity of the emulsion prevent much quantitative data from being drawn. In such a case, the assay is limited to a binary Yes/No response.

Flow Cytometry - **Bulk:** Measuring bulk samples with the cytometer, flow profiles show that is possible to follow aggregations of the bulk sample with the cytometer. Sample non-uniformity prevents gating from being usefully employed to count aggregates using size populations.

Flow Cytometry - Calibrated Emulsion: Using a microfluidic, size calibrated emulsion it is possible to distinguish between singlet and aggregate populations. Variation in absolute counting shows that a more stable response is obtained by plotting aggregate/singlet ratio as a function of analyte concentration. Using this method, agglutination can be quantified to the femtomolar concentration ranges.

Interestingly, there is an apparent limit to reaction efficiency, with a large proportion of single objects remaining even at high analyte concentrations; the cause for this is unknown. Reaction

efficiency also appears to be limited when pure streptavidin is employed as the analyte. Detection limits are consistent with the concentration range found for agglutination of the bulk emulsion. The cause for this is again unknown but suspected to relate to a possible cross-linking mechanism of long spacer ligands at the interface.

The assay protocol is unrefined and not yet optimised. A certain amount of signal is likely to be lost through the shearing of objects. Forces holding the phospholipids at the interface (~20 kT [128]) are weaker than covalent bonds (100 - 300 kT [129]) typically used to functionalise assay particles. A small amount of shear stress applied to agglutinated beads should pull particles apart again (something which must be quantified). This is exacerbated by the large size of the particles (and clusters) which have a large surface upon which shear forces can act. This may be difficult to control since channel flow within the commercial flow cytometer is not well characterized. It may however be possible to study the effects of controlled flow by designing a dedicated microfluidic chip.

It should also be possible to achieve a more narrow size distribution of emulsion droplets, defining more clearly the boundary between singlet and aggregate states. The emulsion used are also not yet fully uniform in size, due to small inhomogeneities of production or aging processes such as evaporation and Ostwald ripening.

There are many additional parameters which should be studied, including the effect of droplet size, initial concentration, reaction kinetics and the role of the magnetic field. Understanding these parameters will help in assay optimisation and ideally allow lower limits of detection to be reached.

Chapter 5 Conclusion & Perspectives

5.1 Conclusion

5.1.1 Formulation & Production

It has been demonstrated that it is possible to create monodisperse magnetic emulsion droplets presenting ligands at a liquid interface for the capture of a target biomolecule. Emulsion stability is controlled by a balance of both chemical and physical forces.

Droplet Functionalisation is achieved by the addition of functionalised phospholipids to the oil. Phospholipid solubility appears to be tied to saturation, indicating CMT dependence. Lipids with higher saturation can be dissolved by mixing them with an excess of unsaturated lipid. Fluorescently tagged lipids are used to verify lipid presence at the interface. To ensure stability, the emulsion requires a co-surfactant in the continuous phase.

Surfactant-phospholipid interactions appear to be dictated by the hydrophobic moiety of the co-surfactant. Short chain aliphatic tail surfactants result in mixed micelle formation and removal of lipids from the interface, conversely several triblock copolymers stabilise the emulsion with minimal disruption to the phospholipid monolayer.

Biotinylation is achieved by using phospholipids grafted with PEG-Biotin. Capture of protein by the ligand at the droplet interface is revealed through the use of fluorescently tagged streptavidin, observed via fluorescence microscopy which, combined with image analysis can be measured to indicate the relative capture efficiency under a range of conditions. Using size calibrated emulsions and a known protein concentration, it is possible to estimate the capture efficiency and compare it to the number of ligands available. By changing the ligand concentration it is found that the droplet saturation point is determined by ligand availability at the interface. Protein/ligand ratio is high, indicating good ligand interfacial ability.

Microfluidic Production: Monodisperse emulsion droplets can be produced using a microchannel membrane step-emulsification technique. The PDMS microchip is susceptible to swelling which greatly disrupts droplet production therefore only certain oils or combinations are possible to use. PDMS swelling oils can be emulsified using a glass device.

Biofunctionalisation is achieved though formulation with biotin conjugated phospholipids; protein interaction is observed through the capture of fluorescently labelled streptavidin, which permits estimation of capture efficiencies. Capture efficiency can be modulated by changing the poloxamer co-surfactant, appearing to relate to the surface activity of the polymer, indicating phospholipid poloxamer competition. Using a fixed poloxamer length, the spacer length of the biotinylated lipid is varied. A threshold is found to exist whereby efficient capture can only occur if the PEG length of ligand spacer exceeds the PEG length of the poloxamer cosurfactant. Capture efficiency dies off at higher ligand length possibly relating to reduced solubility or ease of removal from the interface.

Interfacial fluidity is evidenced by the capture of streptavidin coated beads whose motion can be seen independently of possible bead orientation. Protein diffusion can also be observed through the formation of adhesion plaques at the contact zone between crosslinking droplets.

Magnetic Content: is added to the system using a ferrofluid. Ferrofluid stability is found to heavily influence both emulsion production and stability. Destabilisation during production results in blocking of the microfluidic channel and breakdown of droplet production. Emulsions produced with unstable ferrofluid experience Pickering, resulting in the loss of interfacial fluidity. Stability of the magnetite nanoparticles within the ferrofluid is determined by the nature of the carrier oil and the concentration of oleic acid. Tuning these parameters allows creation of a highly stable ferrofluid emulsion.

Overall: Using the stable magnetic, biotinylated formula with the microfluidic device, it is possible to produce large quantities (mL) of size calibrated magnetic emulsion droplets with good functionalisation.

5.1.2 Particle Application-Agglutination Assay

Particle utility is explored through their application to assay technologies. Simple agglutination of the bulk emulsion demonstrates the necessity of monodisperse populations and the need for sensitive and accurate aggregate counting methods. Cytometry is then explored as a means to quantify emulsions and their specific agglutination in the presence of a target analyte.

Agglutination can initially be observed but not measured using a bulk sample. Measurement of a calibrated emulsion shows an interesting counting discrepancy which relates to measurement flow rate. At a certain flowrate, droplet count reaches a plateau, closely matching theoretical counts. Flowrate-object count dependence is attributed to emulsion creaming, with higher flowrates giving the emulsion less time to cream 'out of reach' of the injection probe, thus giving a more accurate count.

Agglutination of calibrated emulsion droplets is initially performed using magnetic streptavidin coated beads. When passed through the flow cytometer, samples incubated with beads show subpopulations of larger objects not present in blank runs which are attributed to aggregates resulting from specific agglutination. Object counts are obtained by gating the data and when plot as a function of bead concentration (dose-response curves); a positive correlation is observed for the number of aggregate objects whilst a negative correlation is observed for single droplets. Both plots display an apparent counting inconsistency between runs which may relate to sample handling and propensity of the emulsion to cream.

Count inconsistencies are eliminated by calculating the aggregate/singlet ratio and replotting this as a function of bead concentration. Assay efficacy can be determined by measuring the slope of the dose response curve and comparing to an idealised slope based on maximum possible aggregation (1 bead creates 1 aggregate).

The limit of detection (LOD) is calculated from the 3 times the standard deviation (σ) of the noise in a blank run divided by the slope. Using this method, LOD is found to be in the femtomolar range.

Switching to smaller beads (1 μ m and 40 nm) shows a slight increase in capture efficiency, with maxima of aggregation. 3 μ m and 1 μ m magnetic beads analytes show similar assay response. Nonmagnetic 40 nm beads show strong aggregation with some noise at higher concentrations. Agglutination is also performed with pure streptavidin but requires much higher concentrations for an adequate response. With the exception of streptavidin, objects can be detected into the femtomolar range. Sensitivity limit with the pure protein is attributed to a self-crosslinking mechanism with ligands from the same droplet occupying multiple streptavidin reactive sites.

Overall it has been show that emulsions can easily perform as agglutinating particles and can be used to achieve extremely low limits of detection (~fM) with minimal optimisation. This surpasses the standard for magnetic agglutination and matching that of industrial standard. Such sensitivity is attributed to the low noise in blank runs, with typically less than 0.5 % of objects appearing in the aggregate region. This is lower than the noise which is seen using solid beads. In a particle assay this noise usually arises from the presence of non-specific aggregation of the particles. Liquid droplets should be less prone to non-specific aggregation since their refractive index is closer to that of the medium, reducing Van der Waals interaction between the droplets. This is further influenced by geometry, the droplet interface should be perfectly smooth, in comparison to a rough particle surface so there is far less surface area for interaction between adjacent bodies. Low noise is shown to be highly repeatable over multiple blank runs have this results in a low σ value and subsequently low LOD.

Further optimisation may allow the limit of detection to be pushed much further. To do so, further study of the system reaction kinetics is required and creating a stronger link between the number of droplets used and the LOD. Further study should also decipher the aggregation pattern to more easily identify doublets, triplets and higher order aggregates. This technology is now being applied within the DIGIDIAG network, in collaboration with an industrial partner.

5.2 Perspectives

There are a number of additional studies which may be considered to build upon this project:

5.2.1 Agglutination Measured by Flow Cytometry

Fluorescent Flow Cytometry: Using fluorescently labelled droplets, it should be possible to use the fluorescent channels of the cytometer to better separate signal of the emulsion from noise in the sample such as dust or micelles. Fluorescent droplets can be created either using a one of the fluorescently labelled phospholipids (Figure 3.4) or introduction of a fluorescent lipophilic dye into the oil, such as DiD.

Droplet Size: Size is a dominant characteristic in determining the behaviour and reactivity of particle systems. It may be interesting to explore the reactivity of smaller particles produced for example using a couette cell. The couette cell reduces should be able to produce droplets with a much smaller diameter but is likely to result in a higher distribution than that achieved using the microfluidic device.

It is expected that smaller particles (and their corresponding aggregates) will be less sensitive to shear forces and may be a viable strategy for improving the maximum aggregate/singlet ratio in agglutination.

Grafting: It has been show that emulsions combined with flow cytometry are a promising tool for use as an assay technology. Currently, the detection limit of single proteins seems to be a bottleneck, possibly caused by same-droplet crosslinking. Crosslinking may be eliminated by grafting streptavidin directly to the interface. Direct grafting onto emulsions has been performed previously in this lab, however results were difficult to reproduce and the grafted ligands unstable[101]. Grafting has been attempted within the timeframe of this project, introducing carboxylic acid terminated phospholipids (with sufficiently long spacer), with the goal of forming peptide bonds with amine groups within fluorescent streptavidin molecules, via EDC coupling. Grafting attempts have so far been unsuccessful, further optimisation is required. Once streptavidin is grafted, biotinylated reagents can be used to perform agglutination.

Antibodies: This work has concentrated on a model system; the next logical step would be to perform tests using a real antibody and antigen. Strategies for coupling the antibody to the interface will be required. One possibility is to continue with the biotin streptavidin pair; saturating the interface with streptavidin will allow the coupling of biotinylated antibodies. The drawback is that at high coverages, a high coverage of streptavidin may limit interfacial fluidity of the interface, or undergo the proposed self-crosslinking. Grafting, if successful would be an optimal strategy for linking antibodies to the interface.

Non-specific aggregation: Cytometry runs of the emulsion droplets in buffer found consistently low non-specific aggregation of the droplets this is a surprising considering the high salt concentrations used in the buffers (>100 mM). For assay development, further test should be performed to find out if this stability extends to serum, plasma or even full blood.

5.2.1 Alternative Applications

An immunoassay is just one application of these particles, biofunctionalised magnetic emulsion should prove useful for many other applications where solid phase magnetic particles are employed. These particles should work equally well for an assay based around surface capture. The liquid interface should encourage binding to cells for cell sorting [130]. More fundamental work may also be envisaged, such as that of Paul Chaikin and others using both solid and liquid colloids to build complex structures with original optical properties (such as a photonic band gaps [131]) or for self-replication [132].

Materials & Methods

Solubilisation of Phospholipids

Stock solutions of phospholipids in chloroform are stored at -20 °C. An appropriate volume of the stock solution is taken using a 500 μ L glass syringe and injected into a 1.5 mL glass bottle. The chloroform is evaporated under a stream of nitrogen, leaving behind a lipid film. 0.5 mL of oil is pipetted into the bottle. The lipid film is then dissolved via vortexing and sonication (30 minutes minimum).

Preparation of PDMS chips

Sylgard elastomer (Dow Corning) is combined with its crosslinking agent at a ratio of 10:1, for a standard chipset, 44 mL of liquid PDMS is used. The two reagents are mixed thoroughly using a spatula and centrifuged for 20 minutes to remove air bubbles. The PDMS is then poured onto the silicon mould and degassed further under vacuum for another 15 minutes. After degassing the mould is placed into an oven and left at 70 °C for a minimum of 2 hours. The cured PDMS is extracted from the mould, individual chips are cut out using a scalpel and the outlet and inlets are bored with a 0.75 mm boring tool. The bored device is rinsed with isopropanol to remove any dust and PDMS debris and dried using an air pistol. The cleaned surface is then protected with a layer of scotch tape and the device is stored for bonding.

For bonding a microscope slide (76 by 26 mm) is cleaned with isopropanol and dried with an air pistol. The glass slide and the PDMS chip are placed into an oxygen plasma oven and exposed to plasma for 45 seconds. Following exposure the chip is bonded to the glass slide.

5.3 Surface treatment of the PDMS chip

After plasma treatment the PDMS surface has a high degree of hydrophilicity, this is desirable to prevent the oil droplets from wetting the channel and allowing efficient droplet production. Literature has shown that the hydrophilicity can be prolonged by the deposition of a hydrophilic polymer after plasma treatment. To achieve this, a filtered 3 w/v % solution of polyvinylpyrrolidone (PVP K90) is flushed through the chip for 2 hours. Following the polymer solution pure water is flushed through to remove excess unbound polymer. Finally air is introduced through the chip until it is dry; the chip may then be stored and used at a later date.

Microfluidic Emulsification Setup

Fluids are introduced into the chip from falcon tubes using an Elveflow pressure control system. The treated microfluidic chip is placed onto a microscope. Once the Teflon tubing is inserted, initially the continuous phase is flushed through before the oil is gradually introduced. Samples are collected and stored in 10 mL or 4 mL glass bottles. Pressure is controlled using Maesflow or Elveflow pressure control systems. Production was monitored and recorded using a Leica DMI300 B microscope, equipped with a 1024PCI Photron Fastcam.

Microscopy

Microscopy is performed with a Nikon Eclipse E600 microscope fitted with a Photometrics Cascade camera, fluorescence is generated by a Nikon Intensilight C-HFF lamp. Glass slides are prepared with a chamber of double-sided scotch tape. 10μ L of sample is pipetted into the chamber which is then sealed with a glass coverslip (22 x 22 mm).

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